Development and evaluation of a novel and innovative drug sensitivity assay for *Plasmodium falciparum*

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ABSTRACT

Malaria is still the most relevant human parasitic infection in the world – according to the World Health Organization, there were 214 million malaria cases worldwide last year. Antimalarial resistance is what most hampers malaria control; sensitivity testing is hence crucial for resistance detection and further establishment of public health guidelines for malaria treatment. There are several sensitivity tests for *Plasmodium falciparum* – the deadliest human malaria species – but no official global guidelines.

Haemozoin is a crystal with birefringent and paramagnetic properties, produced by the *Plasmodium* parasite while it is developing inside the host red blood cells. This study aimed at the evaluation of a new magneto-optical method, which makes use of the detection of haemozoin, to test the sensitivity of *Plasmodium* to antimalarials.

Several sensitivity assays were performed with commonly used antimalarials and IC50s were calculated. This method proved to be able to detect not only malaria parasites, but also parasite maturation and inhibition effects. Although some variability was observed among replicates with the same drug, this is thought to originate from the assays protocol and not from the method itself.

Hence, although there is the need for protocol optimization in order to obtain less variable results, this Magneto-Optical Test has great potential for further testing with antimalarial drugs and, in the future, for application in the field.

Key-words: malaria, antimalarial resistance, sensitivity tests, haemozoin, magneto-optical test

RESUMO

A malária é ainda uma das doenças parasitárias mais relevantes no mundo – segundo a Organização Mundial de Saúde, houve 214 milhões de casos no ano passado. A resistência a antimaláricos é o que mais impede o controlo da malária; os testes de sensibilidade são, assim, cruciais para a deteção de resistência e posterior estabelecimento de diretivas de saúde pública para o tratamento da malária. Há diversos testes de sensibilidade para *Plasmodium falciparum* – a mais mortal das espécies de malária humana –, embora não haja diretivas oficiais a nível global.

A hemozoína é um cristal birrefringente e paramagnético, produzido por *Plasmodium* aquando do seu desenvolvimento nos eritrócitos do hospedeiro. Este estudo tem como objetivo avaliar um novo método magneto-ótico, que faz uso das propriedades da hemozoína, para testar a sensibilidade de *Plasmodium* a antimaláricos.

Realizaram-se vários ensaios de sensibilidade com antimaláricos comumente utilizados e foram calculadas CI50s. Este método provou ser capaz de não só detetar parasitas da malária, mas também a sua maturação e os efeitos inibitórios dos antimaláricos. Embora se tenha observado alguma variabilidade entre réplicas com o mesmo fármaco, pensa-se que esta seja devida ao protocolo dos ensaios e não ao método.

Consequentemente, e apesar de ser necessária a otimização do referido protocolo de modo a obter resultados menos variáveis, este Teste Magneto-Ótico tem elevado potencial para futuros testes com antimaláricos e aplicação no terreno.

Palavras-chave: malária, resistência a antimaláricos, testes de sensibilidade, hemozoína, teste magneto-ótico

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LIST OF ABBREVIATIONS

ACT: artemisinin-based combination therapy

APAD: 3-acetyl pyridine adenine dinucleotide

CDC: Centers for Disease Control and Prevention

CQ: chloroquine

DELI: double-site enzyme-linked LDH immunodetection

DF: drug-free

DHA: dihydroartemisinin

DHFR: dihydrofolate reductase

DHPS: dihydropteroate synthetase

DHRI: dihydrofolate-reductase inhibitor

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorter

FBC: full blood count

FPPIX: ferroprotoporphyrin IX

G6PD: glucose-6-phosphate dehydrogenase

HLC: haemozoin-like crystal

HPLC: high-performance liquid chromatography

HRPII: histidine-rich protein II

Hz: haemozoin

iRBC: infected red blood cells

LS: lysis solution

MCM: malaria complete parasite medium

MLD: magnetically-induced linear dichroism

MOT: Magneto-Optical Test

NAD: adenine dinucleotide

nHz: native haemozoin

nLS: new lysis solution

OD: optic density

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PfATP4: *P. falciparum* plasma membrane Na^+-ATPase 4

Pfcrt: *P. falciparum* chloroquine resistance transporter

Pfmdr1: *P. falciparum* multidrug resistance gene 1

pLDH: parasite lactate dehydrogenase

PQ: piperaquine

PYR: pyrimethamine
RBC: red blood cells
RDT: rapid diagnostic test
RDT: rapid diagnostic test
sHz: synthetic haemozoin
sLS: standard lysis solution
SP: sulfadoxine-pyrimethamine
UI: uninfected
uiRBC: uninfected red blood cells
WHO: World Health Organization

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I – Introduction

1. Malaria

1.1. History and epidemiology of the disease

Malaria has been noted since ancient history: it is believed to be the main cause of death among primates previous to *Homo sapiens*. The Italian writers from the fourteenth century were the ones naming it “malaria”, meaning “bad air” – since they believed it derived from the vapours emanating from the Tiberian swamps. But this disease is still the most relevant human parasitic infection in the world, being a public health concern to this day. [1-5]

According to the World Health Organization (WHO), although the number of estimated cases decreased 18% already since 2000, there were 214 million worldwide last year – almost 90% of them in the African region, followed by South-East Asia. Just in 2015, there were 438 000 estimated malaria deaths, 90% of those being in Africa – 292 000 children under 5. [6]

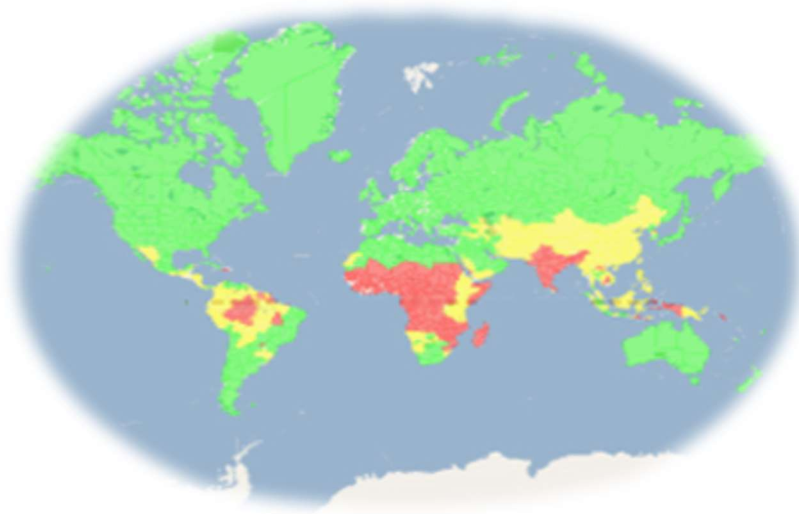

Figure 1 – Malaria transmission throughout the world. In green areas, transmission is not known to occur; in yellow areas, malaria transmission occurs in some parts, and in red areas it occurs throughout. Adapted from CDC.

Most of these statistics may be underestimations due to the fact not only there is no effective control of malaria-endemic countries’ population – births and deaths are often not even reported, let along the causes of death – but also due to the indirect effects malaria has on other aspects of health, such as nutrition and other infections; Nicholas White (in *Manson’s Tropical Diseases*) goes even further and states around 5% of the world’s population is infected with this disease and an astonishing number of more than 600 000 deaths are caused by it each year. [1]

1.2. Cause of disease and life cycle of the parasite

Malaria is caused by an infection with the protozoan *Plasmodium*, which is transmitted by the bite of a female mosquito from the genus *Anopheles*. The infection in humans is caused by one or more of the species *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi*, the first being the cause for almost all malaria deaths and severe disease. [1,2,4,9]

All species of human malaria parasites have a complex life cycle which involves a human host and a mosquito vector – the sporogonic cycle occurs in the mosquito's gut while the pre-erythrocytic (or hepatic) and erythrocytic cycles occur in the human liver cells and human red blood cells, respectively. [1,2,4,9]

The infection starts when a mosquito injects sporozoites into the bloodstream, while taking up a blood meal, which target the liver. There, they infect the hepatocytes and replicate, giving rise to schizonts, containing several merozoites. Schizonts then rupture and release the merozoites to the bloodstream, which infect red blood cells and undergo asexual reproduction. [1,2,4,9] Figure 2 illustrates the blood stage of the parasite's life cycle.

To note that in the case of the erythrocytic cycle of *P. falciparum*, parasites cause red blood cells to exhibit an antigen – *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) – that makes them attach to the vascular endothelium and disappear from the bloodstream – this process is called sequestration. The expression of this antigen increases with the parasite's development and so the more developed forms of *P. falciparum* are not found in the bloodstream, contrary to what happens in other human malaria species. [1]

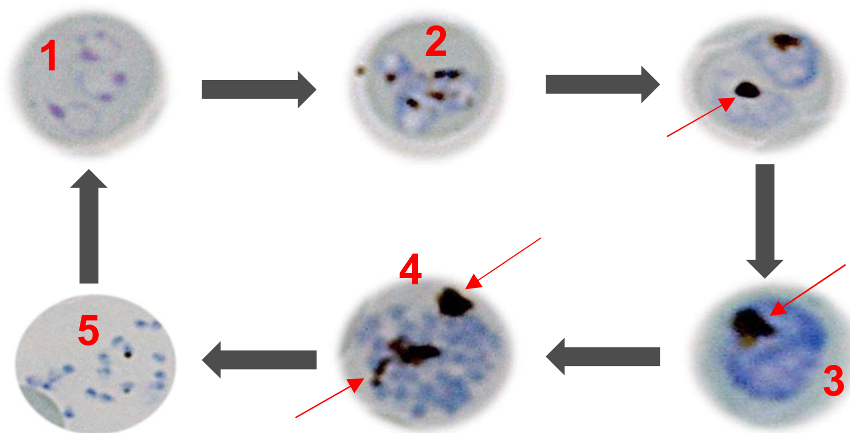

Figure 2 – Maturation of *Plasmodium falciparum* during the blood stage of the infection. When merozoites reach the blood stream, they infect the host red blood cells and undergo asexual reproduction. They develop from ring-form trophozoites (1) and then mature (2), and turn into schizonts (3), with increasing amounts of pigment (red arrows). These rupture the red blood cells (4), releasing the merozoites (5), some of which invade new red blood cells, while others develop into gametocytes – which might be ingested by another feeding mosquito. In *P. falciparum*, the erythrocytic cycle takes around 48 hours. Pictures obtained with a Leica DM2500 microscope.

1.3. The malarial pigment

During the blood stage of the parasite's life cycle a dark pigment called haemozoin (Hz) is formed in the parasite's digestive vacuole. [1,4,8,9,10] This pigment is not exclusive to malaria as other parasites – such as *Haemoproteus* and *Schistosoma* – also synthesize haemozoin. [7,12] Reports of this pigment are even more ancient than the study of malaria itself, since it has been used to detect the disease for a very long time. [1,4,8,10]

As early as in 1717, the Italian physician Giovanni Lancisi was the first to report the presence of a black pigment in the brain and spleen of patients who seem to have died from malaria; in 1847, German anatomist Johan Meckel, while performing a necropsy on a patient who had died in a mental hospital, noted a dark pigment in the patient's blood and internal organs – especially liver and spleen – which he described as being melanin. [1,8,10]

Although this term was wrongly attributed for many years, the malarial pigment aided scientists in making important discoveries regarding the cause for malaria and the life cycle of *Plasmodium*. German physician Rudolf Virchow made the connection between the presence of pigment and malaria and French army surgeon Charles Laveran determined the cause for malaria was a parasite when he found pigmented flagellated bodies in a malaria patient; by 1895, Scottish physician in the Indian Medical Service Ronald Ross had discovered the connection between malaria and mosquitoes, by following the pigment in order to find malaria parasites. [1,2,4,5,13]

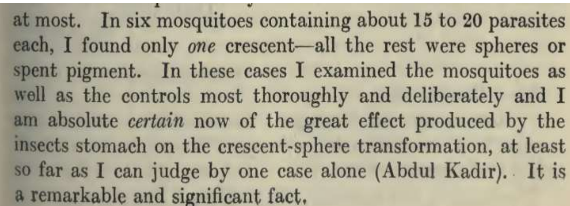
at most. In six mosquitoes containing about 15 to 20 parasites each, I found only *one* crescent—all the rest were spheres or spent pigment. In these cases I examined the mosquitoes as well as the controls most thoroughly and deliberately and I am absolute *certain* now of the great effect produced by the insects stomach on the crescent-sphere transformation, at least so far as I can judge by one case alone (Abdul Kadir). It is a remarkable and significant fact.

Figure 3 – Excerpt from *Memoirs with a full account of the great malaria problem and its solution*, by Ronald Ross.

Despite all these achievements, it was only in 1911 American physician W. H. Brown discovered the origin of this pigment's colour was haem and not melanin; in 1987 Fitch and Kanjananggulpa also showed the pigment was an insoluble aggregate of haematin (ferric form of haem) – also known as ferroprotoporphyrin IX (FPPIX) – which gives it its dark colour. [14,15]

Since then, several studies were made on the molecular structure and chemical composition of haemozoin; Fitch and Kanjananggulpa (1987) stated haem aggregation may involve several types of cross-links such as µ-oxo bridges or anhydride bonds; Slater and colleagues (1991) determined the chemical composition of Hz was similar to that of haematin and its structure to that of haem and concluded haem units are linked by an iron-carboxylate bond. [15, 16]

Bohle's (1997) and Pagola's (2000) teams enlightened the molecular structure of Hz as a microcrystalline cyclic dimer of ferric haem (haematin) – where the iron of each haematin is chelated onto the carboxyl side chain of the next, held in a matrix by hydrogen bonding interactions (see Figure 4). [17,18]

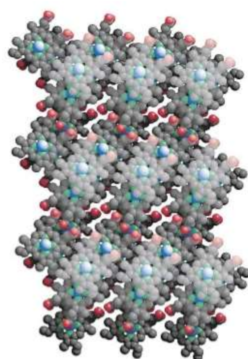

Figure 4 – Crystal structure of haemozoin proposed by Pagola *et al.* (2000). Carbon (C) atoms are represented in grey, oxygen (O) in red, nitrogen (N) in green and iron (Fe) in blue; hydrogen (H) atoms are not shown. Source: Pagola *et al.* (2000)

After this development, Egan and colleagues (2001) then concluded haem was not a polymer as previously stated by many authors and thus the process of its aggregation should be noted as one of crystallization instead of polymerisation; the scientists also demonstrated this process consists firstly of a precipitation of haematin via its dissolution and then of its conversion to haemozoin via reprecipitation. [19]

Several hypotheses were pursued for the process of Hz formation; since haem was thought to be a polymer, haem "polymerisation" was thought by many to be catalysed by an enzyme named "haem polymerase" (Slater *et al.*, 1991; Slater & Cerami, 1992 and others), others thought it to be a spontaneous reaction (Egan *et al.*, 1994) or to be auto-catalysed (Dorn *et al.*, 1995); lipids and histidine-rich proteins (HRPs) were also implicated in this process by several authors. [16,20,21,22,29]

Bendrat and others (1995) claimed phospholipids promoted haem "polymerisation" and might play a role in Hz formation, but Sullivan and co-workers (1996) hypothesised a role for the histidine-rich protein II (HRP II), by binding haem units although they could not conclude about the specific function of the HRP being of enzymatic or nucleation nature. [23,24]

Other authors had alternative theories for the detoxification of haem, which would not lead to haemozoin formation: Ginsburg *et al.* (1998) suggested haem exits the food vacuole and is subsequently degraded by glutathione and Loria and colleagues (1999) suggested haem would react with H_2O_2, destroying both toxic molecules at once. [25,26]

Papalexis *et al.* (2001) showed HRPII indeed facilitates haem degradation by reaction with H_2O_2 and incubation with glutathione induces a rapid decrease in haem absorbance; however, these pathways play a minor role in haem detoxification, being haemozoin formation the main pathway for this purpose. [27]

Campanale *et al.* (2003) identified several FPPIX-binding proteins the parasite uses for protection against it [28] and Huy *et al.* (2003) suggested HRPII may play an important role in the neutralization of toxic haem, by removing haem from haem-bound membranes, hence chelating it. [29]

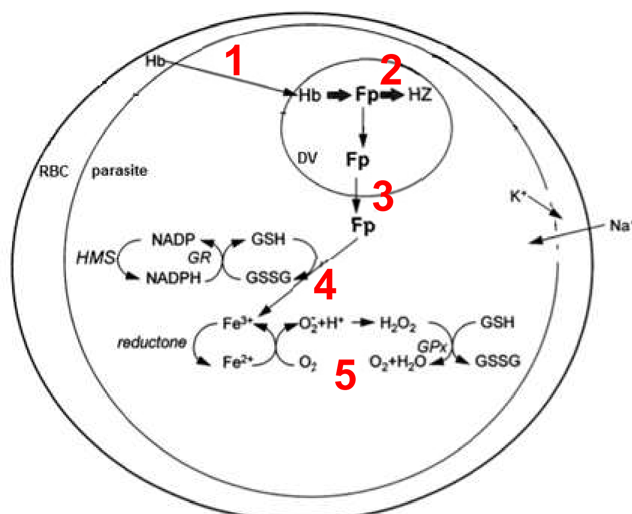

Figure 5 – The glutathione-dependent mechanism of haem detoxification proposed by Ginsburg *et al.* (1998). The parasite ingests haemoglobin (Hb) from the host cell (1), which is digested inside the parasite's digestive vacuole (DV); there, haem (Fp) is converted into haemozoin (HZ) (2) and the resulting free-haem exits to the cytosol (3), where it is degraded by glutathione (GSH) (4). In this process, iron is released, which then enters the redox cycle (5). Adapted from Ginsburg *et al.* (1998).

Dorn and others (1998) concluded both the oxidation state, the pH and the equilibrium of haematin between its monomeric and μ-oxo dimeric forms were main factors in the formation of Hz; they also demonstrated haematin would "polymerise" even in the absence of proteins and this was promoted by lipids, where they would catalyse it by shifting the equilibrium to the haematin monomeric form. [30] Fitch and colleagues (1999) determined that lipids indeed played a part in this process, by co-precipitating with monomeric FPPIX, dissolving it and allowing it to "polymerise". [31]

The involvement of lipids was later also demonstrated by other teams of scientists – Jackson *et al*. (2004) proposed that products from phospholipid breakdown are assembled into triacylglycerols and these serve as important catalysts of haemozoin formation; [32] Egan and co-workers (2006) determined haemozoin formation occurs near lipid-water interfaces. [33]

Pisciotta and colleagues (2007) concluded neutral lipid nanospheres accumulate free haem and promote haemozoin formation at the surface interface, where acidic conditions favour lipid-mediated crystallization; [34] Ambele & Egan (2012) also showed neutral lipids mediate haematin formation. [35]

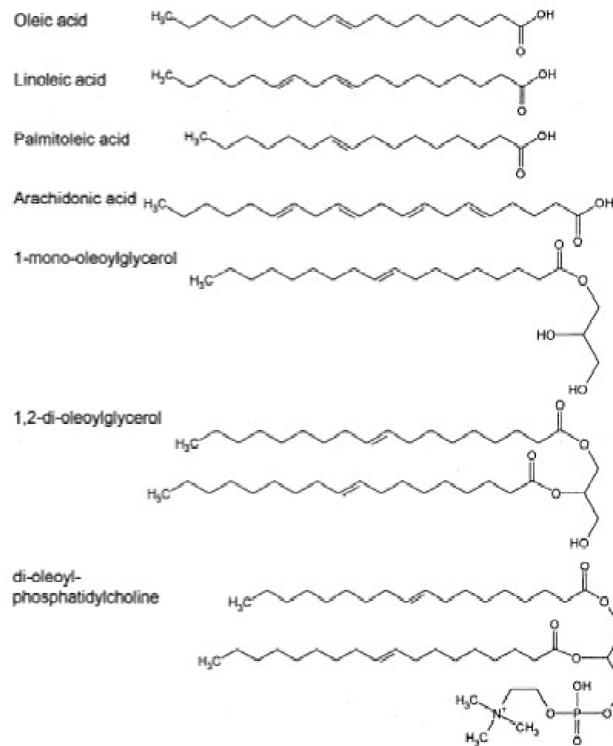

Figure 6 – Lipids that have shown to promote haemozoin formation. Source: Egan (2002)

Jani and colleagues (2008) conducted a study on several malaria parasite proteins and proposed the existence of another way the parasite might detoxify free haem: through a Haem Detoxification Protein (HDP), which would be more potent in catalysing Hz formation than HRPs and lipids; [36] and more recently, Kapishnikov *et al.* (2012) have shown Hz crystals nucleate in an oriented manner at the inner surface of the parasite's digestive vacuole, hence growing within the aqueous phase. [37]

Although haemozoin formation was intensely studied, the mechanism through which haem suffers a process of biocrystallisation is still not completely understood; however, it is now clear this crystal originates in the parasite's digestive vacuole, during the intra-erythrocytic stage of its life cycle. When in the red blood cell, the parasite digests most of the host's haemoglobin and this proteolysis releases reduced haem and globin; while globin is digested by enzymes, the reduced haem is extremely reactive and thus readily oxidized to its ferric form haematin, with consequent production of H_2O_2, a potentially very toxic molecule. [8,10,11,19,38-40,48-50]

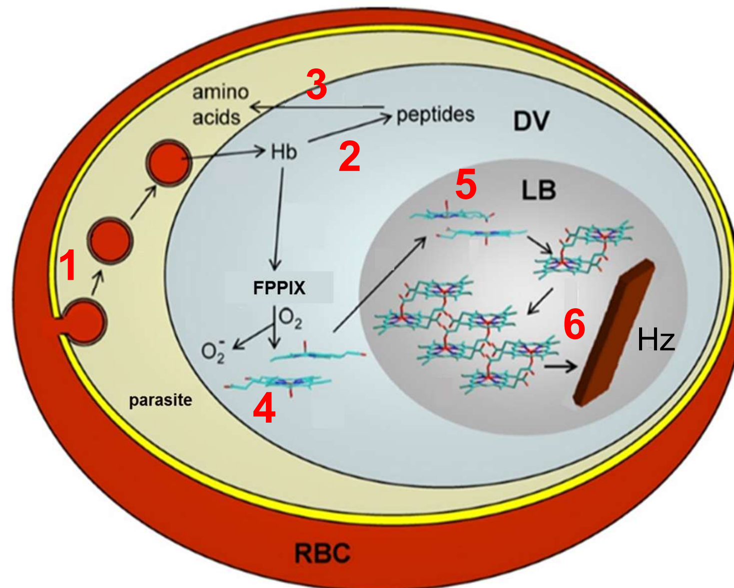

Figure 7 – Schematic representation of haemozoin formation in *P. falciparum* based on several studies. The parasite ingests cytoplasm from the red blood cell (RBC) (1) into the digestive vacuole (DV); there, haemoglobin (Hb) is digested into peptides (2), which are exported and degraded into amino acids (3). Haem (FPPIX) released from Hb is oxidised, generating Fe(III)PPIX and superoxide (4). The oxidised form of FPPIX forms a dimer, which is delivered to the lipid body (LB), also called the lipid nanosphere (5); there, Fe(III)PPIX forms a haemozoin precursor, eventually forming the haemozoin crystal (Hz) (6). Adapted from Egan (2008).

Since haem can cause the disruption of biological membranes, inhibit enzymatic processes, and initiate the chain of oxidative metabolism it might also be potentially toxic to the parasite. As malaria parasites lack haem oxygenase (an enzyme which catalyses haem degradation in all vertebrates), they are unable to cleave haem and thus it is not excreted from the cell. [8-11,17,19]

To prevent its accumulation and bypass said toxicity, the parasite must maintain it as an insoluble compound and thus readily transforms free haem into haemozoin via a process of biocrystallisation. [8-11,17-19] Egan and others (2002) had demonstrated more than 95% of haem iron released from host haemoglobin went into the production of haemozoin – which thus represented the main pathway through which the parasite detoxifies free haem. [38]

Until recently, the digestion of haemoglobin was thought to be merely a way for the parasites to obtain amino acids and iron, since they are not able to synthesise their own. Goldberg and colleagues (1990) even stated malaria parasites obtain most of the nutrients directly from the digestion of haemoglobin. [41] But haemoglobin is known to be a poor source of several important amino acids such as methionine, cysteine and glutamine and even lacks isoleucine; so, parasites must have another reason to degrade haemoglobin other than mere nutrition – this had even been demonstrated by Zarchin and colleagues (1986), by growing parasites in a culture medium with the 20 essential amino acids and noticing they still degraded haemoglobin. [42]

However, several studies showed the parasite's development ceased if proteolysis of haemoglobin had been blocked, even if the parasite had all necessary nutrients available. Also, with the parasite's growth and development the proteolysis of haemoglobin increases but not the amount of parasite protein – according to Krugliak and co-workers (2002), the parasite only utilises around 16% of this proteolysis to synthesise its proteins. [43]

Ginsburg (1990) showed the purpose of haemoglobin digestion was rather preventing the early red blood cells' lysis, which would occur if the parasite did not counterbalance the increase in cell volume; [44] later Esposito and colleagues (2008) supported this hypothesis with mathematical models which fit the experimental data. [45] It is clear now the parasite's development blocks with the ceasing of haemoglobin proteolysis because its food vacuole fills up with the undegraded haemoglobin, leaving no room for it to grow. [17,47-50]

The presence of haemozoin in a malaria parasite marks the evolution of a young-trophozoite (ring-form) with almost no pigment to a densely pigmented schizont. This means the amount of pigment inside a parasite increases with its development within the host's red blood cell – being the erythrocytic schizont the more pigmented asexual form. [8-11,17,47-50]

Therefore, haemozoin might be utilised as an indirect maturation indicator for the parasite itself. This means when haemozoin is being detected for purposes of studying parasite development in certain conditions, parasite maturation might be assessed as well, in order to study the parasite's susceptibility to certain antimalarial drugs. [8-11,46-50]

2. Antimalarials

2.1. History of antimalarials

Antimalarial drugs have usually come onto clinical use slowly; however, malaria has been treated with natural medicines for over a thousand years. The Qinghao plant – *Artemisia annua* – was described in a medical treaty during the second century BC and in the Handbook of Emergency Treatment in 340 AD as infusions to treat fever. [1,2,52-55]

This plant was frequently mentioned as a treatment for agues – the typical malaria fevers – and traditionally used in medical practice in China. The active ingredient, now known as artemisinin, was isolated by Chinese scientists in 1971 and its antimalarial properties were then rediscovered. Several derivatives of artemisinin were developed, which are now very effective antimalarial drugs. [1,2,52-55]

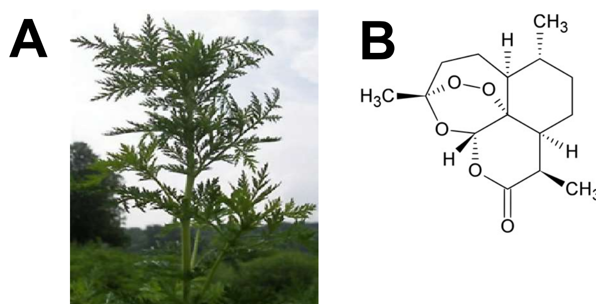

Figure 8 – *Artemisia annua* plant (A), from where the antimalarial artemisinin (B) was isolated. Sources: Miller & Su (2011) and PubChem (CID 20105830).

On the other side of the world, in Peru, another plant caught attention from physicians – the *Cinchona officinalis*; its bark would contain a medicine for the fever. Once the Countess of Chinchon took this natural medicine for her fevers and got immediate results, she requested large amounts of such bark to be sent to her and then gave it to the poor; later, Linnaeus named the tree from which the bark was obtained 'Cinchona' in honour of the Countess. In 1820, the French chemists Pierre Pelletier and Joseph Caventou isolated the active ingredient from the bark, which is now known as quinine. [1,2,54]

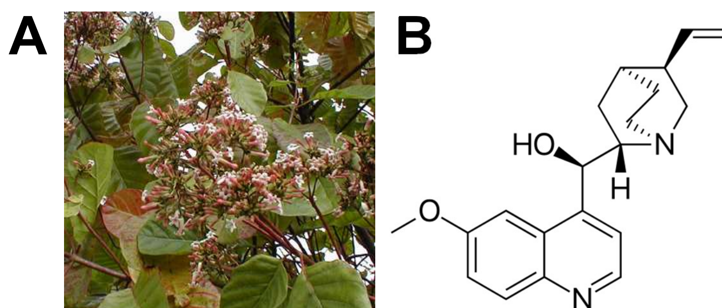

Figure 9 – Plant *Cinchona officinalis* (A), from where the antimalarial quinine (B) originates. Source: US National Library of Medicine, National Institutes of Health, and PubChem (CID 3034034).

Later in Germany, the antimicrobial properties of the newly discovered aniline dyes were investigated, and in 1891, Guttmann and Ehrlich reported the antimalarial properties of what would be the first synthetic antimalarial drug, the thiazine dye methylene blue. The scientists knew this dye would stain malaria parasites and had been used therapeutically in neuralgias; they hence used it on two malaria patients. After this treatment, the patients' fever decreased and parasites cleared from their blood. [1,51]

Armies fighting in tropical theatres of war usually lost more men to malaria than bullets; thus, traditionally, research about antimalarial drugs was promoted in case of war in endemic countries. During World War I, whole armies were immobilized in the Balkans because of malaria and the British and French armies used quinine extensively, so many lives were saved. [1,2,54]

Figure 10 – War-time propaganda against malaria transmission. Source: Marin/Sonoma Mosquito and Vector Control District, in http://www.msmosquito.com/.

After the end of the war, research on antimalarials remerged, and led to attempts to combine the chemical properties of quinine and methylene blue and use these drugs as structural prototypes. Subsequently, an 8-aminoquinoline and a 9-aminoacridine were developed, named pamaquine (also known as plasmoquine) and quinacrine (or mepacrine), respectively. [1,2,54]

Later in 1934, a German scientist from Bayer laboratories developed the structurally related 4-aminoquinoline – and what would be one of the most widely used antimalarial drugs – chloroquine. This drug was initially rejected due to its toxicity and a safer compound was developed, a 3-methylchloroquine named sontoquine. [1,2,54]

Nevertheless, World War II came and, despite clinical studies, these compounds were generally unavailable at its outbreak. Chloroquine was finally recognized and established as a safe effective antimalarial in 1946 by British and American scientists, and later developed into other extensively used antimalarial drugs, amodiaquine and primaquine. [1,2,54]

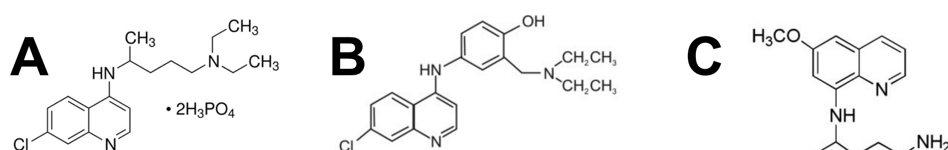

Figure 11 – Molecular structure of chloroquine (A), amodiaquine (B) and primaquine (C). Source: PubChem; CIDs: 2719, 2165, and 4908, respectively.

During WWII, several other drugs were also developed: a different line of research by British scientists led to the development of dihydrofolate-reductase inhibitors (DHRI), such as proguanil in 1945 and pyrimethamine in 1952, for instance. These drugs were generally used in combination with sulfa drugs, like dapsone, sulfadoxine and others. [1,2,54]

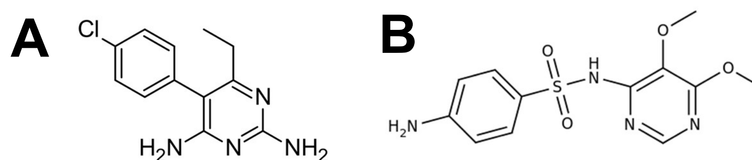

Figure 12 - Molecular structure of pyrimethamine (A) and sulfadoxine (B). Source: PubChem; CIDs: 4993 and 17134, respectively.

In the decade of 1950, the 4-aminoquinolines chloroquine and amodiaquine, and the DHRI pyrimethamine had become widely used for treatment of malaria around the world. Chloroquine, pyrimethamine, and proguanil were used for prophylaxis; primaquine was given to prevent relapses of *P. vivax* and *P. ovale*; quinine and its derivatives were not very used outside of Francophone Africa. [1,2,54]

This, together with the great advent of vector control, made it the highpoint of malaria eradication; the disease disappeared from Europe – in Portugal, the last malaria case was in 1959 – and North America, and even from some areas of the Tropics. This led to a disinterest in continuing research on antimalarial drugs. However, there was no complete eradication in the tropics and in the '60s antimalarial drug resistance emerged; these, together with the Vietnam conflict, made the USA Army lead an extensive research to discover new antimalarials. [1,2,54]

Around 2 millions of compounds were tested, most of them related to the existent quinolones, such as mefloquine and halofantrine. Some antimalarial drugs were developed by the Chinese and later commercialised in the Western World, such as lumefantrine, pyronaridine, and piperaquine. [1,2,54]

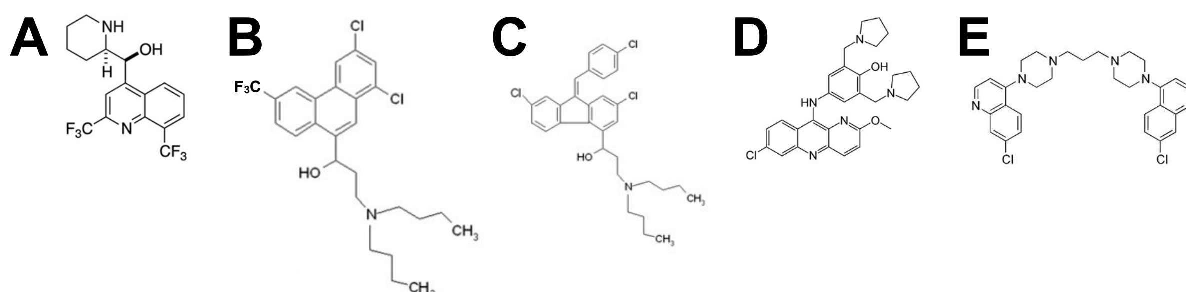

Figure 13 – Molecular structure of mefloquine (A), halofantrine (B), lumefantrine (C), pyronaridine (D), and piperaquine (E). Source: PubChem; CIDs: 40692, 37393, 6437380, 5485198, and 122262, respectively.

Around this time, the hydroxy-naphtha-quinone compound atovaquone arose from a modification on a former compound and together with proguanil formed Malarone^{TM}; this became a very well-known antimalarial because of its action against all stages of malaria parasites and highly

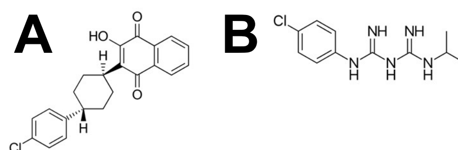

Figure 14 – Molecular structure of the constituents of Malarone^{TM}, atovaquone (A) and proguanil (B). Source: PubChem; CID: 11954242.

effectiveness (atovaquone is not usually used as monotherapy due to the propensity for emergence of high-grade resistance), though very expensive to manufacture. [1,2,54]

In recent years, artemisinin was developed into several derivatives, such as artemether and artesunate and, more recently, dihydroartemisinin. These were widely used in China in the 1980s and when multidrug-resistant *P. falciparum* strains emerged, the Western world became more and more interested in them. By the early 1990s, artemisinin derivatives were being widely used across South-East Asia and they are now the most effective and non-problematic resistance-wise antimalarials known. [52-55]

The rediscovery and development of drugs related to artemisinin is by far the most important development in malaria treatment; besides being unrelated to existing antimalarial drugs, which avoids cross-resistance, they are rapidly effective, well tolerated and safe. Because of their great amount of

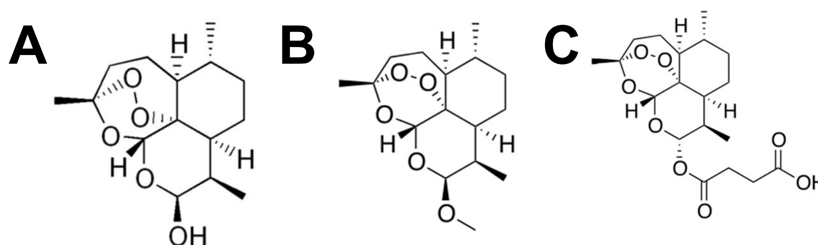

Figure 15 – Molecular structures of the artemisinin derivatives dihydroartemisinin (A), artemether (B), and artesunate (C). Source: PubChem; CIDs: 6918483, 6450800, and 156252, respectively.

advantages, there have been more clinical trials on artemisinin and its derivatives than on any other antimalarials. [52-55]

There are, however, some suspicions on emergent artemisinins resistance, although not confirmed. Hence, for the past years, there was an increased funding and effort to find alternatives to the currently used drugs, in case ineffectiveness arises. [59,68]

This revival of research and development of new antimalarials led to several current studies on new unrelated antimalarials; examples of these are the spiroindolone KAE609 (formerly known as NITD609) and the imidazolopiperazine KAF156, which both had considerable good success in *P. falciparum* and *P. vivax* malaria. [56,58]

Spiroindolones inhibit PfATP4, a parasite plasma membrane Na^{+}-ATPase that regulates sodium and osmotic homeostasis. It was known KAE609 has potent activity against both the asexual and sexual stages of the malaria parasite in vitro; thus, White and colleagues (2014) conducted a phase II study in Thailand with KAE609 in order to assess the initial antimalarial efficacy – in terms of parasite clearance –, safety, and adverse-event profile at a certain daily dose in uncomplicated *P. falciparum* or *P. vivax* malaria patients. [56]

The authors noted KAE609 showed rapid efficacy in the treatment of *P. falciparum* and *P. vivax* malaria, and may even have a faster parasite clearance than artemisinins – which are the most rapidly acting antimalarial agents identified to this date; however, this study was too small to draw conclusions in regards of safety and efficacy of the drug. [56]

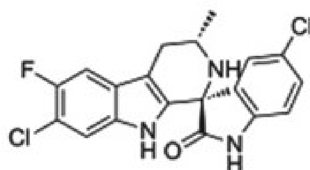

Figure 16 – Molecular structure of KAE609. Source: PubChem; CID: 44469321

Imidazolopiperazines show activity against blood and liver stages of malaria parasites. White and others (2016) conducted a phase II study in Thailand and Vietnam to assess the efficacy, safety, and pharmacokinetic profile of KAF156 in uncomplicated *P. falciparum* or *P. vivax* malaria patients. This antimalarial showed to resolve the illness and rapidly clear parasites from the blood in patients with both malaria species. Although the parasite clearance was slower than those of artemisinins and KAE609, it was still faster than those of sulfadoxine-pyrimethamine, atovaquone-proguanil, quinine, or mefloquine. [58]

Usually, antimalarials with rapid elimination half-life (less than 3 days) cannot cure *P. falciparum* malaria in a single dose; however, the authors noted KAF156 had significant *in vivo* potency, even comparing to that of artemisinin derivatives. The therapeutic responses to KAF156 were similar in patients with mutations associated with resistance and this suggests this antimalarial would be effective even against infections resistant to all currently available antimalarials. [58]

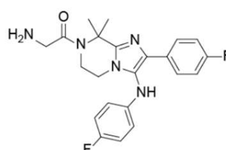

Figure 17 – Molecular structure of KAF156. Source: Flannery *et al.* 2013

2.2. Currently used antimalarials

Malaria is generally divided into uncomplicated or severe disease and according to the *Plasmodium* species when it comes to deciding appropriate treatment. *Plasmodium falciparum* is the usual cause for severe disease due to its ability to cause higher parasitaemias and accumulate in tissues such as the brain. This is thought by some scientists to cause cerebral malaria and consequently coma and death if not treated immediately. [1,2,62]

In the meanwhile, certain signs and symptoms will be a warning for severe disease. According to the WHO, severe malaria can be defined as one or more of a set of signs and symptoms in the presence of asexual hyperparasitaemia (>10% for *P. falciparum*); examples are impaired consciousness, prostration, and multiple convulsions (see Table A.1, Appendix A for more detail). [62]

Malaria treatment has been a fast-developing subject; not very long ago, the standard treatment was 4-aminoquinolines and sulfa compounds and less than 20 years ago these started to fail due to

emergent resistance. Today, after much delay in gaining recognition from the Western countries, the standard treatment recommended by the WHO in most cases includes an artemisinin-based combination therapy (ACT). [59-62]

An ACT is a combination of a rapidly acting artemisinin derivative with a longer-acting partner drug with a different mode of action. The artemisinin derivative rapidly clears most of the parasites from the blood and is also active against the sexual stages of the parasite – meaning it prevents further transmission to mosquitoes and consequently to other humans. The partner drug clears remaining parasites from the blood and provides protection against development of resistance to the artemisinin derivative, while also acting as post-treatment prophylaxis. [62]

Table 1 – Currently recommended antimalarial drugs for the treatment of falciparum and non-falciparum malaria, according to the WHO. Based on Visser *et al.* (2014), Cui *et al.* (2015) and the WHO Malaria Treatment Guidelines (2015).

Class	Drug	Use	Mode of action
4-aminoquinolines	Chloroquine	Treatment of non-falciparum malaria	Uncertain; chloroquine inhibits haem aggregation by binding to FPPIX and also inhibiting glutathione-mediated haem degradation.
	Amodiaquine	Partner drug for artesunate	
	Piperaquine	Partner drug for DHA	
8-aminoquinolines	Primaquine	Radical cure and terminal prophylaxis of *P. vivax* and *P. ovale*	
Aryl-aminoalcohols	Quinine	Treatment of severe falciparum malaria	
	Mefloquine	Partner drug for artesunate	
	Lumefantrine	Partner drug for artemether	
Naphthoquinone	Atovaquone	In combination with proguanil for prophylaxis and treatment of *P. falciparum*	Interference with mitochondrial electron transport; depolarization of mitochondria and consequent blocking cellular respiration.
Antifolates	Pyrimethamine/sulfadoxine	Partner drug for artesunate	Interference with folic acid synthesis, by inhibition of dihydrofolate reductase (pyrimethamine) and inhibition of dihydropteroate synthase (sulfadoxine).
	Proguanil	In combination with atovaquone, for prophylaxis and treatment of *P. falciparum*	Interference with folic acid synthesis, by inhibition of dihydrofolate reductase.
Mannich base	Pyronaridine	Partner drug for artesunate	Uncertain; Initially thought to involve cation (mainly the ferrous ion) mediated generation of carbon-centred free radicals which alkylate critical proteins.
Artemisinins	Dihydroartemisinin	ACT with piperaquine	
	Artemether	ACT with lumefantrine	
	Artesunate	ACT; treatment of severe malaria	

ACT: artemisinin-based combination therapy; DHA: dihydroartemisinin; FPPIX: haem.

If a malaria patient is unable to take oral medication reliably, shows any evidence of vital organ dysfunction or has a high parasite count, the patients is at increased risk of dying, since death from severe malaria usually occurs just within hours of admission to urgent care. Thus, treatment should consist of a highly effective and rapidly-acting antimalarial drug, such as injectable artesunate; if parenteral artesunate is not available, the next suggestion from the WHO is artemether. [62]

This applies to all adults, including pregnant and lactating women, and to all children, including infants. Once the patient has received at least 24 hours of parenteral artesunate/artemether and can tolerate oral therapy, 3 days of an ACT should be given. [62]

Any confirmed malaria patient who does not present any sign or symptom of severe disease is considered to have uncomplicated malaria, hence the purpose of treatment is to avoid the progression to severe disease and, on a more public health scope, to prevent further transmission to others and emergence of resistance to antimalarials; in such cases, the advised treatment is 3 days of an ACT. [62]

There are five possible ACTs recommended by the WHO for adults and children (excluding pregnant women in their first trimester) with uncomplicated malaria caused by *P. falciparum*: artemether with lumefantrine, artesunate with amodiaquine, artesunate with mefloquine, dihydroartemisinin with piperaquine and artesunate with sulfadoxine-pyrimethamine (SP). [62]

When uncomplicated malaria originates in any of the other species, the patient may also be treated with an ACT if in an area with chloroquine-resistant infections; in areas with chloroquine-susceptible infections, this antimalarial can also be used. Mixed malaria infections may also be treated with an ACT, since this is effective against all parasite species. Pregnant women in their first trimester should be treated with a combination of quinine and clindamycin for 7 days in case they have a *P. falciparum* infection. [62]

In case the infection is cause by *P. vivax* or *P. ovale*, the priority is also to prevent relapses after the initial treatment; for that, the WHO recommends a 14-day treatment of primaquine after the initial treatment, since this is the only antimalarial which kills the hepatic forms. In patients who also have glucose-6-phosphate dehydrogenase (G6PD) deficiency, primaquine causes severe haemolysis and so risks and benefits must be assessed. [62]

For pregnant and lactating women, treatment for prevention of relapses should consist in weekly chloroquine until delivery and breastfeeding are completed and then, depending on the G6PD status of the patient, primaquine. [62] Nevertheless, there is very little progress regarding the development of antimalarials which kill hepatic parasites as well as alternatives to primaquine. [59-62]

There are other guidelines for the treatment of malaria, such as Guidelines for Treatment of Malaria in the United States (updated in 2013) and the UK malaria treatment guidelines (2016). These have different recommendations in certain situations, also due to the fact not all antimalarials are legal everywhere in the world. [63,64]

For instance, for uncomplicated *P. falciparum* malaria in areas with chloroquine-sensitive infections, the recommendation in the USA is always chloroquine. If there is chloroquine-resistance in the region, the recommendation is atovaquone-proguanil (Malarone™), a 3-day treatment with an ACT – consisting of artemether-lumefantrine –, quinine or mefloquine. [64]

The UK malaria treatment guidelines, on the other hand, do not recommend mefloquine at all and the recommendation for uncomplicated *P. falciparum* malaria is an ACT – consisting either of artemether with lumefantrine or dihydroartemisinin with piperaquine –, Malarone™ or quinine with doxycycline. Table 2 compares in more detail the three referred guidelines in regards of the first-line treatment for malaria. [63]

Table 2 – First-line treatment for malaria in adults at no risk, according to several guidelines. Source: Malaria Treatment Guidelines from the WHO (2015), UK (2016) and USA (2013).

	Guidelines		
	WHO 2015	**UK 2016**	**USA 2013**
Uncomplicated malaria			
P. falciparum	ACT	ACT*, Malarone™ or quinine+doxycycline	Malarone™ or CQ
P. vivax* or *P. ovale	ACT/CQ+primaquine	CQ+primaquine	CQ+primaquine or quinine**
P. malariae* or *P. knowlesi	ACT or CQ	ACT or CQ	CQ
Severe disease			
P. falciparum	IV artesunate	IV artesunate/quinine***	IV quinidine+DOX/TET/CLIN
non-falciparum	Same as *P. falciparum*	Same as *P. falciparum*	Same as *P. falciparum*

*Only 2 ACTs are licenced in the UK: artemether with lumefantrine and dihydroartemisinin with piperaquine, being the first the drug of choice and the second an alternative; S-P and MQ are not recommended in the UK.
**Quinine is used in case the infection is from CQ-resistant *P. vivax*.
***IV artesunate is unlicensed in the European Union, although it is available in many centres; IV quinine is the alternative.
ACT: artemisinin-based combination therapy; CQ: chloroquine; S-P: sulfadoxine-pyrimethamine; MQ: mefloquine; IV: intravenous; QD: quinidine; DOX: doxycycline; TET: tetracycline; CLIN: clindamycin.

2.3. Resistance to antimalarials

According to the WHO, drug resistance can be defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, provided the exposure is adequate. It arises because of the selection of parasites with genetic changes (mutations or gene amplifications) that confer reduced susceptibility. [1,70,71]

Between 1970 and 2000 the number of malaria cases worldwide and the number of deaths due to malaria steadily increased. This was not a result of a deterioration of the health system in endemic countries, since the number of deaths due to many other infections decreased; many even blame it on the resistance of malaria parasites to used antimalarial drugs. [1,65]

Parasites acquire resistance through a series of different mechanisms, some of which are not yet known, but that are caused by spontaneous chromosomal point mutations or gene duplications. These events are independent of drug selection pressure – which means they do not happen because of malaria treatment – but provide an advantage in the presence of antimalarial drugs. [1,61,65,71]

There are several factors contributing to the spread of this resistance, among which are drug-use patterns, drug characteristics (pharmacokinetics), patients' characteristics, biology of the parasite (frequency of genetic changes; degree of resistance conferred by the genetic change) and others. [1,61,65,71]

P. falciparum has now developed resistance to almost all classes of clinically used antimalarial drugs, while *P. vivax* has acquired resistance to chloroquine and primaquine; the distribution of

resistance varies according to geographic areas. However, antimalarial resistance was not treated with concern until *P. falciparum* developed resistance to chloroquine almost at once in South-East Asia and South America by the end of the decade of 1950. [1,61,65,71]

This may have originated from the erroneous use of chloroquine impregnated salt in an effort of mass prophylaxis. In the years after, chloroquine-resistant *P. falciparum* spread and caused the re-emergence of *P. falciparum* in the tropics. This antimalarial was no longer effective by the beginning of the decade of 1980, when reports of resistance in East of Africa appeared. Since then, chloroquine resistance spread across Africa and today few countries in the tropics are unaffected. [1, 61,65,71]

Pyrimethamine resistance has also worsened and the synergistic combination with sulphonamides is no longer effective in much of East Asia, Southern and Central Africa and South America. Mefloquine resistance has also emerged since 1988, while sensitivity to quinine declined very gradually. [1,61,65,71]

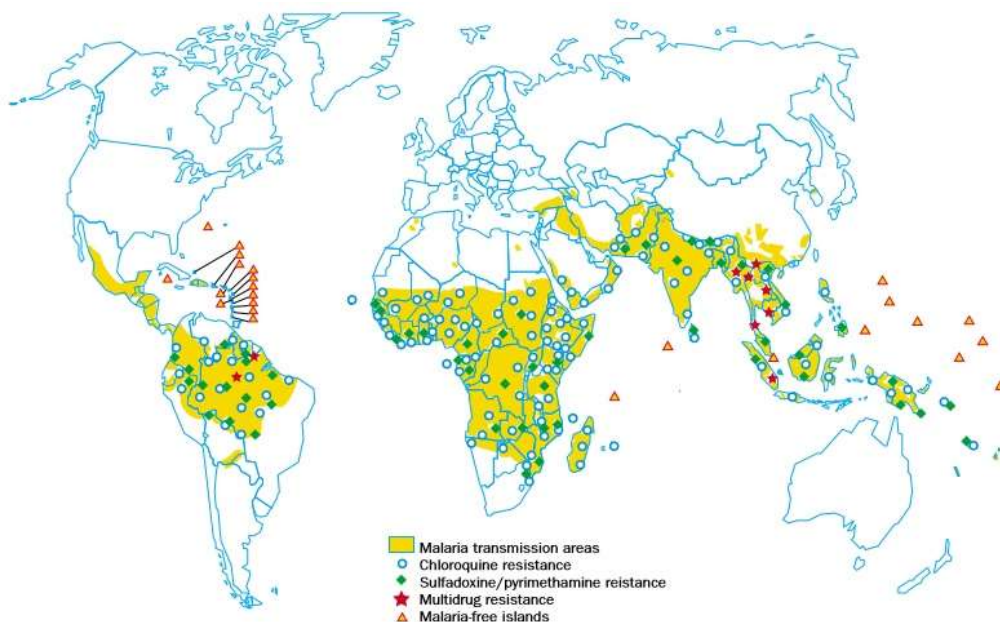

Figure 18 – World distribution of *P. falciparum* resistance, according to the WHO. Source: Wongsrichanalai *et al.* 2002.

In the past few years, resistance to the artemisinins has been a popular concern, since the parasites have shown to clear slower from the blood. This is not yet established since there are many doubts that this is in fact the beginning of artemisinins resistance because the parasites are still cleared from the blood, but at a slower rate. [1,61,65-71]

Nevertheless, the WHO claims *P. falciparum* resistance to artemisinins has been detected in Cambodia, Lao, Myanmar, Thailand and Vietnam and even that a molecular marker associated with this resistance was identified – the Kelch 13 (K13). Mutations in this marker propeller region were associated with the observed delayed parasite clearance, both *in vitro* and *in vivo*. [1,6,61,65-69,71]

However, the WHO only shows concern with the efficacy of artesunate-mefloquine in South-East Asia and South America, noting the efficacy of artesunate-SP and of DHA-PQ is only compromised in areas where there is resistance to the partner drugs (SP and PQ). The other ACTs (artesunate-amodiaquine and artemether-lumefantrine) seem to show no decrease in efficacy anywhere. [1,6,61,71]

Despite any debate on whether malaria parasites are acquiring resistance to the current first-line treatment, antimalarial drug resistance is a major public health concern. It prevents malaria control by enabling perpetuation of transmission, besides increasing morbidity and mortality due to malaria infections. [71]

While the economic costs are of difficult quantification, the development and spread of resistance to antimalarials has greatly increased the global cost of malaria control over time, given the need for continuous development of new antimalarials. Moreover, treatment failure leads to repeated consultations at health facilities for further diagnosis and treatment, resulting in lost work days and absences from school, and increased costs to the health system. [71]

This resistance phenomenon has even been implicated in the spread of malaria to new areas and its re-emergence to where it was previously eradicated. [61,65,71] It is hence of high importance to detect this resistance beforehand as to prevent the further spread of malaria and consequent increased mortality. For that, knowledge of the parasite's susceptibility to currently used antimalarial drugs is essential in deciding appropriate treatment and establishing adequate therapeutic guidelines.

3. Sensitivity tests

3.1. Currently used sensitivity tests

Sensitivity tests are used to assess the susceptibility of parasites to antimalarials. The currently available *in vitro* sensitivity assays can be divided into genotypic – molecular techniques – and phenotypic; the latter are grouped according to the method used to assess parasite growth – direct visual parasite count by microscopy, incorporation of radioisotopes or non-radioactive methods. The latter includes assays with a flow cytometer, fluorometric assays, and ELISA-based methods. [65,71,73,74]

In general terms, the parasites are mixed with growth medium with known concentrations of the studied drug and their growth is assessed in comparison to a drug-free control. Parasite density, haematocrit and development stage are important influencing factors regarding the outcome of these tests; therefore it is important to control these parameters. [73,74]

Extensive description is made below, but Table 3 summarises the currently most commonly used *in vitro* sensitivity assays for *P. falciparum* and their advantages and limitations. All these approaches evaluate intrinsic sensitivity of malaria parasites by directly exposing them to drugs in culture plates and measuring their effect on the growth and development of the parasites; this allows direct and quantitative evaluation of actual resistance of the parasite to the drug.

There are also *in vivo*-*in vitro* (also called bioassays), where the tested compound is administered to healthy vertebrates, their blood is extracted at several timepoints, and *in vitro* assays are performed, based on continuous culture of reference strains of *P. falciparum*. Contrary to *in vitro* sensitivity assays, bioassays are used to test the absorption efficiency of an antimalarial in an animal host and the effects of metabolites – although these cannot be examined separately. [73,74]

The vertebrate host to which a test compound is administered is usually a rabbit, as their serum is compatible with human red blood cells and supports the *in vitro* growth of *P. falciparum*; other larger animals might also be used, such as monkeys. Rabbits are easier to acquire and are commonly used for medical purposes. If the tested drug is clinically approved, serum can be obtained from human volunteers. [74]

Bioassays produce results with less clear scientific value than those attained from other sensitivity assays and, in some authors' opinion, the benefits obtained might not even be worth the harm done to animals. The drawbacks are in high number, which makes these assays increasingly less performed.

It is ethically questionable to administer experimental drugs to larger mammals (as monkeys, for instance) without previous complete toxicological studies in smaller laboratory animals (as mice); this leads to only a few, selected drugs being possible to study in bioassays. Additionally, maintaining larger animals just for the purpose of studying antimalarial drugs is highly expensive and requires great technical support – being even illegal in some countries if a protected animal species is involved.

The pharmacokinetics of the tested drug might even differ from monkeys to humans, which makes it extremely difficult to extrapolate results; this biological variation also hinders the standardization of the bioassays protocols. Nevertheless, these assays have provided important insight

into pharmacodynamic interactions when used to estimate the plasma concentrations of clinically approved drugs and to evaluate the antimalarial activity of the drug and its metabolites in human. [74]

The use of bioassays for indirect measurement of plasma drug concentrations in healthy humans receiving prophylaxis or in healthy volunteers to whom a drug is administered for pharmacological studies might be a simple, affordable alternative to HPLC – which is the current "gold standard" for qualitative and quantitative determination of drug concentrations. [74]

Bioassays might also be performed with the WHO microtest protocol in the field; a small volume of fingerprick capillary blood is obtained from healthy volunteers receiving prophylaxis and the inhibitory effect of serially diluted plasma against laboratory-adapted *P. falciparum* strains is determined. [74]

Several studies were made comparing bioassays with HLPC and the conclusion drawn is the two methods yielded similar results and that bioassays were more sensitive than HPLC. This type of assays might be a useful complement in the field, as so to distinguish different causes for prophylactic failures: drug resistance – implying an adequate plasma drug concentration –, or other causes associated with low plasma concentration – poor compliance, poor absorption, or inadequate hepatic biotransformation. [74]

Sensitivity assays might also be performed *ex vivo*, where a clinically approved drug is administered to malaria-infected patients; blood samples are collected at several timepoints as in bioassays, but to instead obtain infected red blood cells previously exposed to the antimalarial drug. The serial samples are then cultured *in vitro* in order to assess their capability of maturation after drug exposure *in vivo*. [74]

These assays allow the evaluation of stage-specificity of an antimalarial drug, the rapidity of its action and pharmacodynamics in malaria patients under therapy. The results obtained from these assays contribute for the understanding of the mode of action of the tested drugs, being extremely relevant to clinical pharmacology.

Since this procedure requires serial collection of blood samples, it must be performed in a hospital setting or in a hospital-adjacent laboratory. Moreover, as mentioned before, only clinically approved drugs can be assessed through *ex vivo* assays. [74]

In spite of the great amount of sensitivity assays for *P. falciparum*, there are still no guidelines for antimalarials sensitivity testing nor is there an official reference procedure; there are, however, some "favourites" and most commonly used – the WHO microtest and the [^{3}H]-hypoxanthine isotopic assays (see Table 3). This makes it necessary to explore new methodologies feasible in any situation and applicable in every context to draw inhibition profiles for existing antimalarials and to screen antimalarial candidates.

Table 3 – Available *in vitro* sensitivity tests for *P. falciparum* and their advantages and limitations. Based on Noedl *et al.* (2003) and Basco for the WHO (2007).

Assay	Approach	Advantages	Limitations
Phenotypic			
Microscopy (WHO microtest)	Schizont maturation is assessed by counting schizont number total parasite number on thick films prepared from the cellular layer of the cultured samples.	Reflects true resistance; allows multiple tests and to test several drugs; simple to perform; little technical equipment; requires low parasite densities.	Labour-intensive; highly trained personnel.
[^{3}H]-hypoxanthine isotopic assays	Incorporation of radiolabelled DNA precursors in the parasites nucleic acids and measurement of metabolic activity.	Rapid and automated; highly sensitive and accurate; considered the "gold standard".	Use of radioactive material; expensive equipment; long incubation period (42h).
Flow cytometry	Detection of infected red blood cells by measuring DNA content, using DNA-specific fluorochromes.	Rapid and automated; highly sensitive and accurate.	Expensive equipment; highly specialized personnel.
Fluorometric assays	Same as in flow cytometry but measurement is made with a minifluorometer, a fluorescence spectrophotometer or a fluorescence-activated microplate reader.	Less expensive than flow cytometry.	Less sensitive than flow cytometry; longer incubation period.
Antigen detection	Measures levels of specific parasite proteins, such as pLDH and HRPII, by ELISA.	pLDH: sensitive; results comparable to those of isotopic assays; HRPII: easy to perform; 10x more sensitive than isotopic assays; results comparable to those of the WHO microtest and the isotopic assays; does not require specialised personal or much technical equipment.	pLDH: limited monoclonal antibodies supply; HRPII: not enough studies; longer incubation period (48-72h)
Genotypic			
Target-specific assays (molecular techniques)	Investigation markers for association to antimalarial resistance by PCR, for instance.	Small amount of genetic material; independence from host and environmental factors; distinguishes recrudescence from re-infection.	Contradicting reports regarding importance of each marker; major differences regional-speaking; more studies are needed.

WHO: World Health Organization; FACS: fluorescence-activated cell sorter; ELISA: enzyme-linked immunosorbent assay; pLDH: parasite lactate dehydrogenase; PCR: polymerase chain reaction.

3.1.1. The WHO microtest

Rieckmann and colleagues (1968) described a macrotechnique which allowed to assess the development of *P. falciparum* from ring forms into schizonts – and thus named schizont maturation test – in the presence of several concentrations of chloroquine; to monitor the effect of chloroquine in the parasites' maturation, morphological changes would be used to distinguish the sensitive from the resistant. [75]

This assay was later developed by the WHO into a micro-version 24-hour test where schizont maturation of parasites obtained from a finger-prick blood sample and exposed to known amounts of the studied drugs is assessed in microplates; schizonts are counted against the total number of parasites on thick films prepared from the cellular layer of the cultured samples. [71-75]

This assay is very simple to perform, requires little technical equipment and can be done with low parasite densities; however, it is very labour-intensive and requires highly trained technicians to limit subjectivity in the process. [74]

Since this assay has a 24-hour incubation period, drugs which act upon later stages of parasite development – sulfadoxine-pyrimethamine, for instance – cannot be tested by this method. Also, the fact that this assay excludes earlier stages results in a loss of data, since parasites that grow into late-trophozoites but not into schizont within the 24-hour period have the same "value" as parasites that do not show any development whatsoever. [73,74]

For obvious reasons, prodrugs – drugs which require host conversion into active metabolites – or drugs which require any interaction with the host immune system cannot be tested through this method. Nevertheless, this remains one of the most common *in vitro* sensitivity assays to this day, since it accurately reflects true resistance and allows multiple tests to be performed and several drugs to be tested simultaneously. [74]

3.1.2. Radioisotopic assays

The incorporation of radiolabelled DNA precursors can also be used to assess parasite maturation – by measuring metabolic activity –, being radiolabelled hypoxanthine the preferred precursor. [74] Desjardins *et al.* (1979) were the first to develop a sensitivity microtest based on the measurement of the amount of tritium-hypoxanthine incorporated into the parasites nucleic acids. This allows an accurate and sensitive measurement of the inhibitory effect on parasite maturation. [76]

These assays are automated and thus fast to perform, with less variability resulting from human factors. They also allow for longer incubation periods hence might be used to test a broad number of drugs – although this might also be a drawback. [74]

Before the assay, cultures must be maintained under optimal conditions by dilution with uninfected red blood cells every 2 to 3 days and by limiting the parasitaemia to 2%. Samples are then prepared in microplates and incubated with the drugs for 24 hours; [^{3}H]-Hypoxanthine is added to the samples after this incubation period. [73,74,76]

After an additional 18-hour incubation period, the assay is terminated and incorporation of [^{3}H]-hypoxanthine is quantified with a liquid scintillation counter. [^{3}H]-hypoxanthine incorporation occurs

essentially in developing mature trophozoites and schizonts of the first blood cycle (parasites in ring stage at the beginning of the assay). [74,76]

Desjardins *et al.* (1979) initially designed this assay for drug screening in well-characterized, laboratory-adapted *P. falciparum* clones. This assay has now been adapted for epidemiological studies on fresh clinical isolates and is now one of the most commonly used assay methods in well-equipped malaria research laboratories, thanks to its versatility – being considered the "gold standard" for antimalarial drugs sensitivity assays. [73,74,76]

The use of radioactive material is one of the biggest disadvantages of this methodology; although the radioactivity of tritium results in weak emissions – and thus is blocked by plastic tubes and culture plates, not representing direct danger to the handler –, the half-life for radioactive decay of tritium is around 11 years. Therefore, special care is still necessary regarding waste disposal. [73,74]

The other main disadvantage of this method is the fact the equipment is highly expensive; it also requires initial high parasite densities, limiting it to culture-adapted parasite strains or field samples with high parasitaemias. [73,74]

3.1.3. Flow cytometry

Parasite growth might also be measured by assessing the increase in percentage of infected red blood cells in the culture, after a 48 to 96-hour incubation period. [73,74] This allows the testing of antimalarial drugs which act on the later developmental stages. It was originally done through microscopy as well, thus it would be very labour-intensive and prone to subjectivity; however, it was developed into methods which allow automated reading of results such as flow cytometry or fluorometric assays. [73,74]

Flow cytometers can be used to detect and measure DNA content of infected red blood cells using DNA-specific fluorochromes, while also differentiating the developmental stage, according to the amount of DNA – uninfected red blood cells do not have DNA and thus are not fluorescent, while fluorescence intensity increases proportionally to parasite maturation. [74]

Depending on the purpose of the analysis, the sample is fixed and tagged with different compounds. Several DNA-specific fluorochromes are available, including thiazole orange, acridine orange, Hoechst 33258 and Hoechst 33342, DAPI, and SYBR Green I. The latter is preferred since it does not require flow cytometers to have UV lasers – which are quite expensive – as Hoechst 33258 or 33342 do, and is a double stranded DNA-specific dye – contrary to thiazole and acridine orange, which bind to DNA and RNA – excluding the problem of RNA present in the reticulocytes (immature red blood cells). [74]

Once the cells are fixed and stained, the entire procedure can be completed in less than 3 hours, including the processing and analysis of the resulting data, which is done by a computer linked to the cell-sorter. These results were shown to be similar to those obtained with microscopic examination and the radioisotopic assay. Additionally to being rapid and automated, this method is highly accurate and sensitive, besides being non-radioactive. [74]

The main drawbacks of FACS is its high cost and need for very specialised personal – whether it is for operation or for maintenance. Moreover, this method is not adapted for installation in a tropical

climate, unless the laboratory is well-equipped with air conditioners and a constant electrical source, which makes it too sophisticated for field application – and thus not appropriate for malaria-endemic countries. [74]

3.1.4. Fluorometric assays

Fluorometric assays are also based on DNA fluorochrome-labelling but fluorescence is instead assessed with a mini-fluorometer, fluorescence spectrophotometer or fluorescence-activated microplate reader. This makes this assay less expensive than flow cytometry, although also less sensitive and requiring longer periods of incubation. [74]

After incubation, the red blood cells are lysed, washed and then dissolved. The cells are then stained with DNA-binding fluorochromes; if SYBR Green I is used, lysing and washing of red blood cells is not necessary. The DNA extraction protocol must be optimised to ensure complete recovery of DNA from each sample and this protocol must be performed by a highly specialised technician. [74]

The toxicity of some of the available dyes must also be taken into account; ethidium bromide, for instance, is highly mutagenic and thus must be handled with extreme caution and requires a special disposal system. [74]

Nevertheless, fluoroassays are still accurate, rapid and simple. Therefore, and although fluoroassays need further improvement as so to be used as alternative sensitivity assay suitable for field use, it is still very promising for antimalarial sensitivity assays in malaria-endemic areas.

3.1.5. ELISA-based assays

Detecting certain antigens from the parasites is also a means to assess their maturation. For this, enzyme-linked immunosorbent assays (ELISA) are performed; these assays can be performed in microplates as radioisotopic methods, with the advantage that no radioactive compound is necessary. [73,74]

The parasite lactate dehydrogenase (pLDH) was one of the first enzymes from malaria parasites shown to be electrophoretically, immunologically and kinetically distinct from that of the host. pLDH is a terminal enzyme in the Embden–Meyerhof pathway (glycolysis) of the human malaria parasites and plays an important role in the anaerobic carbohydrate metabolism of the parasites; since parasites rely on anaerobic glycolysis, they require the regeneration of nicotinamide adenine dinucleotide (NAD) for the continuous flux of glucose through this pathway. [73,74]

Since the levels of pLDH are proportional to parasite density, this enzyme serves as an indicator for the presence of malaria parasites and its production and accumulation are indicators to the parasites' viability, thus it can be an indicator for drug activity. [73,74] Makler and colleagues (1993) were the first to develop an assay which measures the enzymatic activity of pLDH, using the analogue of NAD, 3-acetyl pyridine adenine dinucleotide (APAD). Pyruvate is formed in the presence of LDH and APAD, resulting in the formation of reduced APAD, which in turn reduces blue tetrazolium, forming a blue product measurable by spectrophotometry. [73,74,77]

The enzymatic activity of pLDH is a direct reflection of the parasites metabolic activity, corresponding to the number of surviving parasites with intact glycolytic activity. This detection system

uses specific antibodies for pLDH in an antigen capture immunochromatographic assay, where antibodies are immobilised on a solid surface; this is hence a highly specific technique, which is also rapid and simple to perform. [73,74,77]

After a 48-hour incubation period, aliquots of the contents of each well are transferred to another 96-well microplate for washing and ELISA reading, including a series of incubations with a biotinylated monoclonal antibody that reacts with *Plasmodium* LDH, streptavidin-peroxidase conjugate, and peroxidase substrate (tetramethylbenzidine), with several washes between each incubation. The resulting colour reaction is quantified with a spectrophotometer at 450 nm. [74,77]

These several washing steps are the main disadvantage of the ELISA-based assays, since it can be tedious to perform. On the other hand, the required reagents and basic equipment are relatively cheap and widely available in moderately equipped laboratories – being promising field-wise. Nevertheless, this method requires parasite densities of 1 to 2% thus it is not sensitive in the field. [74]

This method was thus developed into an approach which measures pLDH levels in a double-site enzyme-linked LDH immunodetection (DELI) assay. After the same 48-hour incubation period, the samples are frozen-thawed 3 times to ensure complete haemolysis and liberation of LDH. The lysate is then diluted and transferred into another 96-well micro plate, which is previously treated with the first monoclonal antibody that specifically binds to *P. falciparum* LDH; the subsequent steps are those of a typical ELISA assay. [74]

This procedure is very laborious and time-consuming, due to the several freeze-thaw cycles and numerous washes and incubation periods. This is particularly problematic if an automated ELISA plate washer is not available, which is the case in most field laboratories. Moreover, before the OD of the entire microplate can be measured, an extra step of trial-and-error dilution is required to adjust the absorbance readings of drug-free control wells. [74] The main disadvantage continues to be the use of monoclonal antibodies, which are not of easy supply; this in turns limits the validation of this method.

The most recent addition to ELISA-based sensitivity tests uses the levels of the HRPII, developed by Noedl and others (2002). [74,78] HRPs appear to be produced specifically by *P. falciparum,* and not by other *Plasmodium* species. HRPII is known to be secreted into the host erythrocyte and the extracellular compartment; in synchronized laboratory-adapted strains, its levels are low during the first 48 hours of development, reaching its peek at 60-72 hour. Inhibition of parasite growth *in vitro* in the presence of a drug arrests parasite metabolism and consequently HRPII production; HRPII levels are thus highly correlated to parasite density and maturation, while also allowing the testing of longer-acting antimalarial drugs. [74,78]

This assay is also performed in microplates, with an incubation period up to 72 hours. Red blood cells are then lysed by two freeze-thaw cycles and the lysate transferred to another microplate, previously treated with HRPII-specific monoclonal antibodies; the samples are then incubated for 1 hour. After four successive washes, a conjugate is added and the plate is incubated for another hour; after another 4 washes, chromogen is added and samples incubated for 15 minutes. The reaction is then stopped, and the absorbance is measured with an ELISA plate reader. [74,78]

This procedure has fewer steps than the DELI assay and has been simplified for field use. Data obtained so far suggest results obtained with the HRPII-based sensitivity assay are comparable to those

obtained with the WHO microtest and the isotopic assays; [74,78] nevertheless, there are not enough studies to assure this test can replace the traditional *in vitro* tests in assessing resistance of malaria parasites to existing antimalarial drugs, although it is a good choice to test potential new antimalarials.

3.1.6. Target-specific assays

In cases where the molecular targets of candidate drugs are known, these can be purified and exposed directly to the drugs, instead of whole living parasites being used, as in the previously described assays. These are called target-specific assays and are especially useful for drug screening, when the drug belongs to a class known to inhibit the target. Currently, several substances are potentially promising molecular targets; some examples are haemozoin for quinolines, and dihydrofolate reductase (dhfr) for antifolate drugs. [71,73,74]

Quinoline-containing drugs – such as chloroquine – are thought to inhibit haemozoin formation; however, the precise target – enzymes, haematin, haemozoin – and mode of action are still not completely known. *In vitro* assays for inhibition of formation of β-haematin, the synthetic equivalent of naturally occurring haemozoin, are important alternative tools for accelerated, high throughput drug screening. *P. falciparum* chloroquine resistance has now been associated with the substitution of a threonine for a lysine at position 76 on the chloroquine resistance transporter (pfcrt), thanks to these assays. [73,74]

Enzymes specifically from malaria parasites – i.e., which differ considerably from those of their human counterparts – are also particularly fit for target-specific assays; DHFR is an example. Its gene can be amplified by PCR, inserted into an expression vector, and transformed into bacteria or yeast; mutagenesis is used to introduce point mutations. The produced protein is purified, and the enzyme activity is determined with natural substrates and drug candidates. Pyrimethamine resistance has now been associated with point mutations on the dhfr gene, and this approach has been used to screen new antifolates that inhibit mutant *P. falciparum* DHFR associated with pyrimethamine resistance. [73,74]

Sulfadoxine resistance has also been associated with point mutations on the dihydropteroate synthetase (DHPS) gene and the presence of certain polymorphisms is also associated with resistance to mefloquine; the relationship between specific polymorphisms and certain antimalarials has yet not been completely understood. There is also evidence suggesting a role for the multidrug resistance gene 1 of *P. falciparum* (pfmdr1) on the resistance to several antimalarials, including the new artemisinins. [73,74]

Molecular techniques allow the study of potential molecular markers associated with antimalarial drug resistance. Additionally, genetic data might provide important information regarding potential drug and vaccine targets. This can thus become of much value to antimalarials sensitivity testing and play a part in discovering the origins of resistance to antimalarials; however, there is still some struggle in finding the true importance of each molecular marker, since there are contradicting reports and major differences regional-speaking. [73,74]

The main disadvantage of these methods is that the current assay systems are designed to detect only those drugs that have mechanisms of action similar to those of the quinoline-type antimalarial drugs and antifolates. These *in vitro* assays are hence only complementary methods for primary drug

screening; they have little relevance for field workers, whose main concern is to track drug-resistant parasites. [74]

3.2. Haemozoin as an indicator for sensitivity testing

Since its discovery, malariologists have studied several ways to detect haemozoin – and therefore malaria parasites – and several methods, including microscopy, have been developed for the identification of haemozoin in blood and other tissues. In the process, some peculiar characteristics of haemozoin have been studied and used as diagnostic tools – haemozoin paramagnetic and birefringent properties.

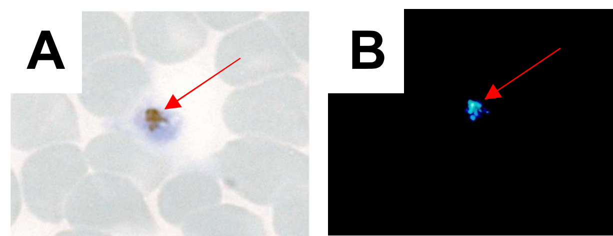

Figure 19 – *P. falciparum* parasites stained with Giemsa's. Schizonts are the asexual forms that contain the most amount of haemozoin (red arrows). Because of its birefringence, haemozoin appears as brownish-black under bright field microscopy (A), and it glows as white-blue while under polarizing light (B). Pictures obtained with a Leica DM2500 microscope.

As said before, parasite maturation might be assessed using haemozoin as an indirect indicator, in order to study the parasite's susceptibility to certain antimalarial drugs. But for almost a century the only practical application was the use of its birefringent properties for darkfield microscopy. Recently, several new methods for malaria parasite detection based on the detection of the malarial pigment were developed, using the malarial pigment's characteristic properties.

In 1936, Pauling and Coryell had already talked about the magnetic properties of haemoglobin and its derivatives [79] and, later in 1977, Pauling published a review emphasising the previous study; [80] Paul and colleagues (1981) noted haemozoin was birefringent and paramagnetic; the authors conducted a preliminary investigation, where the results indicated that high gradient magnetic separation was a useful tool for the rapid concentration of malarial parasites in red blood cells. [81] This proved parasitized cells had paramagnetic properties.

Moore *et al.* (2006) have confirmed that throughout the parasite development in red blood cells, there are characteristic changes of the magnetic properties of these cells; the authors noted magnetophoresis could be used to study free haem-haemozoin, which is an important parameter to parasite survival inside the red blood cell. [82] Zimmerman and colleagues (2006) have also developed a magnet-based approach to concentrate malaria parasites and increase the sensitivity of microscopy for detection of malaria parasites, which is useful in the detection of malaria infections with low parasitaemias. [83]

Packer (1945) and Jamjoom (1983) used dark-field microscopy to detect malaria parasites, indicating haemozoin had a distinct scattering of the incident light with thus allowed easy detection of the parasites; [84,85] Lawrence and Olson (1986) proposed examining blood with polarised light would

be an accurate, sensitive, specific, and practical method of identifying malaria parasites, noting the malarial pigment was brightly birefringent. [86]

Grooth and colleagues (1987) proposed light-scattering polarization measurements as a new parameter in flow cytometry [87], which was later implemented in a haemotology analyser; Mendelow and colleagues (1999) evaluated an automated malaria diagnostic tool, using a full blood count (FBC) technology with incorporated analysis of depolarized laser light and concluded it has high sensitivity and specificity. [88]

A year later, Hänscheid and others proposed malaria diagnosis by automated detection of haemozoin, using FBC analysers such as flow cytometers; when samples of malaria patients were analysed through FBC, lobularity–granularity plots were abnormal, which seemed to be caused by the presence of birefringent, depolarizing haemozoin in monocytes and neutrophils. [89]

Men *et al.* (2012) developed a method based on the colorimetric quantification of haemozoin as a sensitivity microtest, which produced very reproducible results; [90] since this assay was simple, easy and fast to perform, and much less expensive than traditional methods, it turned out to be a useful high throughput screening method for candidate antimalarials.

Rebelo and others (2013) developed a haemozoin detection assay based on flow cytometry, where a common flow cytometer was adapted to detect light depolarization caused by Hz; this strategy allows for the assessment of not only parasite maturation but also inhibitory antimalarials effects and it has proven to produce similar results to those of other more standardized methods. [91]

In 2015, Tempera and colleagues published on the characterization and optimization of the haemozoin-like crystal (HLC) assay, which was developed by Thomas *et al.* (2012). Although HLC is not structurally identical to Hz, the inhibitory effects appear consistent with those obtained with synthetic Hz assays. [92,93]

The HLC assay proved simple and inexpensive to perform, besides being very robust and reproducible; these qualities made this method feasible in rudimentary laboratories with a simple visual positive/negative read-out and also a good *in vitro* sensitivity assay. [93]

Overall, it is by now demonstrated haemozoin has the potential to be the optimal maturation and growth marker for malaria parasites; not only it increases with the development of the parasite but also has very specific characteristics that allow its easy and unquestionable identification and differentiation.

Since it is such a good indicator, haemozoin can be easily suited for existing antimalarials sensitivity testing and for inhibition profiles of candidate drugs. Eventually, haemozoin might even turn out to be part of the reference standard of *in vitro* sensitivity testing.

3.3. An alternative method: The Magneto-Optical Test (MOT)

Traditionally, the emergence of new antimalarials sensitivity tests has come from the adaptation and optimisation of existing diagnostic methods. Lately, there has been the need for the development of new diagnostic methods.

Recently, there has been an increasing number of new techniques, using the malarial pigment as the target material, being the magneto-optical detection one of them. The idea to take advantage of

the unique magnetic properties of the malarial pigment and to use it as an alternative target of optical diagnosis has been proposed by several groups.

In 2008, Newman and colleagues proposed a new diagnostic method for malaria that would make use of both paramagnetic and birefringent properties of the malarial pigment. The authors noted when suspended in a fluid, the long axes of the haemozoin crystals would randomly orientate; however, when applying a magnetic field to said fluid, the crystals would orientate along the applied field direction – this phenomenon is referred to as the Cotton-Mouton effect (in physical optics, the Cotton–Mouton effect refers to birefringence in a liquid in the presence of a constant transverse magnetic field) (see Figure 20). [94]

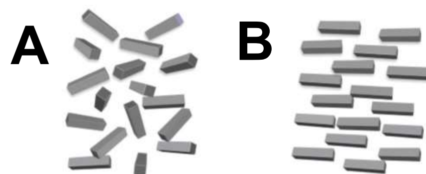

Figure 20 – Cotton-Mouton effect haemozoin suffers when suspended in a fluid and a magnetic field is applied. When there is no magnetic field (A), haemozoin crystals are randomly orientated; when a magnetic field is applied (B), the crystals become aligned along the direction of the applied field. Adapted from Mens *et al.* (2010).

When the haemozoin-containing fluid would be interrogated with polarized light it would express preferred direction of optical absorption, producing an optical modulation signal directly proportional to crystal concentration – and this could be used to differentiate a haemozoin-containing sample from one which did not.

An instrument operating under these principles was developed and blood samples were tested for *Plasmodium* infection and results compared to those of a standard rapid diagnostic test (RDT); there was an excellent correlation between the MOT and the RDT results, and clinical confirmation. Although certain details of this process were not optimized yet – such as sample volume or optical path length – this assay had a performing time of around one minute, having thus the potential of high throughput screening. [94]

This was the start of what could be a simple and precise magneto-optical method to diagnose malaria, even in resource-limited contexts, since it could work without electricity and it was relatively inexpensive. Two years later, Mens and others published a study describing the field evaluation of the MOT. A more elaborate prototype was developed (see Figure 21) and extensively evaluated with several stored clinical samples. [95]

Figure 21 – Diagnostic device developed by Men *et al.* (2010). A polarised laser beam (1) passes through the sample (2), which is placed between the poles of a magnet (3) supplying a magnetic field and the transmitted intensity is recorded using a photodetector (4).

The authors concluded the sensitivity and specificity were not comparable to other diagnostic tests – such as PCR and RDT – but it had the potential to rapidly screen patients for malaria in endemic and non-endemic countries. According to the authors, the major flaws of this study were the lack of fresh samples and limited amount of endemic controls; additionally, the influence of hand assembly of samples could disguise the true potential of this new device. They hence suggested further evaluation with the MOT with fresh blood samples and protocol optimisation. [95]

Butykai *et al.* (2013) also further investigated the unique properties of haemozoin and developed a new magneto-optical test, which they thoroughly evaluated, testing its sensitivity through many parameters. The authors referred to magnetically-induced linear birefringence/dichroism and polarization-dependent light scattering as a whole as magnetically-induced linear dichroism (MLD), which is what the MO instrument measures. In the used configuration (see Figure 22), a laser beam would pass through a polariser and probe the sample, which would be under the influence of a magnetic field. [96]

An arrangement of permanent magnets in a ring-shaped structure – called Halbach-cylinder – was developed, which would surround the blood sample and rotate with an adjustable frequency; this would generate a uniform magnetic field at the sample position, causing the efficient co-alignment of the Hz crystals. The light beam was then divided into two parts with orthogonal polarizations by a Rochon prism and the characteristic polarising profile would in turn be detected by a balanced photodiode bridge; this would largely reduce the intensity background noise, meaning that an ordinary laser diode is sufficient as light source. [96]

The authors concluded the MOT sensitivity would be largely reduced due to the strong absorption and light scattering by the blood components, thus they used lysed blood in their samples in further evaluation; this haemolysis would favour the diagnosis since haemozoin-containing red blood cells would liberate it into the blood plasma, making it become detectable by the MOT. [96]

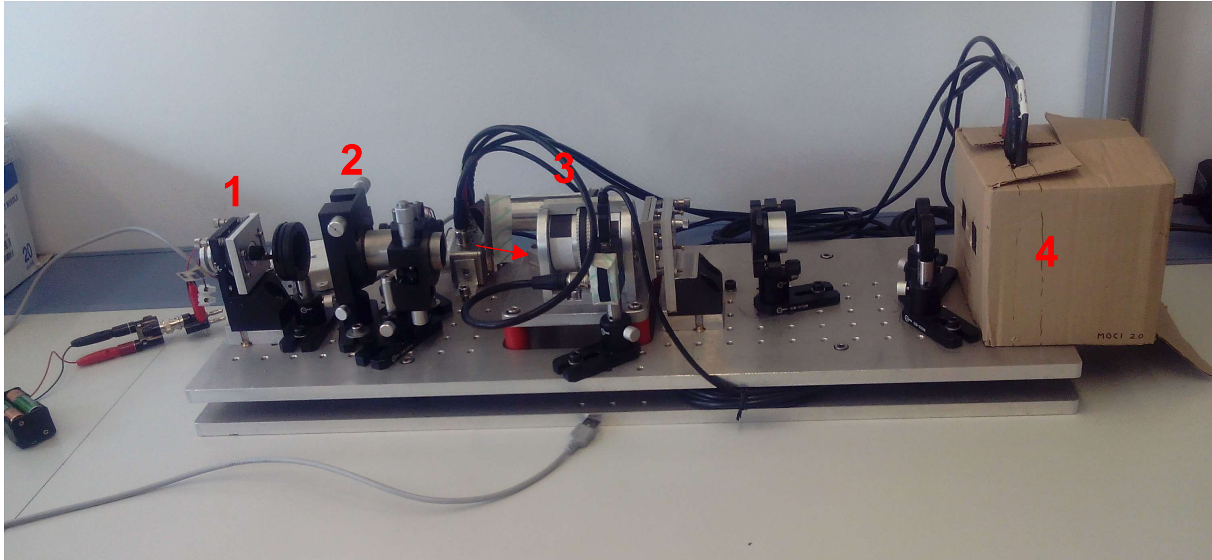

Figure 22 – Ensemble of the Magneto-Optical Instrument. The beam from a laser diode (1) passes through a polarizer (2), becoming vertically polarized. Then it goes through the sample holder, which is located within the center (red arrow) of the magnet (3). The beam is then detected (4) and the data are visualised in a computer.

This study revealed an estimated parasitaemia less than 1 parasite per μL of blood would still be detectable by this method, being its estimated threshold concentration equivalent to 30 parasites per μL of infected blood, which would exceed the performance of RDT and approach the detection limit of microscopic observation. [96]

Although these tests were made with haemozoin suspensions and not infected blood samples, the authors assumed the sensitivity of the detection in hemolyzed blood would be close to that in water; they thus expected no major reduction of sensitivity when using infected blood samples, since their lysis would release the haemozoin within the red blood cells. Hz crystals aggregate with one another, forming clumps of pigment and, since the MOT only detects crystals which are liberated in the sample suspension and can be magnetically rotated, this could influence this method's sensitivity. [96]

To establish the usefulness of the MOT for field trials and its further application in a diagnostic setup, Orbán and others (2014) conducted a study where the aim was to address some issues such as the difference in haemozoin suspensions and true infected blood samples and how these behave in the MOT setup. For that, the authors evaluated the MOT performance using synchronized laboratory cultures of *P. falciparum* and investigated the limit of detection in samples with low levels of parasitaemia. The detection limit obtained with this study was 40 parasites per μL of blood for ring-forms and 10 parasites per μL of blood for schizonts, which is explainable by the fact that schizonts contain larger amounts of haemozoin than early trophozoites. [97]

Since this was obtained with thawed blood samples, the authors believed this detection limit had the potential to be improved while measuring freshly lysed blood samples; still, these limits are below those obtained with RDT – 100 parasites per μL of blood – and within the same range as those obtained with microscopy – 5-50 parasites per μL of blood. [97]

This method has some limitations intrinsic to haemozoin properties; there can be false positives due to the presence of Hz within white blood cells after an infection is cleared or there can be false negatives in cases where there are only very early ring-forms, which contain almost no Hz at all. [97]

Nevertheless, this magneto-optical method promises to be inexpensive, rapid and highly sensitive, thus having the potential to fulfil current needs in malaria research, not only diagnostic-wide but also in terms of sensitivity testing. [97]

Preliminary results showed this MO method could provide an efficient tool to assess sensibility of *Plasmodium* parasites to novel or clinically relevant antimalarial drugs by detecting haemozoin as an indirect indicator of parasite development. However, further development and optimisation should be conducted in order to judge the value of this method. [97]

II – Objective

The main purpose of this project was to further evaluate this magneto-optical test, as a novel and rapid sensitivity test for *Plasmodium* spp. The present study evaluated the detection of haemozoin through this new magneto-optical method and its usefulness as a novel, inexpensive, real time assay to assess antimalarial drug effects on *P. falciparum.*

III – Materials and Methods

1. Reagents

1.1. Malaria Complete Parasite Medium (MCM)

The parasite growth medium consisted of RPMI 1640 culture medium supplement (no L-glutamine; with $NaHCO_3$) supplemented with gentamycin 50 mg/mL, L-glutamine 200 mM, HEPES buffer solution 1 M and Albumax II solution; MCM was stored at 37ºC.

Albumax II solution consisted of RPMI powder (with L-glutamine, without $NaHCO_3$), gentamycin 50 mg/mL, HEPES buffer powder, $NaHCO_3$ powder, glucose powder and Albumax II powder; the Albumax II solution was sterilised with a 0.22 µm MilliPore filter and stored at -20ºC.

RPMI 1640, HEPES, gentamycin and Albumax II were purchased from Gibco (Carlsbad, USA); L-glutamine, $NaHCO_3$, glucose, and hypoxanthine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2. Lysis solution (LS)

The lysis solution (LS) consisted of 20 mM NaOH and 0.063% Triton in distilled water.

A stock solution of 475 mM NaOH was prepared by dissolving 1.9 g in 100 mL distilled water. A stock solution of 10% Triton was prepared by taking 1 mL of Triton X-100 and diluting it in 9 mL distilled water. Both reagents mentioned above were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.3. Giemsa stain

Giemsa's azur eosin methylene blue solution was diluted 1:10 in PBS 1X. Giemsa's solution was purchased from Merck (Darsmstadt, Germany) and PBS 1X from Gibco (Carlsbad, USA).

1.4. SYBR green I

SYBR green I solution 10000X was diluted to 1X in PBS 1X. SYBR green I was purchased from Invitrogen (Carlsbad, USA).

1.5. Sorbitol

5% sorbitol in distilled water was used for culture synchronization. Sorbitol was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Culture Maintenance

2.1. Culture conditions

Cultures were always managed in a sterile environment and maintained at 5% haematocrit and parasitaemias lower than 2%. The cultures were incubated at 37ºC and 5% CO_2. The culture medium was changed at least every two days and if parasitaemias were higher than 1.5%, they were diluted with uninfected red blood cells (uiRBC).

The parasitaemia of the culture was assessed by bright field microscopy. A small volume of the infected red blood cells (iRBC) was taken from the culture and a smear was made and stained with Giemsa's stain; parasitaemia was estimated by counting the number of total red blood cells and infected red blood cells, under oil immersion with a 100X objective;

2.2. Preparation of uninfected red blood cells (uiRBC)

Healthy blood was provided by the Instituto Português do Sangue e Transplantação (IPST) with ethical approval. The isolation of uiRBC was performed by centrifuging the blood, removing plasma and white blood cells and washing it with RPMI until the supernatant was clear; this was then stored at 4ºC.

2.3. Thawing frozen infected red blood cells (iRBC)

1.6% NaCl and 12% NaCl were used for this process; NaCl (s) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and solutions were made with Milli-Q water.

A vial with frozen RBC infected with *P. falciparum* 3D7 or Dd2 was thawed at 37ºC for a few minutes and the volume of iRBC was measured and transferred to a tube. 0,1V of 12% NaCl and then 10V of 1.6% NaCl was added, always drop by drop, while swirling the tube. This was then centrifuged and the pellet transferred into a culture flask and MCM added accordingly to its volume.

2.4. Giemsa staining

Giemsa staining of blood smears was performed by fixing air-dried blood smears in absolute methanol, air-drying them and staining them with Giemsa 1:10 for 10 minutes.

2.5. Culture synchronization

Culture synchronization kills the schizont forms by adding the detergent (sorbitol), leaving only young trophozoites in the culture.

The culture medium (MCM) is taken out of the culture flask and the same amount of 5% sorbitol was added; this volume was transferred to a tube and incubated at room temperature for 10 minutes and then centrifugated; the supernatant was discarded and the iRBC were washed with RPMI; the volume of iRBC is measured and cultured again with the correspondent amount of MCM.

3. Antimalarial drugs sensitivity testing with the Magneto-Optical Test (MOT)

3.1. Antimalarial drugs preparation

The drugs tested were dihydroartemisinin (DHA), piperaquine (PQ), chloroquine (CQ), and pyrimethamine (PYR). DHA, CQ and PYR were purchased from Sigma-Aldrich (St. Louis, MO, USA) and PQ was kindly given by Sigma-tau (Pomezia, Italy).

In order to have the correct final concentration in the mixture of iRBC culture and drugs, drugs had to be prepared twice as concentrated, since they were going to be diluted by half. Intermediate and working solutions were all prepared in MCM. The tested drug concentrations were chosen based on previous works with these drugs. Table 4 summarizes the prepared concentrations of each drug.

Table 4 – Prepared solutions of the studied antimalarials.

Drug	Stock solution (solvent)	Intermediate solution (µM)	Working solutions (nM)
DHA	1.758 mM (DMSO)	10	0.24, 1, 4, 16, 64 (growth-curve assay) 8, 16, 32, 64, 128 (confirmation assays)
PQ	1 mM (Milli-Q water)	1	12, 36, 108, 324, 972
CQ	77.5 µM (distilled water)	1	12, 25, 50, 100, 200
PYR	1 mM (absolute ethanol)	10	25, 50, 100, 200, 400

DHA: dihydroartemisinin; PQ: piperaquine; CQ: chloroquine; PYR: pyrimethamine; DMSO: dimethyl sulfoxide.

3.2. General protocol

Drug sensitivity assays were prepared with four antimalarial drugs – dihydroartemisinin (DHA), piperaquine (PQ), chloroquine (CQ), and pyrimethamine (PYR). These assays were performed with two laboratory strains of *P. falciparum*, 3D7 and Dd2. The first is sensitive to all these drugs, while the second is chloroquine-resistant.

The assays were divided into two sets: the first set of assays consisted in assessing the growth of the *P. falciparum* strains over 48 hours. The negative controls consisted of uninfected samples, while the positive controls consisted of drug-free samples. The infected samples were prepared from continuous cultures of either *P. falciparum* 3D7 or Dd2, which were synchronized every 2 days – at least twice – so they consisted mainly of ring-form parasites.

Before every assay, the parasitaemia of the cultures was assessed by flow cytometry as described below. The samples were prepared in 96-well plates in triplicate and each well contained a final volume of 160 µL. The wells of infected samples contained 80 µL parasite culture at 5% haematocrit and 80 µL drug solution (or 80 µL RPMI in drug-free controls); the wells of uninfected samples contained 80 µL uiRBC with MCM at 5% haematocrit and 80 µL RPMI.

The second set of assays consisted of repetition assays performed with DHA, PQ, and CQ with the 3D7 strain. Sample preparation followed the same procedure as before.

3.2.1. SYBR green I staining and flow cytometry analysis

5 µL iRBC of the culture was stained with SYBR green I 1X for 20 minutes in the dark; the sample was analysed by flow cytometry using a 535/45 nm bandpass filter in front of the detector. The parasitaemia was then adjusted to 1%.

3.3. Data acquisition

The MLD signal of all samples was measured (0-hour timepoint) and the plates were incubated at 37ºC. For the assays of DHA, PQ, and CQ, samples were measured again at 6, 8, 10, 14, 20, 24, 30, 36, and 48 hours of incubation; the PYR assay followed until 96 hours of incubation, with extra timepoints at 60, 72, 84, and 96 hours of incubation. To note that a plate was prepared for each timepoint, which means it was not the same plate being measured every time.

To analyse the samples with the MO instrument, the content of each well was removed from the plate at each timepoint and put on microtubes with 160 µL lysis solution and then each well was washed with more 160 µL lysis solution. At the end, each microtube had 160 µL of the sample and 320 µL lysis solution. This volume was then transferred to sample holders prepared specifically for the MO instrument and the MLD signal was measured. The signal was converted into numerical values and observed in a computer software, also prepared specifically for the MO instrument.

In the second set of assays, sample measurement was only at the beginning of the assay and 24 hours after incubation, but followed the same procedure as before. Whenever it was possible, an extra plate was also prepared to be analysed by depolarization flow cytometry, for purposes of confirmation.

3.4. Data analysis

The MLD values resulting from these assays were transformed into files in a workable format and analysed with the software Microsoft Office Excel (in the case of the time-curves) and GraphPad Prism (for repetition assays), where the mean of the measurements, together with the standard deviation, was calculated and used as the value for each triplicate set. IC50 values were calculated through a nonlinear regression model (sigmoidal dose-response/variable slope) with SigmaPlot - Systat Software (Chicago, IL, USA).

4. Variability tests

Samples were always prepared in triplicated and measured with the MO instrument at the beginning of every assay and again 24 hours later; subsequent data was analysed as in sensitivity assays.

4.1. Comparing lysed (L) samples with non-lysed (NL) samples

Two stocks of synthetic haemozoin (sHz) and one of native haemozoin (nHz) were used, previously prepared by others and of which initial concentration is unknown. Stock 1 of sHz was diluted to 1:10 and 1:100, and stock 2 was diluted to 1:1000 and 1:10000; nHz stock was diluted to 1:10 and 1:100. All dilutions were made in PBS.

160 µL of each dilution was added to 320 µL of lysis solution (LS) or PBS the same way as in sensitivity assays. For the lysed set of samples the control was 160 µL PBS with 320 µL LS and for the non-lysed it was 480 µL (=160+320) PBS.

4.2. Assessing the influence of MCM

Stock 1 of sHz was diluted to 1:10 and 1:100 and tested in two sets. In the first set, samples consisted of uiRBC with 1% sHz, in PBS at 2.5% haematocrit. The negative control was uiRBC without sHz, in PBS at 2.5% haematocrit. The second set followed the same procedure, but MCM was used instead of PBS.

4.3. Comparing different types of storage

A running culture of *P. falciparum* 3D7 was used for this test, without adjusting the parasitaemia; the haematocrit was adjusted to 2.5% with MCM. The negative control consisted of 2.5% uiRBC in MCM. 160 µL of each was added to 320 µL of LS in two sets. One was left at room temperature between measurements and the other was stored at 4 ºC as usual.

4.4. Assessing the influence of red blood cells (RBCs)

sHz was used and prepared in two sets, where the dilution was made in PBS or MCM, with 2.5% uiRBC. The negative controls consisted of 100 µL sHz in PBS or MCM, without RBC. All samples were lysed as in sensitivity assays.

4.5. Comparing different lysis solutions

A new lysis solution was prepared with Triton 0.063 % in distilled water. A running culture of *P. falciparum* NF54 was used for the infected samples, without adjusting the parasitaemia. All samples were at 2.5% haematocrit in MCM. Two sets of samples were prepared and one was lysed with the standard lysis solution (sLS), while the other was lysed with the new lysis solution (sLS).

IV – Results

1. Antimalarial drugs sensitivity testing with the Magneto-Optical Test (MOT)

1.1. Growth curves

This first set of assays consisted in assessing the growth of the *P. falciparum* strains over 48 hours, in order to determine the best timepoint for 50% inhibitory concentration (IC50) calculation. DHA, PQ and CQ were followed until 48 hours of incubation because they act on the first blood cycle of the parasites, while PYR was followed until 96 hours, given that it is a slow-acting drug and hence its effects only begin to show after the second life cycle of the parasite.

The MO method discerns between an uninfected sample and an infected one, even at the beginning of each assay, since the first always presents MLD values under 1 and the latter always presents much higher MLD values.

For all the tested antimalarials, the MO instrument could assess drug effects, according to the different drug concentrations and, at the 24-hour timepoint, it was already possible to distinguish two groups of samples; one consisting of the two higher concentrations of drug and the other comprising the three lower concentrations (see Figure 23).

For the pyrimethamine assay, this is only visible at the 60-hour timepoint, as this is a slow-acting drug (see Figure 24). For the assay with chloroquine with the Dd2 strain, all samples grew as a drug-free sample, expected, since this strain is chloroquine-resistant (see Figure 25).

The protocol was standardised, meaning at the beginning of each assay the parasitaemia and the haematocrit, and the amounts of each component in the working mixture were always the same; however, the initial MLD values were never the same, sometimes being twice as high.

There was some variation between triplicates from certain samples – sometimes a 2-fold difference –, which resulted in very wide standard deviations.

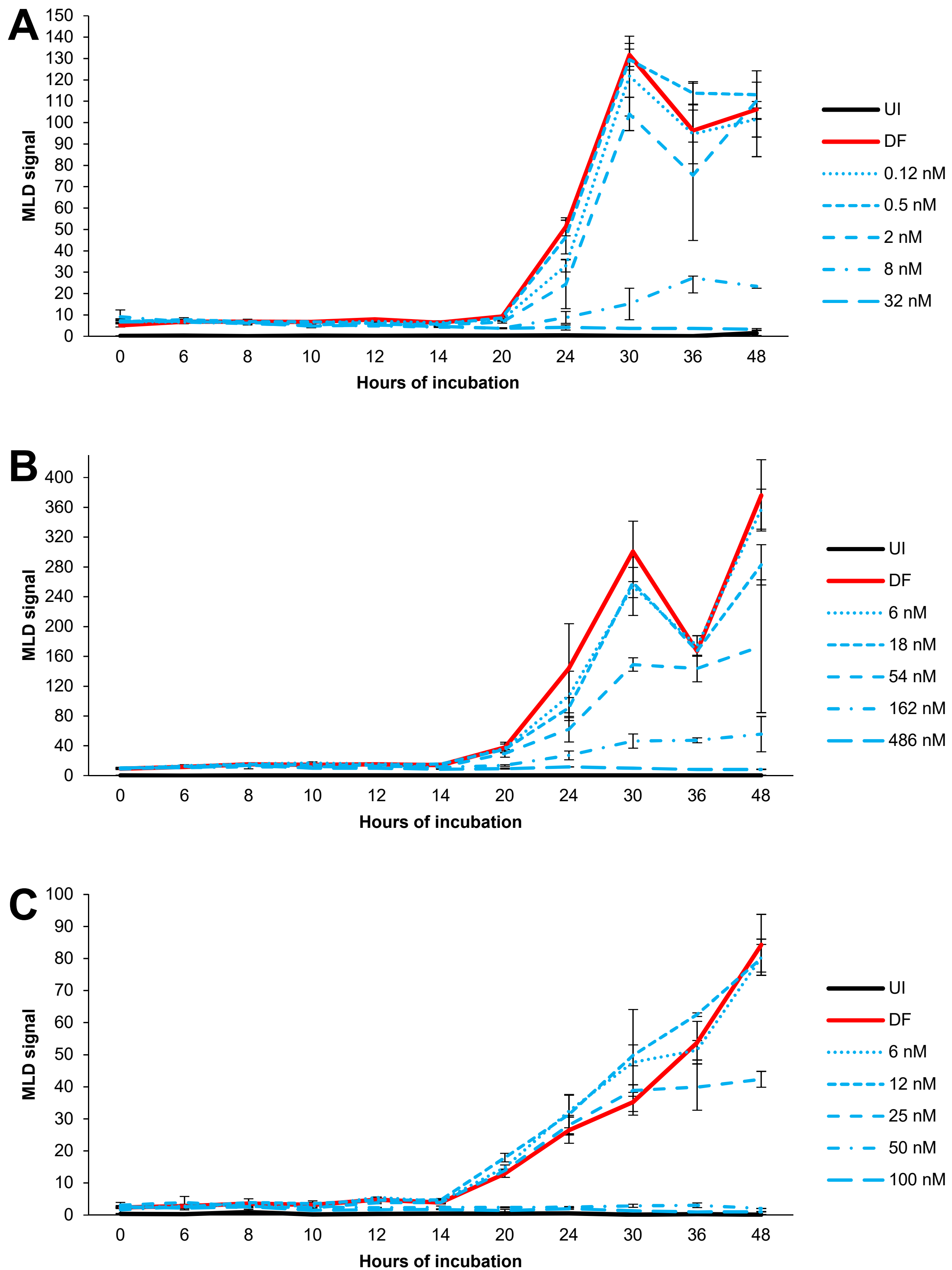

Figure 23 – Growth curve of *P. falciparum* 3D7 in the presence of several concentrations of dihydroartemisinin (A), piperaquine (B), and chloroquine (C). The means and standard deviations of triplicates are represented. The negative control was an uninfected (UI) sample and the positive control was a drug-free (DF) sample.

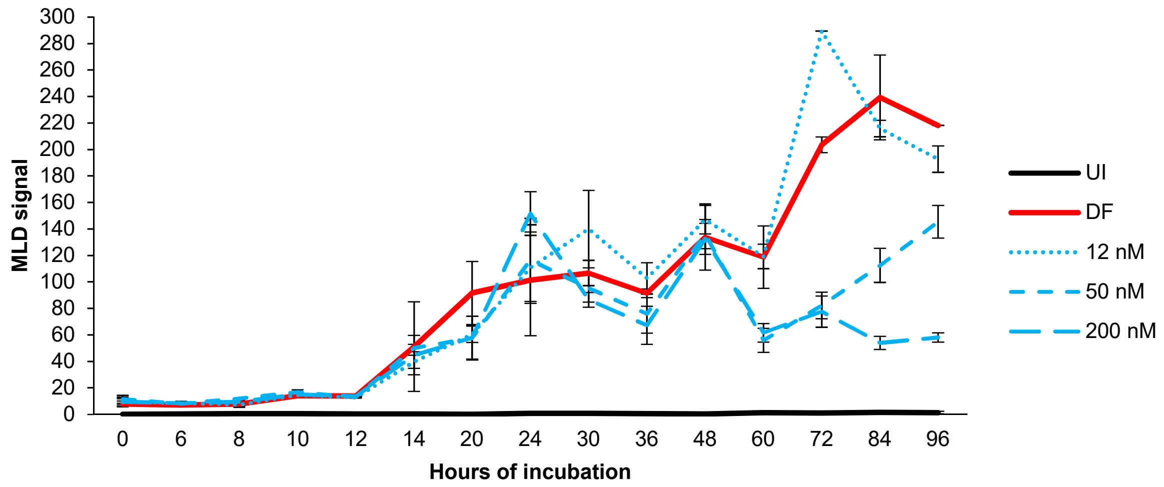

Figure 24 – Growth curve of *P. falciparum* 3D7 in the presence of several concentrations of pyrimethamine. The means and standard deviations of triplicates represented. The negative control was an uninfected (UI) sample and the positive control was a drug-free (DF) sample.

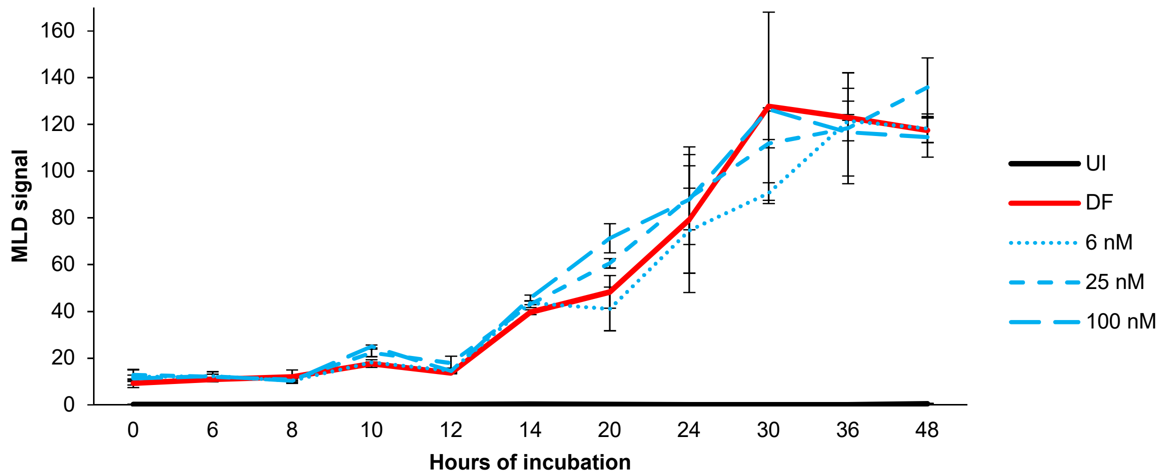

Figure 25 – Growth curve of *P. falciparum* Dd2 in the presence of several concentrations of chloroquine (CQ). The means and standard deviations of triplicates are represented. The negative control was an uninfected (UI) sample and the positive control was a drug-free (DF) sample.

1.2. Confirmation assays

This second set of assays was performed with the goal of assessing the robustness of the MO method and the reproducibility of its results. CQ with the Dd2 strain and PYR were not followed for practical reasons. The 24-hour timepoint was chosen for these assays, since it was where inhibition effects were visible for the tested drugs on the previous assays.

Figure 26 shows the obtained results from the confirmation assays of DHA, PQ, and CQ. Table 5 summarises the IC50 estimated from each assay and respective growth curve, and flow cytometry values, when applicable.

There was some variability among replicates of the same drug, whether at the 0-hour timepoint – despite the standardised initial conditions – or at the end of the assays – meaning the inhibition effects were not the same in every replicate with the same drug, which reflects in the diverse IC50.

Nonetheless, inhibition effects are visible on all assays. CQ was the most consistent drug in terms of inhibition effects and subsequent IC50, with the smaller IC50 range (25-41 nM), while PQ originated the most variable results, with IC50 ranging from 18 nM to 62 nM.

Table 5 – Comparison between estimated IC50 from growth curves and confirmation assays of dihydroartemisinin (DHA), piperaquine (PQ), and chloroquine (CQ). IC50 from MO values were calculated from the 24-hour timepoint and IC50 from flow cytometry values from the 48-hour timepoint, with SigmaPlot. IC50 from flow cytometry values are represented in square brackets.

	IC50 (nM) from MOT [flow cytometry]					
Drug	**Growth curve**	**Confirmation assays**				
DHA	2 [5]	13	26	7	5.5 [12]	8
PQ	31 [50]	54	62 [43]	18	41	-
CQ	37 [33]	41	33 [32]	25	25	-

IC50: 50% inhibitory concentration; DHA: dihydroartemisinin; PQ: piperaquine; CQ: chloroquine

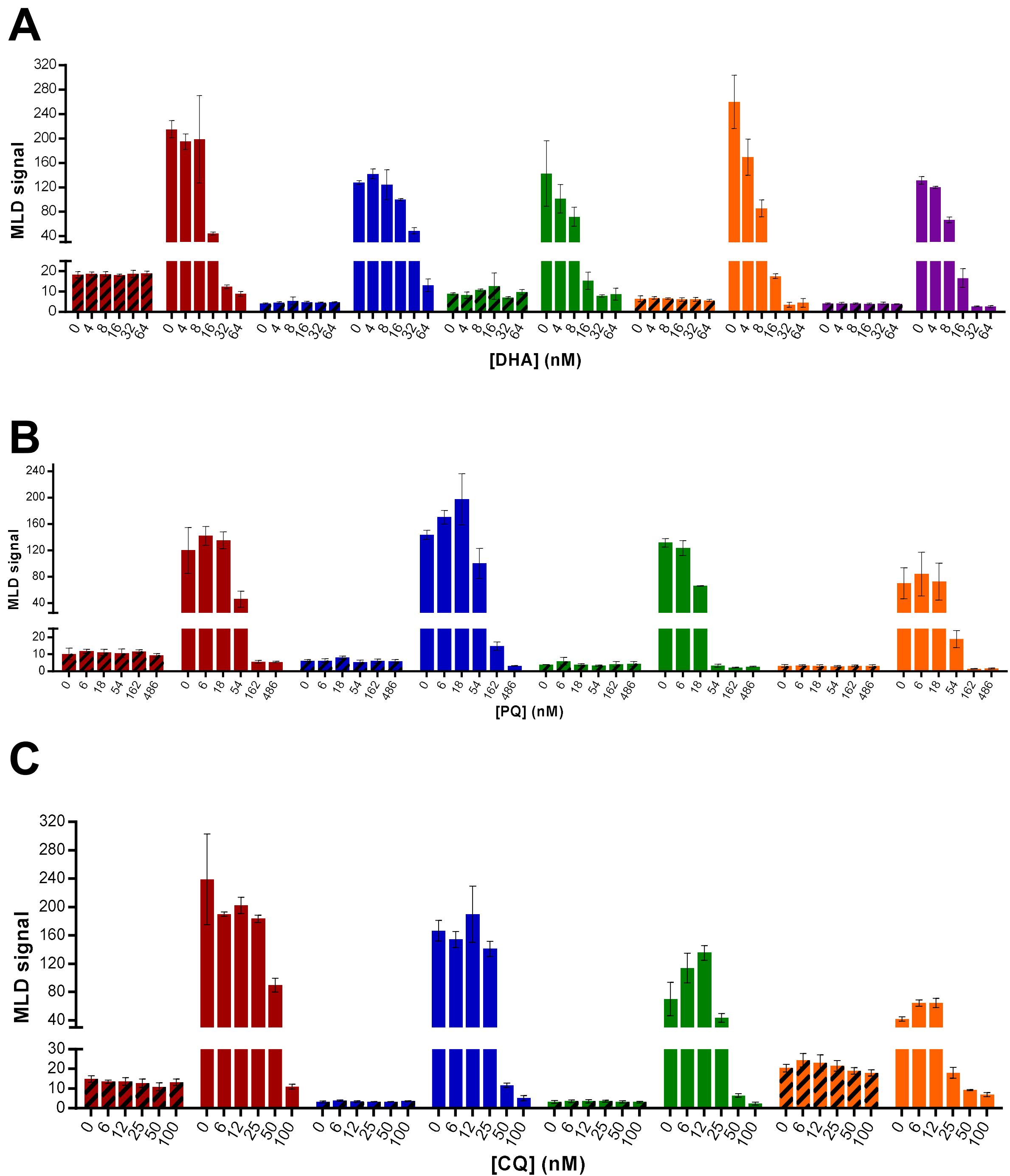

Figure 26 – Repetition assays of the sensibility of *P. falciparum* to (A) dihydroartemisinin (DHA), (B) piperaquine (PQ), and (C) chloroquine (CQ). The means and standard deviations of triplicates are represented. The first replicate is represented by the red bars, the second by the blue, the third by the green, the fourth by the orange, and the fifth and last (only in the case of DHA) by the purple bars. Striped bars represent the 0-hour timepoints of each assay, while solid bars represent the 24-hour timepoint.

2. Variability tests

After the antimalarial drugs sensitivity tests were performed, several additional tests were carried with the MO instrument, in order to test which steps of the previous procedure would add more variability to the resulting data.

2.1. Comparing lysed (L) samples with non-lysed (NL) samples

To test whether lysing the samples would produce different results than not lysing them, synthetic and native haemozoin were used in two sets of samples, one lysed as to mimic previous assays and the other where lysis solution was not added (see Figure 27).
Both lysed and non-lysed negative controls behaved as uninfected (UI) samples, keeping the MLD signal under 1 at both timepoints, as expected.
In all assays, MLD values from lysed samples decreased significantly from the 0-hour timepoint to the 24-hour timepoint. This did not verify with the non-lysed samples, which maintained a somewhat constant MLD value.

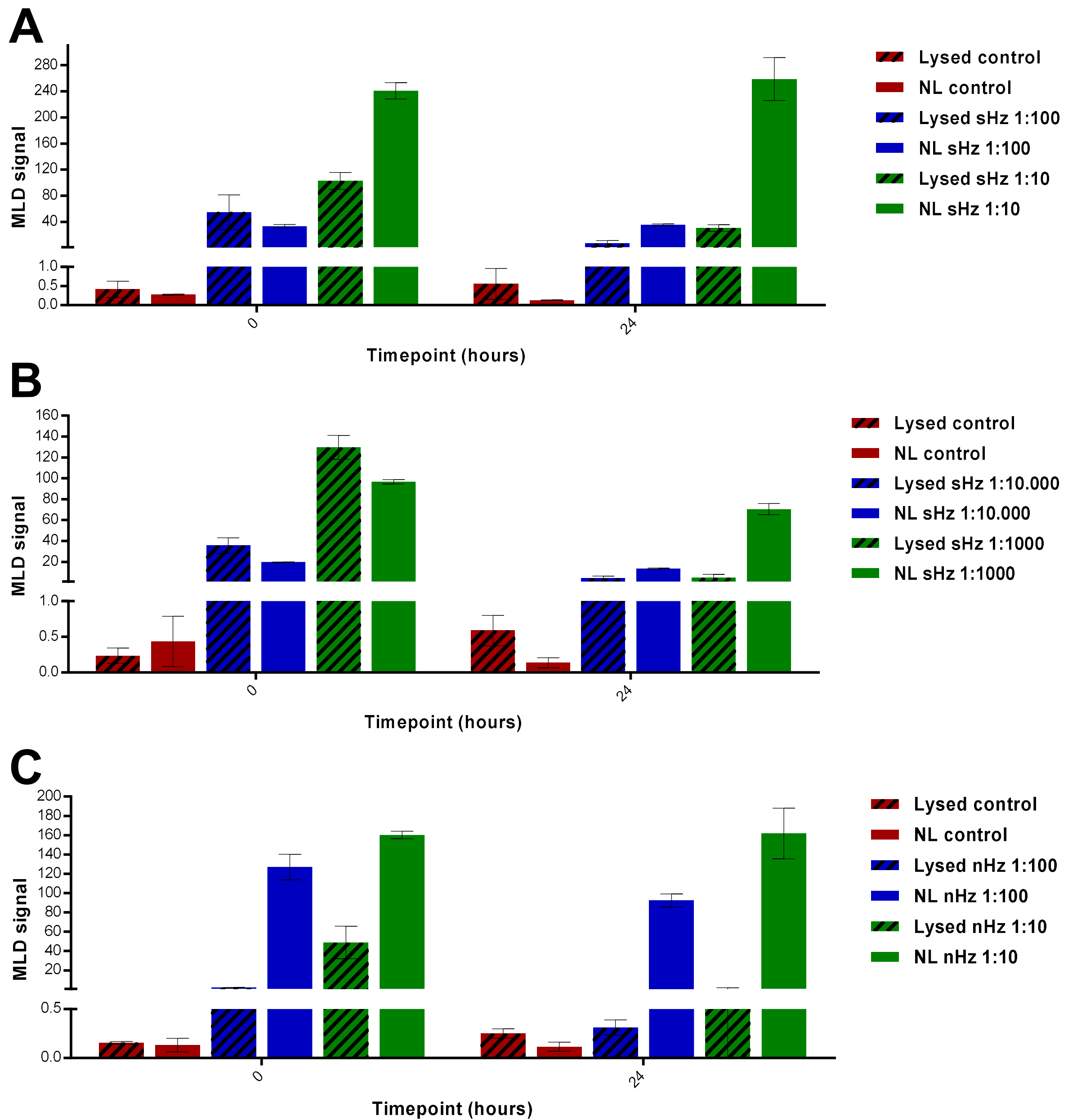

Figure 27 – Comparison between lysed and non-lysed (NL) samples from stock 1 (A) and 2 (B) of sHz, and from nHz (C). The means and standard deviations of triplicates are represented. The negative controls are represented by the red bars, the most concentrated samples of Hz by the blue, and the less by the green bars. Striped bars represent lysed samples, while solid bars represent NL samples. sHz: synthetic haemozoin; nHz: native haemozoin; NL: non-lysed.

2.2. Assessing the influence of MCM

To test whether the outcome of an assay would be different if samples contained PBS or MCM, stock 1 of sHz was used (see Figure 28). To mimic the most a drug assay, uiRBC were also used.

Both negative controls behaved as UI samples, keeping the MLD signal under 1 at both timepoints, as expected.

Samples with MCM had lower MLD values than their counterparts with PBS. All samples with MCM had MLD values under 1 at both timepoints.

Both sHz 1:100 samples had MLD values under 1 at both timepoints. Sample sHz 1:10 with PBS had a much higher MLD signal than its counterpart with MCM.

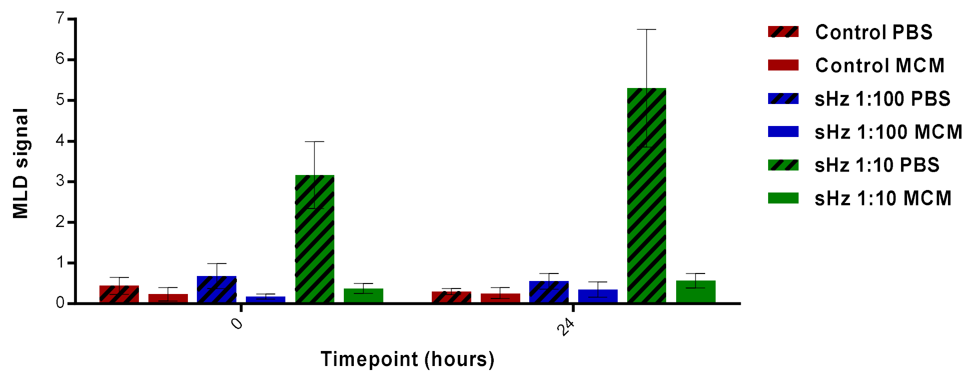

Figure 28 – Comparison between samples with PBS and samples with MCM. The means and standard deviations of triplicates are represented. sHz: synthetic haemozoin; PBS: phosphate buffer saline; MCM: malaria complete parasite medium.

2.3. Comparing different types of storage

Since in previous assays samples were stored at 4ºC between measurements and this type of storage could influence the outcome of the assay, a different storage was tested (see Figure 29).

Both negative controls behaved as UI samples, keeping the MLD signal under 1 at both timepoints, as expected.

At the beginning of the assay, both 3D7 samples had a similar MLD signal, clearly distinguishable from the negative control; at the 24-hour timepoint, both had decreased their MLD signal, but still maintained similar. There was hence no difference between storing samples at room temperature or at 4ºC.

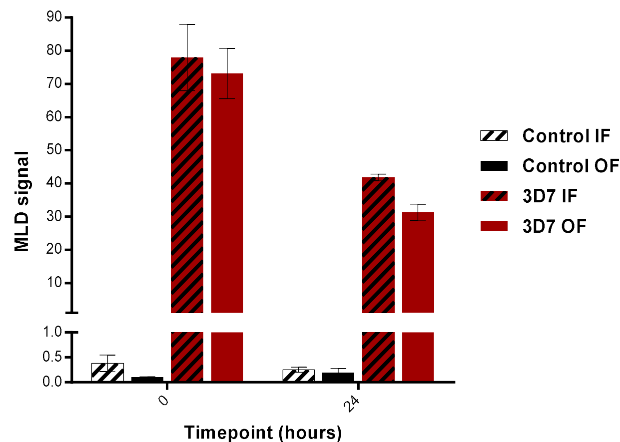

Figure 29 – Comparison between different types of sample storage, with *P. falciparum* 3D7. The means and standard deviations of triplicates are represented. IF: inside fridge (4ºC); OF: outside fridge (room temperature).

2.4. Assessing the influence of red blood cells (RBCs)

To assess if RBCs had a significant influence on the MLD signal of a sample, whether the samples were in PBS or in MCM, a stock of sHz was used (see Figure 30).

The control with PBS behaved as if it was an UI sample, although it contained the same amount of sHz than the remaining samples; its counterpart with MCM had an MLD signal around 200-fold higher at the 0-hour timepoint.

Samples with RBC appear to return much higher MLD signal than samples with no RBC. Additionally, at the 24-hour timepoint, variability among triplicates of the same sample seems to be much higher than at the 0-hour timepoint, where standard deviations are relatively small.

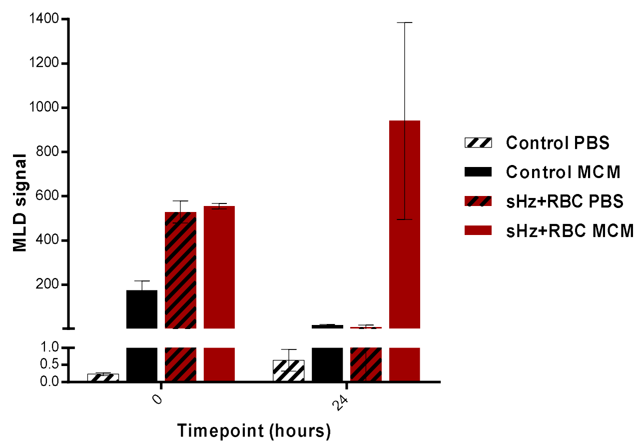

Figure 30 – Assessing the influence of red blood cells (RBC), with synthetic haemozoin (sHz). The means and standard deviations of triplicates are represented. sHz: synthetic haemozoin; RBC: red blood cells; PBS: phosphate buffer saline; MCM: malaria complete parasite medium.

2.5. Comparing different lysis solutions

To understand if the regularly used lysis solution (LS) was too strong and had a detrimental influence on the outcome, a different lysis solution was made (see Figure 31).

Both negative controls behaved as UI samples, with MLD signals under 1 at both timepoints, as expected.

All samples maintained a somewhat constant MLD signal over time; nonetheless, NF54 with the new lysis solution had an MLD signal around 17-fold lower than its counterpart with the standard lysis solution, at both timepoints.

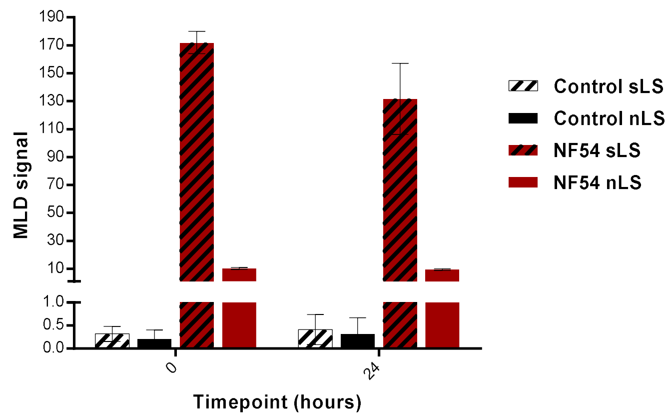

Figure 31 – Comparison between the regularly used lysis solution (sLS) with a new lysis solution (nLS), without NaOH, with *P. falciparum* NF54. The means and standard deviations of triplicates of each sample are represented.

V – Discussion

1. Can the Magneto-Optical Test (MOT) detect maturation of *Plasmodium* parasites?

By performing the first drug assays with *Plasmodium falciparum* cultures and obtaining the respective growth curves, it was confirmed this method indeed detects maturation of the parasites. Since the assays start with parasites in the initial forms of the blood stage development (ring forms), at 24 hours of incubation, the parasites are schizonts and hence full with haemozoin. This haemozoin will be liberated into the culture well and merozoites will invade new red blood cells and produce more haemozoin until the next 24 hours of incubation.

Therefore, the exponential increase of signal – indirect indicator of the amount of haemozoin – until 48 hours of incubation was expected. Consequently, after 24 hours of incubation, it is already possible to see growth in all drugs, except for pyrimethamine, and to easily distinguish a grown sample from a non-grown sample. In fact, with piperaquine and chloroquine – which, being quinolones, interact with haemozoin production [9,98,106,108-110] –, growth is already seen at 20 hours of incubation.

For pyrimethamine, growth is observable at 14 hours and clear distinction from inhibited samples is possible at the 60-hour timepoint; for chloroquine with the *P. falciparum* Dd2 strain, since none of the drug concentrations influence parasite growth, growth is observed already at the 14-hour timepoint.

Since the purpose was to further evaluate this method as a sensitivity test for Plasmodium, the 24-hour timepoint was chosen for subsequent confirmation assays, since this is where the inhibition effects start to diverge.

2. Can the MOT detect inhibition effects from antimalarials? Is it reproducible?

This MO method promised to overcome some of the mentioned drawbacks for current sensitivity tests, while still being an effective tool in assessing inhibition effects of known antimalarials. Orbán and colleagues' (2014) preliminary data indicated it might be an efficient tool to assess the amount of hemozoin produced by the different parasite stages in synchronized cultures; therefore, it could eventually be used for testing the susceptibility of *Plasmodium* parasites to antimalarial drugs. [97]

With this study, it was possible to verify the MOT indeed detects inhibition effects of some of the most commonly used antimalarials nowadays. These effects were, for the most part, easily assessed and showed a correlation between drug concentrations and inhibitory effects. However, they did not occur the same way in all confirmation assays with the same drug; in other words, it seemed as the same concentration of a drug did not behave the same way in all replicate assays.

This variability was reflected on the diversity of IC50 obtained for the same antimalarials – for instance, the IC50 for DHA raged from 2 nM to 26. This variability could be due to the MOT or to the drug assay itself. The fact at the beginning of each replicate assay, all samples have somewhat the

same signal, indicates the observed variability might me due to the whole process before the MOT measurements – since the difference is between 0-hour timepoint of replicates and not between samples of the same replicate.

Ring and early trophozoites are thought to convert about 3-5% and 15-20% of host haemoglobin, respectively, and schizonts about 50–70%; [48,50] there is hence no precise haemoglobin digestion and consequent haemozoin production rate, so these could vary along these ranges within the same population of parasites. This variation would implicate a wide variation on haemozoin amount in each well – which would mean triplicates of the same sample would behave differently.

Despite the initial conditions being standardized, the initial MLD values were never the same for correspondent samples. Since at each timepoint a different microplate was measured – meaning there were not the same parasites being assessed at different timepoints –, the different proteolysis and subsequent haemozoin production rates would translate in such different results, where similar amounts of haemozoin should be produced, theoretically.

Additionally, the assays where the initial MLD values were higher were not necessarily those where the final MLD values were also the higher. Thus, this seems to be the most likely reason for most of the obtained variability. Therefore, reproducibility of the MOT does not seem questionable at this point. Nevertheless, being just a prototype, the instrument might also have introduced some variability in the assays' results.

Since this prototype consisted of several optical components screwed to a steady platform, these components got progressively loosened due to extensive use; this would turn the instrument increasingly unstable, which would obviously introduce variability while measuring the same sample. Even if the components were screwed back, this replacement was obviously not completely accurate and hence the photodetector could possibly detect differences in MLD signal.

The protocol of the sensitivity assays might also not be optimised to obtain the best results possible. Basco (2007) summarised several parameters which could influence the outcome of a sensitivity assay, some intrinsic to *in vitro* sensitivity assays. These range from the use of Albumax in the composition of MCM, going through the fact different solvents were used for different drugs, to the tendency of some drugs to bind to specific types of materials. [74]

Desjardins *et al.* (1979) recommended preparation of stock solutions in 70% ethanol and further dilutions in RPMI 1640 with HEPES, $NaHCO_3$ and 10% human plasma/serum; [76] this would standardise this parameter and eliminate subsequent variability. However, each antimalarial has its own optimal solubility and requires an appropriate solvent; despite all working solutions being prepared in MCM, each stock solution was prepared with a different solvent.

Lell, Binh & Kremsner (2000) studied the effects of alcohol on malaria parasite growth; the authors concluded the presence of ethanol inhibited parasite growth by 20-30% during a 48-hour incubation. When fresh drug dilutions containing DMSO or ethanol – which was the case for DHA and PYR, respectively – the final concentrations of these solvents should hence not overcome 0.1%, in order to avoid toxic effects on the parasites which will obviously alter growth-effects. [99]

Most of the used materials in the drug assays were plastic and some drugs highly adsorb in plastic materials; since the final drug concentrations are in the nanomolar range, this could lead to

extremely inconsistent results and therefore increased estimated IC50. According to Basco (2007), chloroquine is the only used drug not to bind to plastics at the assays' temperatures.

According to Wein *et al.* (2010), reliability of antimalarial sensitivity tests also depends on drug mechanisms of action. [100] This means a sensitivity test based on haemozoin detection might assess inhibition effects of haemozoin-affecting antimalarials, such as chloroquine, more reliably; this, together with the fact that chloroquine does not bind to plastics, could explain why the results obtained for this antimalarial were less variable than the other used drugs.

Chloroquine was, indeed, the antimalarial which produced the most stable results and, consequently, IC50. IC50 calculated from flow cytometry values and found in the literature were very similar to those obtained from MOT values, unlike what happened with other drugs.

Lastly, Orbán *et al.* (2014) believed freshly lysed blood samples would have an improved detection limit with the MOT, and the performance of the method was limited by a residual MLD signal due to some part of the lysed cell suspension – since the used samples were kept frozen and only thawed at the time of measurements.

In fact, the variability test performed to assess the influence of red blood cells in the signal of a sample, showed samples with no red blood cells had a much lower signal than their counterparts with red blood cells – although they all contained the same amount of synthetic haemozoin –, indicating the presence of red blood cells disguises the true lower signal derived from the present haemozoin.

Overall, with this method, the researcher is able to assess inhibition effects of some common antimalarials on a *Plasmodium falciparum* strain, in a laboratory context, seemingly producing reproducible results.

3. Is this method practical?

The whole process of the sensitivity assays produced a high amount of waste, mainly plastic. Since each timepoint was assessed with a different microplate, and each sample was prepared in triplicate and then each triplicate transferred to a microtube, the amount of plastic waste produced was massive. This is obviously not feasible field-wise, since the amount of available material is not as much as in a laboratory in a developed country.

The process was also very time-consuming; it took a considerable amount of time between taking the plate from the incubator, transferring wells' content to the microtubes for sample lysing, transferring this to the sample holders, performing the measurements, and cleaning the materials. This would cause some samples to be in contact with the lysis solution for more time than others, until measurement. The different lysing times could also introduce a confounding factor to the end result, since the lysis solution seems to have a detrimental effect over time in the signal obtained – as it was observed in the variability tests.

Additionally, electrical power was used for the rotation of the magnet and batteries for the laser; this would not be feasible in malaria-endemic countries, where electric power is not available at all times. if this method was to be used in malaria-endemic countries in the future, this would also have to be optimised for field-use.

Overall, the assay process is not very practical, besides being laborious; nonetheless, the used protocol could be optimised in order to facilitate the process and subsequent measurement of a great number of samples. The amount of plastic material used should be decreased by cutting steps in the protocol and directly transfer the content of each well to the sample holders. These should also be developed to a more practical form, by automated filling with samples, instead of the manual filling by the handler.

VI – Conclusions

There are currently some sensitivity assays available for *P. falciparum*; although some are suitable to inform the authorities of inhibition effects in the context of creating public health guidelines and antimalarial drug development, each has its inherent disadvantages. The radioisotopic assay with [^{3}H]-hypoxanthine and the WHO microtest are the most widely accepted methods for sensitivity testing and subsequent IC50 determination.

Although the radioactive assay has become the global reference sensitivity test in developed countries, it cannot be the reference method for most malaria-endemic countries; thus, there is the need for standard non-radioactive sensitivity tests which are feasible even field-wise in malaria-endemic countries.

On the other hand, the WHO microtest is field-applicable and the required incubation period is of at least 24 hours. However, it is also labour-intensive and has the need for highly trained personnel, which still does not eliminate subjectivity issues; again, in malaria-endemic countries, highly trained personnel are scarce. Additionally, the WHO has recently discontinued the production of this sensitivity test – although it is still used.

Although molecular techniques are increasingly gaining recognition in the sensitivity tests field, and more molecular markers for drug resistance are being discovered, *in vitro* sensitivity testing is still crucial for drug screening and for the study of drug resistance of currently used antimalarials.

This Magneto-Optical Test has thus great potential for the use in malaria parasites detection for sensitivity testing, since it can in fact differentiate a non-infected sample from an infected one, and among infected between different amounts of haemozoin; however, it is still far from being at its optimal form.

Therefore, this method is still not feasible field-wise; nevertheless, when the instrument is optimised into a more portable version and the process of drug assays is further developed into a more practical – and less waste-producing protocol –, this method has the potential to be the new reference sensitivity test, since it would be automated, fast, and inexpensive.

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VIII – Appendices

Appendix A

Table A.1 – Signs and symptoms used to identify severe falciparum, vivax and knowlesi malaria, according to the WHO. Source: WHO Guidelines for the treatment of malaria, 3rd edition.

Sign or symptom	Parameter	Range
Impaired consciousness	Glasgow coma score (adults)	< 11
	Blantyre coma score (children)	< 3
Prostration	Generalised weakness: patient is unable to sit, stand or walk without assistance.	
Multiple convulsions	Number of episodes	> 2 within 24 hours
Acidosis	Base deficit	> 8 mEq/L
	Plasma bicarbonate level	< 15 mmol/L
	Venous plasma lactate	≥ 5 mmol/L
Hypoglycaemia	Blood/plasma glucose	< 2.2 mmol/L (40 mg/dL)
Severe anaemia	Parasite count (mandatory for *P. falciparum* and *P. knowlesi)*	> 10000/µL
	Haemoglobin concentration	≤ 5 g/dL (children under 12) < 7 g/dL (adults)
	Haematocrit	≤ 15% (children under 12) < 20% (adults)
Renal impairment	Plasma/serum creatinine	> 265 µmol/L (3 mg/dL)
	Blood urea	> 20 mmol/L
Jaundice	Parasite count (mandatory for *P. falciparum* and *P. knowlesi*)	> 100 000/µL (*P. falciparum*) > 20 000/ µL (*P. knowlesi*)
	Plasma/serum bilirubin	> 50 µmol/L
Pulmonary oedema	Radiologically confirmed	
	Oxygen saturation and respiratory rate	< 92 % on room air and > 30/min
Significant bleeding	Recurent/prolonged from the nose, gums or venepuncture sites; haematemesis and melaena	
Compensated shock	Capilary refill	≥ 3 s
	Temperature gradient on leg but no hypotension	
Decompensated shock	Impaired perfusion (mandatory)	Cool peripheries or prolonged capillary refill
	Systolic blood pressure	< 70 mm Hg (children) < 80 mm Hg (adults)
Hyperparasitaemia	Parasitaemia	> 10 % (*P. falciparum*)
	Parasite density	> 100 000/µL (*P. knowlesi*)

Note: for severe *P. vivax* malaria there are no parasite density thresholds.