Host and parasite factors influencing clinical malaria in African children

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Christian Ngouadjio Nguetse

aus Batouri, Kamerun

Tübingen

2016

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	06.12.2016
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Peter G. Kremsner
2. Berichterstatter:	Prof. Dr. Andreas Peschel

Table of Contents

ZUSAMMENFASSUNG

Seit einigen Jahren gibt es eine deutliche Abnahme sowohl in der Mortalität als auch in der Morbidität von Malaria in endemischen Gebieten. Dies ist der Einführung den Artemisinin-basierten Kombinationstherapien (ACTs) zu verdanken. Dennoch gehört Malaria nach wie vor zu einem der größten Gesundheitsprobleme in Afrika, vor allem südlich der Sahara. Das klinische Bild der Malaria wird sowohl vom Parasiten als auch von genetischen Faktoren des Wirtes stark beeinflusst. Aus diesem Grund setzt sich die vorliegende Arbeit mit möglichen Wirts- und Parasitengenen, die die Malariaanfälligkeit und das Behandlungsergebnis erheblich beeinflussen, auseinander. Im **ersten Kapitel** meiner Arbeit beschreibe ich eine einfache und schnelle Methode zur Extraktion nuklearer DNA aus Parasiten und habe eine Schleifen-vermittelte isothermale Amplifikations (loop mediated isothermal amplification (LAMP)) Methode etabliert, um Malariaparasiten zu detektieren. Diese Methode ist robust und schnell. Innerhalb von 45 Minuten werden aus Nukleinsäuren visuelle Ergebnisse - mit einem sehr niedrigen Detektionslimit von nur einem *Plasmodium falciparum* Parasiten/µL. Im **zweiten Kapitel** meiner Arbeit habe ich den humanen *FOXO3A* Varianten, der in der Lage ist das Immunsystem zu modulieren, genetisch untersucht. Die mögliche Verbindung des untersuchten *FOXO3A* Genvarianten rs12212067 T>G wurde in Personen mit klinischer Malaria ermittelt. Der untersuchte *FOXO3A* Genvariant konnte mit der Schwere der Malaria in Zusammenhang gebracht werden. Im **dritten Kapitel** meiner Arbeit bewertete ich den Zusammenhang zwischen Parasiten Genpolymorphismen mit der therapeutischen Wirksamkeit von Artesunate, welches Afrikanischen Kindern entweder in einer dreifachen Dosis oder in einer fünffachen Dosis verabreicht wurde. Es konnte gezeigt werden, dass eine dreifach Dosis, die intramuskulär verabreicht wird, nicht schlechter ist als die konventionelle fünffache Dosis. Darüber hinaus zeigten die Analysen, dass der *PfMDR1* N86Y Polymorphismus mit einer verspäteten Parasitenbeseitigung einhergeht, unabhängig von dem Behandlungsschema. Im **vierten Kapitel** untersuchte ich den Glukose-6-Phosphate Dehydrogenase (G6PD) Mangel, eine genetische Funktionsstörung, die zu einem partiellen Schutz gegen Malaria beiträgt. Anhand der Ergebnisse kann festgehalten werden, dass schwerer G6PD Mangel mit einer geringeren Hämoglobinkonzentration einhergeht und keine Verbindung zur Parasitendichte besteht. Zusammenfassend lässt

sich sagen, dass diese Arbeit unterschiedliche Wirts- und Parasitenfaktoren beleuchtet, die sowohl die klinische Malaria als auch die therapeutische Wirksamkeit des antimalaria Medikamentes Artesunate beeinflussen könnten.

ABSTRACT

In recent years, there has been a considerable reduction in malaria morbidity and mortality in endemic regions, following the implementation of artemisinin-based combination therapies (ACTs) as a first line treatment. Nevertheless, malaria yet remains a major public health problem in sub-Saharan Africa. The clinical course of malaria is essentially influenced by both parasite and host genetic factors. This dissertation aims to investigate possible host and parasite genes that may largely influence the malaria susceptibility and treatment outcome, respectively. In the **first chapter** of my thesis, I have described a simple and rapid method for extraction of nuclear DNA from parasites and established a loop mediated isothermal amplification (LAMP) methodology to detect malarial parasites. This methodology is robust with a turnover time of 45 minutes from nucleic acids isolation to visual readout of results, with a limit of detection to as low as one *Plasmodium falciparum* parasite/µL. In the **second chapter** of my thesis, I have investigated the human *FOXO3A* genetic variant which is known to modulate immune responses. The possible associations of the studied *FOXO3A* gene variant rs12212067 T>G were determined in a cohort of individuals with clinical malaria phenotypes. The investigated *FOXO3A* gene variant was associated with the severity of malaria. In the **third chapter** of my dissertation, I evaluated the association of parasite gene polymorphisms with therapeutic efficacy of parental artesunate administered to African children either as a three or a five dose regimen. The investigations led to the findings that three dose intra-muscular regimen was not inferior to the conventional five dose regimen. In addition, the analysis revealed that the *PfMDR1* N86Y polymorphism is associated with delayed parasite clearance irrespective of treatment regimen. In the **fourth chapter**, I investigated the glucose-6-phosphate dehydrogenase (G6PD) deficiency, a genetic disorder that is demonstrated to confer partial protection from malaria. The investigations led to the conclusion that severe G6PD deficiency was associated with a lower hemoglobin concentrations and there was no association with parasite densities. Taken together, the dissertation provides added value on different host and parasite factors that could influence clinical malaria and therapeutic efficacy of antimalarial drug artesunate.

LIST OF PAPERS

This doctoral dissertation is based on the following four original publications:

I. Port JR[#], **Nguetse C[#],** Adupko S, Velavan TP. A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification. *Malaria Journal*. 2014 Nov 24; 13:454. [#]Equal contribution. PMID: 25421401

II. **Nguetse CN,** Kremsner PG, Velavan TP. FOXO3A regulatory polymorphism and susceptibility to severe malaria in Gabonese Children. *Immunogenetics*. 2015 Feb; 67(2):67-71. PMID: 25421486

III. Kremsner PG, Adegnika AA, Hounkpatin AB, Zinsou JF, Taylor TE, Chimalizeni Y, Liomba A, Kombila M, Bouyou-Akotet MK, Mawili Mboumba DP, Agbenyega T, Ansong D, Sylverken J, Ogutu BR, Otieno GA, Wangwe A, Bojang KA, Okomo U, Sanya-Isijola F, Newton CR, Njuguna P, Kazungu M, Kerb R, Geditz M, Schwab M, Velavan TP, **Nguetse C,** Köhler C, Issifou S, Bolte S, Engleitner T, Mordmüller B, Krishna S. Intramuscular artesunate for severe malaria in African children: A multicenter, randomized controlled trial. *PLOS Medicine*. 2016 Jan 12; 13(1). PMID: 26757276

IV. **Nguetse CN,** Meyer CG, Adegnika AA, Agbenyega T, Ogutu BR, Kremsner PG, Velavan TP. Glucose-6-phosphate dehydrogenase deficiency and reduced Haemoglobin levels in African children with severe malaria. *Malaria Journal*. 2016 Jul 7; 15(1):346. PMID: 27388012

LIST OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
CYP2A6	Cytochrome P450 2A6
FOXO3	Forkhead box O3
FOXP3	Forkhead box P3
G6PD	Glucose-6-phosphate dehydrogenase
Hb	Hemoglobin
HIV	Human immunodeficiency virus
IFA	Immunofluorescence antibody
IFN-γ	Interferon gamma
IL-	Interleukin
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnancy
K13	Kelch propeller domain on parasite chromosome 13
LAMP	Loop mediated isothermal amplification
PCR	Polymerase chain reaction
PfATP6	*Plasmodium falciparum* Ca (2+)-adenosine tri-phosphate 6
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PfMDR1	*Plasmodium falciparum* multidrug resistance 1
PfMDRP1	*Plasmodium falciparum* multidrug resistance protein 1
RBCs	Red blood cells
RDTs	Rapid diagnostic tests
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UGT1A9	Uridine di-phosphate glycosyltransferase 1A9
UGT2B7	Uridine di-phosphate glycosyltransferase 2B7
WHO	World health organization

1. INTRODUCTION

1.1. Malaria: Generalities, epidemiology and causative agents

Malaria is a major public health concern in endemic areas. The disease is a vector-borne infection caused by an intracellular protozoan parasite belonging to the genus of *Plasmodium* and is transmitted to humans through the infective bite of a female *Anopheles* mosquito [1]. Classified as one of the ancient and widespread infectious diseases [2], malaria has evolved both biologically and phylogenetically along with the humans [3].

Although malaria-related mortality rates and case incidence have considerably reduced worldwide by 60% and 37%, respectively between 2000 and 2015, the disease remains a major threat with an estimated 3.2 billion people in 95 countries still at risk [4]. In 2015, World Health Organization (WHO) reported approximately 214 million cases and about 438,000 deaths occurring in the world with the highest morbidity and mortality rates (~90%) observed in Africa, especially among children under the age of five years [4].

The seasonal activity and the spatial limits of distribution of malaria are strongly influenced by meteorological factors, including temperature, rainfall and humidity [5, 6], and as well as by the local capacity to control the disease [5]. Malaria transmission occurs at higher rates in the tropical and sub-tropical countries (Figure 1). However, following the Global Malaria Eradication Program launched by the WHO in the 1950s, 79 countries successfully eliminated malaria. This was mostly achieved in the extra-tropics (Eurasia, northern America, most of northern Africa, and Australia) where malaria transmission was highly seasonal due to temperate climatic conditions and mainly caused by *P. vivax* [7]. Nevertheless, a possible risk of re-introduction of the disease in these countries is constant [7].

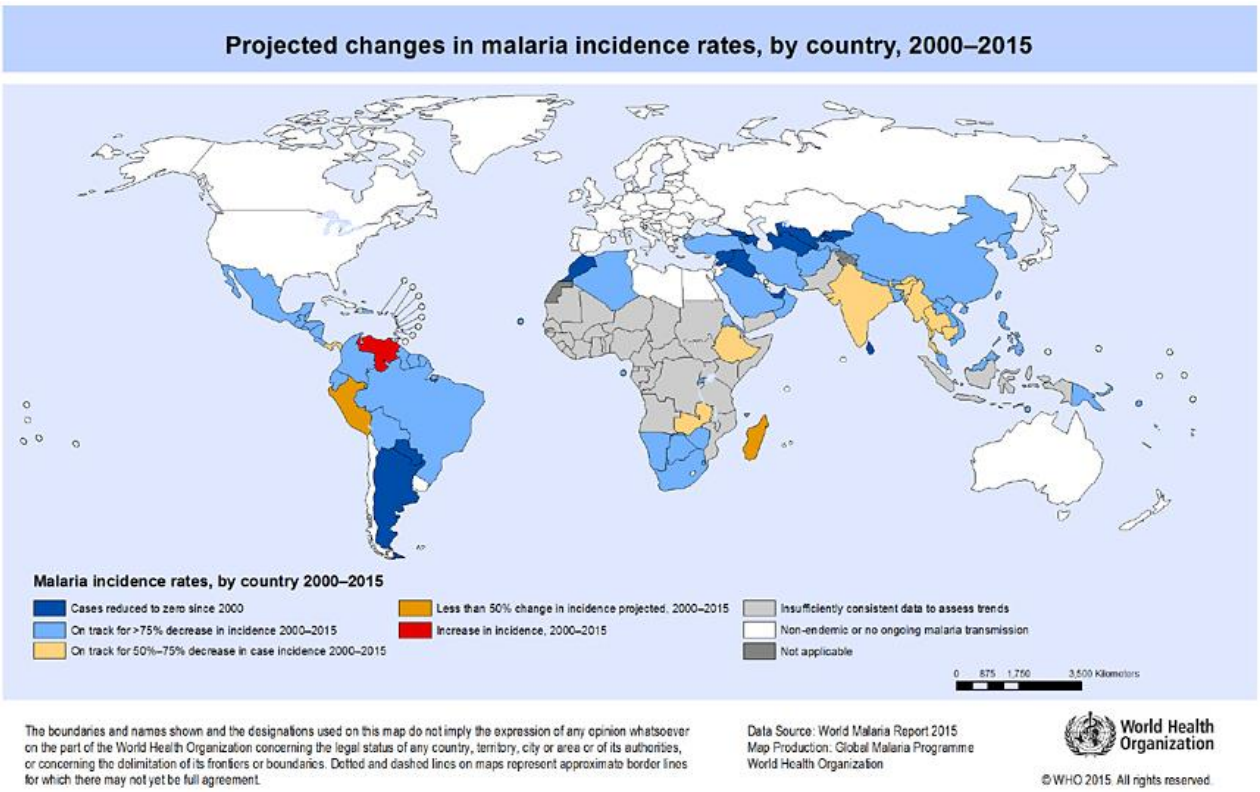

Figure 1: Malaria incidence in countries or areas at risk of transmission, 2015.
Source: World Malaria Report 2015, WHO [4].

For decades, only four *Plasmodium* species, *malaria*e, *ovale*, *vivax* and *falciparum*, were known to cause human malaria. Later a new species, *knowlesi* reported to infect humans was identified in South-East Asia [8-12]. In 2008, WHO recognized it as the fifth human *Plasmodium* species [13-15]. Among these species, *P. falciparum* is the most virulent and associated with the higher morbidity and mortality observed in affected countries [16]. For the disease to be transmitted, a vector, which is a female mosquito of the genus *Anopheles*, is required.

So far, 465 *Anopheles* species are properly identified [17]. Out of these 465 anopheline species, 70 are reported to be the vectors of human malaria [18] with 52 characterized as dominant vector species with the ability to transmit malaria at a level of major concern to public health [19]. Among these vectors, three namely *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* are reported to be the most efficient malaria vectors in the world [20, 21]. *Anopheles gambiae* being the main vector of *P. falciparum* [22].

1.2. Life cycle of Plasmodium spp.

The life cycle of all *Plasmodium* species is very similar following fundamentally the same process with many stages. The development of the *Plasmodium* involves a host change from an arthropod vector to a vertebrate host (Figure 2). The infection starts with the bite of an infected female *Anopheles* mosquito and the injection of sporozoites from the salivary glands into the host's bloodstream. In about 30 minutes, the sporozoites invade the hepatocytes and undergo asexual replication that results in the production of schizonts. The hepatocytes burst after approximately five days later, releasing merozoites which then invade the red blood cells (RBCs), initiating another asexual replication phase in the erythrocyte [23]. During nine days, the invaded merozoites develop from the trophozoite to the schizont containing 16 to 32 new merozoites which are released and re-invade new RBCs. The duration of the cycle in the erythrocyte is species dependent and varies between 24 hours (*P. knowlesi*) and 72 hours (*P. malariae*) [24]. Meanwhile, some parasites differentiate into the sexual forms, the male and female gametocytes, which are taken up by a female mosquito during her blood meal. Inside the mosquito mid-gut, the male gametocyte undergoes a nuclear division, producing eight flagellated microgametes that will fertilize the female macrogamete. At the end of the zygote development, hundreds of sporozoites are formed, and migrate to the mosquito salivary awaiting injection into the human host [24].

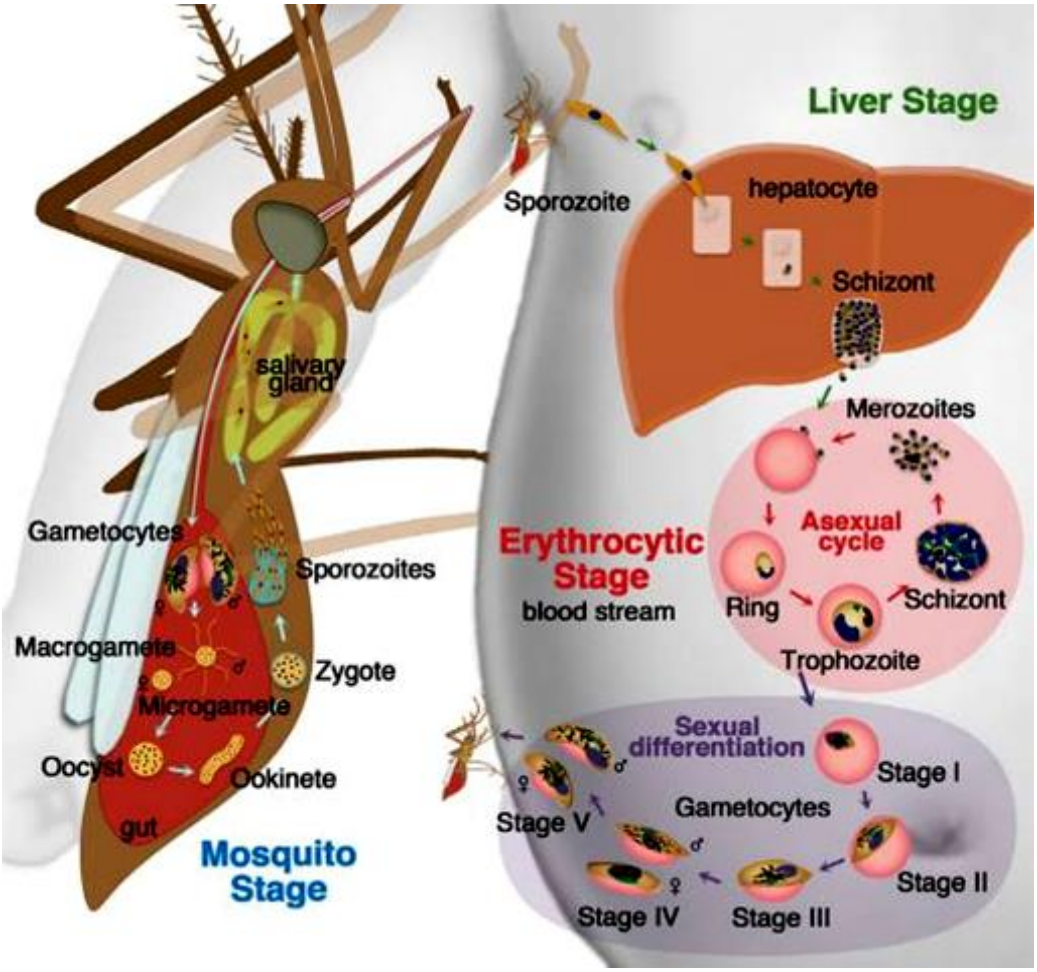

Figure 2: The life cycle of *Plasmodium* parasites. Source: Biamonte *et al*. 2013 [23]. Reprinted with permission from Elsevier Limited.

1.3. Malaria pathophysiology

With a complex pathogenesis, malaria infection has a very broad outcome [25]. The clinical presentation of the disease ranges from asymptomatic to severe and complicated forms through mild and uncomplicated malaria [26, 27].

Asymptomatic malaria

The definition of asymptomatic malaria remains a major problem due to the lack of standard diagnostic criteria. Many definitions involve the detection of asexual or sexual

parasites and an absence of any acute clinical symptoms, usually fever, during a specified time frame. Without being always explicit, asymptomatic malaria refers to bloodstream infections and does not include dormant liver stages [28]. On another hand, some studies have rather used parasitemia thresholds to define asymptomatic malaria. Only cases of fever with parasite densities above the predetermined cutoff were therefore considered to be symptomatic malaria cases [29]. However, the most widely used diagnosis criteria are presence of parasites in peripheral thick blood smears, an axillary temperature < 37.5°C, and an absence of malaria-related symptoms [30-33]. In the absence of transmission, the asymptomatic carriers constitute a major reservoir of the disease [34] and thus remain a significant obstacle in malaria elimination [35]. This is due, in addition to the obvious lack of clinical symptoms, and the presence of a submicroscopic parasitemia [36].

The adaptation of the malaria infection to an asymptomatic status is mediated by the acquisition of a partial immunity which can lead to a reduction in the acute clinical symptoms of the disease [28]. The development of this partial immunity is antigen-specific and mediated by exposure to genetically distinct parasite clones [37]. Therefore, in settings with high malaria transmission, constant exposure to malaria parasites leads to semi immunity and thus being symptom free after infection [38].

The contribution of host factors to this disease state is not fully elucidated. The main reason is that the majority of studies are rather focused either on the susceptibility or resistance to severe malaria [25, 39]. Earlier investigations reported that mutations in mannose binding lectin are significantly associated with the control of parasitemia in asymptomatic adults [40].

Uncomplicated malaria

According to WHO, uncomplicated malaria is a symptomatic disease without signs of severity or clinical/laboratory evidence of vital organ dysfunction [41]. Although the disease has some clinical manifestations, they are non-specific and mimic that of many other infections [42]. The symptoms include a vague absence of wellbeing, fever, fatigue, headache, muscle aches and abdominal discomfort [43]. These clinical signs are

influenced by the degree of the acquired immunity, parasite factors, and the previous exposure [44].

The association of host polymorphisms such as erythrocyte disorders with uncomplicated malaria remains less clear [45].

Severe malaria

The severity of malaria is mostly caused by *P. falciparum*. *P. vivax* and *P. ovale* can also produce serious complications leading to death. However, this is rare [46]. The definition of *P. falciparum* severe malaria follows the WHO criteria [27]. Symptoms and mortality depend on the age and malaria transmission [47]. Severe anemia and hypoglycemia are more common in children, whereas acute pulmonary edema, acute kidney injury, and jaundice are prevalent in adults. Coma (cerebral malaria) and acidosis occur in all age groups [48]. However the detailed information supporting the age-dependent severity is very scarce [49]. Different phenotypes related to severe malaria have been defined. They include severe malarial anemia and cerebral malaria [50].

The influence of the human factors on susceptibility and resistance to severe malaria has been extensively studied and several genetic polymorphisms have been identified to play a vital role [51-55].

1.4. Diagnosis, treatment and prevention

The diagnostic of malaria is still a challenge in many affected countries [56]. It is sometimes only based on clinical signs and symptoms although this method cannot differentiate malaria from other tropical diseases [57]. As the disease can quickly progress to a severe form, an effective diagnosis based on laboratory findings is necessary in order to reduce both complications and mortality. Microscopic examination of stained thin and thick blood films using Giemsa remains the gold standard. To improve the quality management of the disease, especially in remote areas, WHO has recommended the use of rapid diagnostic tests (RDTs), which are based on the detection of the malaria parasite

antigens, to supplement light microscopy [58]. Serological methods such as the immunofluorescence antibody testing (IFA) have been also used and are based on the detection of antibodies against asexual blood stage of parasites. Molecular techniques, e.g. polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) are highly sensitive and sophisticated tools for malaria diagnosis [57].

Malaria is a curable disease. Currently available antimalarial drugs include: cinchona alkaloids (quinine and quinidine), 4-methanolquinolines and amyl alcohols (mefloquine, lumefantrine and halofantrine), 4-aminoquinolines (chloroquine and amodiaquine), 8-aminoquinolines (primaquine and tafenoquine), antifolates (sulfadoxine, pyrimethamine, dapsone and proguanil), naphthoquinones (atovaquone), non-antifolate antibiotics (clindamycin, doxycycline, azythromycin and fosmidomycin) and artemisinins (artemisinin, dihydroartemisinin, artemether and artesunate) [59].

The prevention and control of malaria rely on a combination of antivectorial measures, including the use of insecticide-treated bed nets and indoor residual spray, and the chemoprophylaxis. In addition to the chemoprophylaxis allowed to international travelers, WHO also recommend a seasonal malaria chemoprevention for children and pregnant women as intermittent preventive treatment in infants (IPTi) and intermittent preventive treatment in pregnancy (IPTp), respectively [60].

Amongst the prevention measures, the vaccines are the most important tools. Unfortunately, there is currently no licensed malaria vaccine. The main reasons are the absence of immune correlate of protection and the high genetic diversity of parasites. [61]. So far, RTS,S, a pre-erythrocytic vaccine, is the most advanced candidate [62-65].

1.5. Antimalarial drug resistance

In 1973, WHO defined antimalarial drug resistance as the parasite strain ability to multiply and/or survive despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject

[66]. A decade later, the definition was amended to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" [67].

Resistance to antimalarial drugs arises from genetic events resulting from spontaneous and rare mutations, including gene duplications [68]. The mechanisms are known to vary and include alterations of drug transport and permeability, conversion of the drug to a form with lower activity, increased expression of the drug target, and alterations of the drug target that lowers its binding affinity [69].

The resistance of *Plasmodium* to antimalarial drugs started with the appearance and spread of mutations on the target of chloroquine [70]. As a consequence, sulfadoxine-pyrimethamine, an alternative to chloroquine at that time, was introduced. Unfortunately, resistance to this drug rapidly evolved, occurring at high frequency in malaria settings [71]. Following this additional failure, several other antimalarial drugs , including mefloquine, amodiaquine and quinine have since been deployed to fight resistant parasites [72]. Presently, artemisinin-based combination therapies (ACTs) highly efficacious drugs are used to improve the treatment efficacy and reduce the resistant parasite strains. Unfortunately, resistance to ACTs characterized as prolonged parasite clearance is already reported in South-East Asia [73].

1.5.1. Host genetics

Although antimalarial drug resistance is particularly associated with mutations in the parasites, host genetic factors are also known to play a significant role. The contribution of host factors, especially those linked with antimalarial drug metabolism remains poorly investigated [74]. Variations in the genes that metabolize drugs may possibly influence its clearance through differences in parasite exposure (time and plasma concentrations), and consequently prime for drug resistance [75]. Therefore, functional studies investigating the absorption–distribution–metabolism–excretion (ADME) properties are needed as they

may improve our knowledge on the pharmacokinetics and metabolic pathways of these drugs.

1.5.2. Parasite genetics

Drug resistance undeniably hinders the battle against malaria and subsequently increases the morbidity and mortality rates. The major cause associated with this phenomenon is the mutations found on the parasite protein-target genes. Various parasite proteins, including *P. falciparum* chloroquine resistance transporter (*PfCRT*) and the multidrug resistance-1 (*PfMDR1*) on the digestive vacuole [76-79], *P. falciparum Ca(2+)-ATP6* (*PfATP6*) located in the endoplasmic reticulum and suggested to be the artemisinin target [80], *P. falciparum* multidrug resistance protein-1 (*PfMDRP1*) found on the parasite plasma membrane [81], and the Kelch propeller domain (*K-13*) [82, 83] are associated with decrease parasite susceptibility to antimalarial drugs. However, there are no specific markers that can be widely used to monitor artemisinin resistance. In that regard, polymorphisms in the *K-13* gene have been suggested as perfect ones [82]. Unfortunately, the mutations in *K-13* associated with delayed parasite clearance in South-East Asia are absent in Africa [84, 85].

1.6. Immune response and severe malaria

The host immune system through both the adaptive and innate responses plays an essential role during malaria infection. After a repeated exposure to infection, individuals in endemic settings acquire some mechanisms which limit the inflammatory response and kill or inhibit the parasite replication [86]. This suggests that the outcome of malaria infections is probably determined by a delicate balance between pro-inflammatory and regulatory responses. The timing, the source and the site of these reactions being critical [87]. In response to *P. falciparum* malaria infection, various elements of the immune system such as macrophages and other important cellular effectors, molecules and cytokines will activate the immune system [88].

Among these immune elements, two cytokines, interferon γ (IFN-γ) and tumor necrosis factor (TNF) have been extensively studied as there are considered to be important for the destruction of the parasites [89]. Concerning the regulatory T cells (Tregs), key mediators of immune homeostasis, their role has recently received more focus [90].

During malaria infection, the induction of soluble immune mediators such interleukin-2 (IL-2), IL-10 and the transforming growth factor-beta (TGF-ß) via the induction and expansion of Tregs by the parasites has been reported to down-regulate the inflammatory responses [91]. Such specialized functions of the Tregs are mediated by the transcription factor FOXO3 [92], which is also involved in the regulation of several immune functions in dendritic and T cells [93] as well as in macrophages [94].

As a consequence of selection arising after exposure to various pathogens and also the need to prevent a self-destruction, the immune system genes are very polymorphic [95]. Genetic mutations in loci associated with Treg activity have been suggested to underlie both susceptibility to infection and level of Treg expression [96]. Previous studies of Hanel *et al.* [97] on the transcription factor FOXP3 revealed an association of promoter polymorphisms with the level of Treg expression and the susceptibility to parasitic infection. Recently, a polymorphism in the intronic *FOXO3A* region was identified and linked to the differential outcomes in inflammatory and infectious diseases [98].

2. AIMS

This thesis is a work that was part of the German-African cooperation project. The project was a sub-study of a large multicenter clinical trial study comparing the therapeutic efficacy of a simplified intravenous (i.v) and intramuscular (i.m) regimens of artesunate to the WHO-recommended regimen in African children with severe *P. falciparum* malaria. The major aim was to systematically investigate the contributions of the parasite and host genetic factors to clinical malaria and therapeutic efficacy.

To achieve these goals, distinct studies were performed using samples of individuals with asymptomatic infection, and severe *P. falciparum* malaria. The effects of the host genetics on the susceptibility to severe malaria, and the contribution of the parasite genetic factors to the therapeutic response were investigated.

More specifically, the objectives of the work presented in this dissertation are as follows:

- To standardize and implement a method for rapid isolation of nucleic acids using microwave irradiation and identification of *Plasmodium* spp using LAMP assay (**Paper I**)

- To investigate the contribution of host genetics to the differential disease prognosis during *P. falciparum* malaria (**Paper II**)

- To evaluate the efficacy of simplified regimens of artesunate, and determine the relationship between parasite genetics and therapeutic response (**Paper III**)

- To investigate the association of host genetics with the clinical parameters during severe *P. falciparum* malaria (**Paper IV**)

3. RESULTS

The results of each paper are summarized into this section. The references are made to the tables and figures in the different publications.

3.1. Paper I

A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification

Julia R Port[#], **Christian Nguetse[#]**, Selorme Adupko, Thirumalaisamy P. Velavan.
[#]Equal contribution.

***Malaria Journal* 2014, 13(1):454.**

An appropriate diagnosis of malaria is important for the substantial reduction of avoidable deaths. So far light microscopy remains the gold standard. However, rapid molecular diagnostic tests appear as an important alternative.

In this study, the microwave-based irradiation source was transformed into a standardized extraction-based technique for nucleic acids isolation from human blood and malaria parasite. In addition, a loop-mediated isothermal amplification (LAMP) methodology for the detection of the parasites was implemented. Blood samples collected in heparinized tubes or by finger prick and stored on sterile Whatman filter paper from Gabonese patients with severe *P. falciparum* malaria were used to validate the novel assay. The sensitivity of the conventional PCR and LAMP assays was established based on repeated dilution series till negativity of ring stage parasites from 3D7 cultures.

Following microwave irradiation, DNA containing vapor droplets were used for successful amplification of the *PfMDR1* gene of *P. falciparum* (Figure 2 - Paper I). The limit of detection for conventional PCR was up to five parasites/µL (Figure 3 – Paper I) whereas

the LAMP methodology was capable to detect as low as one parasite/µL (Figure 4 - Paper I) with a turnover time of 45 minutes.

In this study, a new standardized method for malaria diagnosis is presented. With the lack of sophisticated equipment in developing countries, this methodology may improve the diagnostic and the therapeutic interventions of malaria.

3.2. Paper II

FOXO3A regulatory polymorphism and susceptibility to severe malaria in Gabonese children

***Christian N. Nguetse**, Peter G. Kremsner, Thirumalaisamy P. Velavan.*

Immunogenetics 2015 Feb; 67(2):67-71.

In individuals affected with malaria, the clinical course of the disease varies. The genetic factors of the human host have been reported to influence the prognosis of malaria. Following an infection, an array of pro- and anti-inflammatory molecules are released. Among the molecules involved in the modulation of these inflammatory responses, the role of the transcription factor FOXO3 in mediating distinct functions of the regulatory T cell populations has been recently acknowledged.

The possible associations of the *FOXO3A* variant rs12212067T>G which has been associated with the prognosis of distinct inflammatory and infectious diseases were investigated in this study. A group of Gabonese children infected with *P. falciparum* were enrolled into the study, and the severe cases of malaria were compared to those with asymptomatic/mild cases.

The statistical analyses revealed that the *FOXO3A* polymorphism rs12212067T>G was significantly associated with the severe malaria phenotype following the allelic (OR=1.54, 95% CI=1.15–2.05, *P*=0.0028) and dominant (OR=1.94, 95 % CI=1.36–2.77, *P*=0.0002) models (Table 1 – Paper II).

This study unravels the pivotal role of the FOXO3-dependent pathway in malaria as it demonstrates the contribution of the *FOXO3A* variant rs12212067T>G to the increased inflammatory responses to *P. falciparum* malaria.

3.3. Paper III

Intramuscular artesunate for severe malaria in African children: a multicentre, randomised controlled trial

Peter G. Kremsner, Akim A. Adegnika, Aurore B. Hounkpatin, Jeannot F. Zinsou, Terrie E. Taylor, Yamikani Chimalizeni, Alice Liomba, Maryvonne Kombila, Marielle K. Bouyou-Akotet, Denise P. Mawili Mboumba, Tsiri Agbenyega, Daniel Ansong, Justice Sylverken, Bernhards R. Ogutu, Godfrey A. Otieno, Anne Wangwe, Kalifa A. Bojang, Uduak Okomo, Frank Sanya-Isijola, Charles R. Newton, Patricia Njuguna, Michael Kazungu, Reinhold Kerb, Mirjam Geditz, Matthias Schwab, Thirumalaisamy P. Velavan, ***Christian Nguetse****, Carsten Köhler, Saadou Issifou, Stefanie Bolte, Thomas Engleitner, Benjamin Mordmüller, Sanjeev Krishna.*

PLOS Medicine 2016 Jan 12; 13(1).

The antimalarial drug artesunate is now approved as the front-line treatment for severe malaria in both children and adults. A five-dose regimen administered intramuscularly (i.m) or intravenously (i.v) is recommended by WHO. However in resources limited-settings, its practicability is a great challenge as finding and maintaining i.v access, especially in children to ensure that correct doses are given on time is difficult. Therefore a simpler regimen is really needed and would be preferable.

A simplified (once daily during 3 days) dose regimen i.v artesunate has been previously investigated and reported to be non-inferior to the complex 5-dose regimen. However, its appraisal has since remained a long debate urging to investigate the i.m route as this has received so far or not little attention.

Intramuscular artesunate (total dose 12 mg/kg) split into simplified once daily 3-dose regimen (4 mg/kg) was compared for non-inferiority to the WHO-recommended 5-dose regimen in African children with severe malaria enrolled into a multicenter clinical trial. In addition to the primary and secondary objectives investigating the non-inferiority of the

simplified regimens, and comparing the tolerability and safety, respectively, analysis of the influence of parasites genetic factors on parasite clearance was done.

The results of the primary efficacy endpoint defined as the proportion of patient with ≥ 99% parasite reduction from the baseline parasitemia at 24±1 h demonstrate that in both the per protocol and intended to treat populations 3-dose i.m of artesunate was non-inferior to the 5-dose standard i.m regimen (Figures 3 and 4 – Paper III). In addition, the time to 99% parasite clearance was comparable between the treatment groups.

The investigation of the influence of the parasite genetics on the parasite clearance (PC) estimates revealed that *PfMDR1* N86Y was significantly associated with delayed time to 100% and 99% parasite clearance of 4.8 h (95% CI 1.9 – 7.6 h; $P< 0.001$) and 2.8 h (95% CI 0.9 – 4.8 h; $P= 0.005$), respectively.

The major drawback associated with parenteral artesunate is the occurrence of delayed hemolytic anemia happening between one to two weeks after treatment. Our investigations revealed a significant association between delayed anemia with the leukocyte count ($P<0.001$). Patients with delayed anemia had a significantly higher leukocytes count at Day 7 (Figure 8 – Paper III).

3.4. Paper IV

Glucose-6-phosphate dehydrogenase deficiency and reduced haemoglobin levels in African children with severe malaria.

Christian N. Nguetse, *Christian G. Meyer, Ayola Akim Adegnika, Tsiri Agbenyega, Bernhards R. Ogutu, Peter G. Kremsner, Thirumalaisamy P. Velavan.*

Malaria Journal 2016 Jul 7; 15(1):346.

Red blood cell polymorphisms, including glucose-6-phosphate dehydrogenase (G6PD) deficiency confer relative protection against severe malaria. G6PD is a key enzyme involved in the pentose phosphate pathway and provides a reduced form of nicotinamide adenine dinucleotide phosphate which helps the erythrocytes to counterbalance the oxidative stress. G6PD deficiency is an X-linked inherited disorder with three most common variants [376A (G6PD type B), 376G (G6PD deficiency type A^+) and 202A (severe G6PD deficiency type A^-)] prevalent in the African populations. We undertook this study to assess the effect of the 202A allele on the clinical presentation of severe malaria among African children.

The analysis revealed an overall prevalence of G6PD deficiency of 13% [36/278; 3% (4/132) female homozygous and 22% (32/146) male hemizygous]. The frequency of the other phenotypes was 14% (40/278) of female heterozygous and 73% (202/278) of G6PD normal [67% (88/132) females and 78% (114/146) males] children (Table 2 – Paper IV). The multivariate regression analysis showed that the moderately (type A^+) and severely (type A^-) deficient alleles were significantly associated with lower hemoglobin concentrations according to the baseline data [P<0.0001, (G6PD heterozygous: P<0.0001), (G6PD deficient: P=0.009)] without leading to SMA (P=0.66). There was no association of the *G6PD* alleles with the baseline parasite densities.

4. GENERAL DISCUSSION AND CONCLUSION

An important factor for the efficient management of malaria is the accurate and prompt diagnosis. The observation of Giemsa-stained blood smears using light microscopy remains the gold standard. However the technique is time and labor consuming and sometimes inaccurate as it requires considerable training and experience. As an alternative, immunochromatography-based rapid diagnostic tests (RDTs) were introduced but they showed limitations in detecting lower parasitemia. Molecular techniques such as the polymerase chain reaction (PCR) assays are highly sensitivity but require long time to get a result, expensive and sophisticated equipment. This makes their use difficult for point of care diagnostic tool in poor settings. The development of an alternative method that can combine speed and sensitivity will be advantageous. In that regard, a loop-mediated isothermal amplification (LAMP) method was developed. It amplifies the DNA with high specificity, efficiency and rapidity under isothermal conditions [99]. LAMP was primarily applied to pathogens causing food-borne disease before assays for a variety of viruses, including those responsible for severe acute respiratory syndrome (SARS), influenza, measles, human papilloma virus disease, and mumps, diseases of resource-limited settings such as human immunodeficiency virus (HIV), tuberculosis and African trypanosomiasis were developed [100]. The first primer sets for malaria targeted the 18S ribosomal RNA gene [101] and subsequently the mitochondrial DNA [102].

Together with high quality DNA, molecular assays are modern tools to improve detection of malaria cases [103]. As the extraction method is critical for the downstream applications, different methods have been developed to remove the inhibitory effect of blood during sample preparation [104-106]. However, they are labor-intensive, time-consuming, sample specific, subject to potential loss of the targeted microorganism or nucleic acid, and finally not suitable for automation [107].

In the first study, in attempts to simplify the procedure for DNA extraction and purification, we described a rapid and reliable procedure for nucleic acids isolation from human blood and malaria parasites using microwave irradiation. Additionally, we described a LAMP methodology for the molecular detection of malarial parasites.

The application of the microwave irradiation technique for the extraction of DNA and detection of some microorganisms such as HIV virus has been reported some decades ago [108-110]. Additional reagents or distilled water were used to facilitate the cytolysis, dilute the hemoglobin content and thus reduce its inhibitory effect on PCR [108]. In our study, we used phosphate buffered saline (PBS) solution only to elute the blood stored on filter paper during microwave irradiation treatment and alternatively for enduring storage. Although the use of PBS caused a certain decrease of sensitivity due to the dilution, the sensitivity levels were comparable to that of commercial kits. Besides, we were able to isolate DNA from fresh and archived blood samples and test by PCR and LAMP assays.

In malaria endemic settings, the prevalence of submicroscopic and asymptomatic parasitemia is very common, and these cases are sometimes misdiagnosed by the microscopy. To improve the malaria control strategies, there is therefore a need for a diagnostic tool able to detect subclinical infections such as the asymptomatic careers who contribute in maintaining the disease transmission and the endemicity [111-113]. In that regards, LAMP for malaria has been shown to reliably detect low parasitemic infections [114]. Another positive point of the LAMP methodology is its rapidity in achieving the result. Unlike the microscopy and other molecular diagnostic tools such PCR, LAMP is very fast as we demonstrated in our study. In fact, we achieved a final visual readout in 45 minutes. In developing countries and malaria endemic areas where the evolution of the disease to a severe case with a fatal outcome is very fast, we believe that LAMP will be a best alternative diagnostic tool.

However, like other molecular techniques, LAMP is also subject to contamination which can lead to false positive results. We therefore proposed to use the hydroxynaphthol blue (HNB) which is directly added to the primary reaction mixture. Nevertheless, the use of HNB may be a potential limitation of the LAMP methodology described here. In fact, this dye is sensitive to changes in pH and metal ion concentrations. Therefore, it is important to respect the concentrations of calcium, magnesium and pH of the master mix, as well as the DNA extraction method which may content reagents that affect the pH and metal ion concentrations [115].

Exposure of humans to changing environments associated with the differences in climate and pathogens, acted as selective forces, obliging them to adapt in order to survive [116-118]. Understanding of the type and intensity of the selection acting on human genome can help to identify the genes involved in rare, severe diseases or in susceptibility to complex diseases [119]. A plethora of studies have identified multiple signatures of selection in response to malaria (Review in [120]).

In malaria-affected individuals, the outcome of the disease will significantly vary and little is known about the mechanisms leading to this differential prognosis. The host immune system is suggested to play an essential role. Accumulated evidences show that malaria parasites trigger the secretion of many immune soluble mediators through the induction and expansion of regulatory T cells (Tregs) which will in turn down-regulate the inflammatory responses [91]. During infection, Tregs play an important role in controlling the immune responses and contributing to the stability of the immune homeostasis [121, 122]. This is mediated by the suppressive mechanisms of Tregs done via cell-cell interactions and/or production of suppressor cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-ß) [123], cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [124] and the programmed death (PD-1) [125, 126]. This mediation being strongly modulated by the FOXO3 transcription factor [93].

In the second study, we investigated the impact of the *FOXO3A* mutation rs12212067T>G on the outcome of malaria in the Gabonese population. Interestingly, our results showed that the minor allele *G* of this intronic variant was significantly associated with the increased risk of severe malaria. This suggests that FOXO3A interfere with the inflammatory responses in humans. Such association of the *FOXO3* mutations with a fatal disease outcome was previously reported with parthenogenesis and ovarian cancer in mice [127]. The underlying mechanisms are suggested to be via monocytes, important immune cells involved in the inflammatory responses during infectious diseases [98, 128]. In the case of severe malaria, FOXO3 is reported to limit the inflammatory responses in monocytes, which through the TGF-ß1, restricts the production of pro-inflammatory

cytokines, including tumor necrosis factor alpha (TNFα) and increases the production of anti-inflammatory cytokines such as IL-10 [98].

P. falciparum severe malaria accounts for the higher rates of mortality and morbidity observed in endemic settings. Till today there is not licensed vaccine to improve the malaria control strategies. Therefore, treatment with efficacious antimalarial drugs remains an appropriate solution for the management of the disease [129]. Based on the results of two large multicenter randomized controlled trials [130, 131], parenteral artesunate has been recommended as frontline treatment for severe malaria. However, population pharmacokinetic analyses showed that the drug properties may be or are altered by pregnancy, acute malaria infection [132], and body weight [133]. Therefore the selection of the route of administration is important. In that regard, studies to optimize the dosing regimens are needed, especially as the artesunate regimens for severe malaria are complex [129].

The WHO-recommended 5-dose regimen is practicably challenging in resources-limited settings. A simpler regimen has the advantages that it will help the healthcare workers to ensure that the correct doses are administrated on time.

In the third study, we investigated the efficacy of simplified 3-dose i.v and i.m regimens in African children with severe malaria. A particular focus was on the i.m regimen as there are limited pharmacokinetic data on i.m artesunate with respect to this population [133]. For comparison to the standard regimen, pharmacodynamic endpoints based on parasite clearance, a visible measure of the effectiveness of the drug, were used instead of pharmacokinetic variables [134]. The results demonstrated that the simplified i.m regimen, associated with a faster parasite clearance kinetics, is as efficacious as the 5-dose i.m regimen. Therefore, it is a preferable route for administration of antimalarial drugs in small children [135].

The pharmacokinetics of antimalarials might be influenced by numerous factors including the disease and the host parameters such as age, sex and weight [132, 133, 136]. However, in our study the analyses showed that the 3 regimens were of comparable

efficacy. These findings are in agreement with previous studies [133, 137]. They support the idea that a simplified once daily artesunate in higher dose (4 mg/kg) is sufficient to significantly reduce parasitemia.

We also assessed the effects of *P. falciparum* mutations on the drug susceptibility. We found that the *PfMDR1* N86Y mutation was associated with decreased parasite sensitivity to artesunate *in vivo*. Mutations in the *PfMDR1* gene have been associated with contradictory results ranging from no effect, decreased, or increased sensitivity to artemisinins in *in vitro* assays [138-141]. Our findings suggest a constant monitoring of parasites susceptibility to artemisinin compounds.

Although highly efficacious for treating severe malaria, many reports have revealed the appearance of delayed hemolysis between one to four weeks after treatment with parenteral artesunate [142]. The drug quality was suspected to be the plausible explanation [143]. However, a recent case of severe delayed hemolysis linked with GMP-certified artesunate [144], suggested no relation with the drug quality.

In our study, 22 % of children had delayed anemia defined as Hb ≤ 7g/dL seven days or more after admission. The analyses did not show any relationship with pharmacokinetic parameters for artesunate, dihydroartemisinin or dihydroartemisinin glucuronide (DHAG), sickle cell disease and G6PD deficiency. On the contrary, a higher leucocyte (neutrophil) count on Day 7 was significantly associated with delayed anemia. These findings suggest that delayed hemolysis might be related to the drug toxicity [145] and drug-dependent antibodies through an immune-related mechanism [146] rather than sickle cell disease and G6PD deficiency [147].

The investigations on susceptibility and protection against malaria have recognized the contribution of host genetic factors. These factors largely involve the red blood cell polymorphisms, including the glucose-6-phosphate dehydrogenase (G6PD) deficiency. As a consequence of the strong evolutionary pressure of malaria on the human genome, the frequencies of G6PD deficiency have raised [53] and a plethora of previous studies have reported a positive correlation between the appearance of G6PD deficiency and

malaria endemicity [reviewed in [148]. However, their true impact on clinical parameters during severe malaria remains not fully understood.

In the fourth study, we aimed at determining the frequency distribution of G6PD deficiency in malaria African children, and investigating the effects of *G6PD* genotypes on parasite densities, hemoglobin levels and severe malarial anemia during admission. Although many mutations in the *G6PD* gene have been identified and associated with low enzyme activity [149, 150], the most relevant three variants [376A (G6PD type B, no deficiency), 376G (moderate G6PD deficiency type A^+) and 202A severe G6PD deficiency type A^-] in Africa [151] were genotyped to determine the prevalence of G6PD deficiency in this study. The other variants being reported at a substantially lower frequencies [152, 153]. Our analyses revealed that the prevalence of G6PD deficiency substantially increased in our study in comparison to previous reports [39, 154-156]. The presence of G6PD deficiency and consequently its rise in prevalence in Africa hinder the world strategy of malaria elimination as it prevents the use of primaquine, an antimalarial drug effective for both transmission blocking of *P. falciparum* and anti-relapse treatment against *P. vivax*. In fact, primaquine is known to cause hemolytic anemia in G6PD deficient individuals [157].

During malaria infection, the elimination of parasitized red blood cells by the spleen contributes to the reduction of the number of circulating erythrocytes and consequently leads to anemia. We may then expect that in severely G6PD deficient individuals, the removal of infected erythrocytes associated with the reduced lifespan of red blood cells due to subclinical hemolysis [158] might significantly decrease the hemoglobin concentrations and cause severe malarial anemia. Interestingly, our results reveal that lower hemoglobin concentrations based on baseline data were significantly associated with type A^+ and type A^- *G6PD* genotypes. However, this lower hemoglobin levels did not lead to severe malarial anemia. Our findings are partially in agreement with the results of one of the largest and most comprehensive case-control study conducted in Kenya [159]. Like in our study, they found a significant association between G6PD deficiencies with lower hemoglobin concentrations at the time of hospital admission. However, in contrast to our findings they observed that G6PD deficient children who had lower hemoglobin

levels also had an increased risk for severe malarial anemia [159]. The likely explanation could be due to the sample size which was rather small in our study.

According to published studies suggesting the impaired ability of parasites to grow and replicate, or the rapid clearance of infected erythrocytes [160, 161], we hypothesized that the parasite densities might be lower in the G6PD deficient individuals. Unfortunately, our analyses did not detect a difference by G6PD status. However, severely G6PD deficient children had a lower parasitemia in comparison to others. This finding once again suggests that the underlying mechanisms of protection with G6PD deficiency are still to be elucidated [159].

CONCLUSION

In the first paper, the standardization and implementation of a reliable and rapid methods that improve the extraction of DNA, and the detection of plasmodia is described. These new techniques can easily be used in resources-limited settings to help diagnose the low-density malaria parasitemia. In the second paper, the host factors associated with severe *P. falciparum* malaria phenotype were studied. The findings improve our understanding of the genetics of infectious diseases such as malaria. In the third paper, simplified once daily 3-dose regimens were investigated for non-inferiority. Interestingly, they showed comparable efficacy in the reduction of parasitemia as the standard 5-dose regimen. The 3-dose i.m appeared to be a recommendable route for parenteral artesunate in febrile children. However, the parasite clearance kinetics were impaired by the *PfMDR1* N86Y mutation. In the last paper of this dissertation, G6PD deficiency was significantly associated with lower hemoglobin concentrations during severe malaria at the time of hospital admission. However, this was not related to the severe malarial anemia experienced by some individuals. In addition, there was no association of G6PD deficiency with parasitemia. Taken together, these studies expand our knowledge on the contribution of host and parasite genetic factors to malaria susceptibility and progression, and on another hand their associations with the treatment response.

5. PERSONAL CONTRIBUTIONS

The achievement of the aims and objectives of this thesis was a combined effort of all authors, including myself. However, my own contribution to the papers included:

Paper I: (*Malaria Journal* Published): A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification.

- I standardized and implemented the LAMP and microwave irradiation methodologies,
- I performed the laboratory experiments,
- I drafted, reviewed and approved the manuscript for publication.

Paper II: (*Immunogenetics* Published): *FOXO3A* regulatory polymorphism and susceptibility to severe malaria in Gabonese children.

- I contributed to the study design,
- I performed the laboratory experiments,
- I analyzed and interpreted the datasets,
- I drafted, revised and approved the manuscript for publication.

Paper III: (*Plos Medicine* Published): Intramuscular artesunate for severe malaria in African children: a multicentre, randomised controlled trial.

- I performed the laboratory experiments on parasite and host genes,
- I performed the pharmacogenomics analyses,
- I contributed to the revision and approval of the manuscript for publication.

Paper IV: (*Malaria Journal* Published): Glucose-6-phosphate dehydrogenase deficiency and reduced haemoglobin levels in African children with severe malaria.

- I performed the laboratory experiments,
- I analyzed and interpreted the datasets,
- I drafted, reviewed and approved the manuscript for publication.

6. REFERENCES

1. Cox, F.E., *History of the discovery of the malaria parasites and their vectors.* Parasit Vectors, 2010. **3**(1): p. 5.
2. Carter, R. and K.N. Mendis, *Evolutionary and historical aspects of the burden of malaria.* Clin Microbiol Rev, 2002. **15**(4): p. 564-94.
3. Sabbatani, S., R. Manfredi, and S. Fiorino, *Malaria infection and human evolution.* Infez Med, 2010. **18**(1): p. 56-74.
4. WHO, *World Malaria Report*. 2015. 280.
5. Caminade, C., et al., *Impact of climate change on global malaria distribution.* Proc Natl Acad Sci U S A, 2014. **111**(9): p. 3286-91.
6. Alemu, A., et al., *Climatic variables and malaria transmission dynamics in Jimma town, South West Ethiopia.* Parasit Vectors, 2011. **4**: p. 30.
7. Feachem, R.G., et al., *Shrinking the malaria map: progress and prospects.* Lancet, 2010. **376**(9752): p. 1566-78.
8. Singh, B., et al., *A large focus of naturally acquired Plasmodium knowlesi infections in human beings.* Lancet, 2004. **363**(9414): p. 1017-24.
9. Lee, K.S., et al., *Plasmodium knowlesi from archival blood films: further evidence that human infections are widely distributed and not newly emergent in Malaysian Borneo.* Int J Parasitol, 2009. **39**(10): p. 1125-8.
10. Sulistyaningsih, E., et al., *Diagnostic difficulties with Plasmodium knowlesi infection in humans.* Emerg Infect Dis, 2010. **16**(6): p. 1033-4.
11. Putaporntip, C., et al., *Differential prevalence of Plasmodium infections and cryptic Plasmodium knowlesi malaria in humans in Thailand.* J Infect Dis, 2009. **199**(8): p. 1143-50.
12. Jiang, N., et al., *Co-infections with Plasmodium knowlesi and other malaria parasites, Myanmar.* Emerg Infect Dis, 2010. **16**(9): p. 1476-8.
13. Cox-Singh, J. and B. Singh, *Knowlesi malaria: newly emergent and of public health importance?* Trends Parasitol, 2008. **24**(9): p. 406-10.
14. White, N.J., *Plasmodium knowlesi: the fifth human malaria parasite.* Clin Infect Dis, 2008. **46**(2): p. 172-3.
15. Cox-Singh, J., et al., *Severe malaria - a case of fatal Plasmodium knowlesi infection with post-mortem findings: a case report.* Malar J, 2010. **9**: p. 10.
16. Perkins, D.J., et al., *Severe malarial anemia: innate immunity and pathogenesis.* Int J Biol Sci, 2011. **7**(9): p. 1427-42.
17. RE, H., *Genus Anopheles Meigen, 1818. Mosquito Taxonomic Inventory. Book Genus Anopheles Meigen, 1818 Mosquito Taxonomic Inventory City.* . 2011.
18. Service MW, T.H., *The Anopheles vector. In: Gilles HM, Warrell DA, editors. Essential Malariology.* Fourth edition ed. London: Arnold; ed. 2002.
19. Hay, S.I., et al., *Developing global maps of the dominant anopheles vectors of human malaria.* PLoS Med, 2010. **7**(2): p. e1000209.
20. Tonnang, H.E., R.Y. Kangalawe, and P.Z. Yanda, *Predicting and mapping malaria under climate change scenarios: the potential redistribution of malaria vectors in Africa.* Malar J, 2010. **9**: p. 111.
21. Collins, F.H. and N.J. Besansky, *Vector biology and the control of malaria in Africa.* Science, 1994. **264**(5167): p. 1874-5.
22. Cohuet, A., et al., *Evolutionary forces on Anopheles: what makes a malaria vector?* Trends Parasitol, 2010. **26**(3): p. 130-6.
23. Biamonte, M.A., J. Wanner, and K.G. Le Roch, *Recent advances in malaria drug discovery.* Bioorg Med Chem Lett, 2013. **23**(10): p. 2829-43.

24. Faik, I., E.G. de Carvalho, and J.F. Kun, *Parasite-host interaction in malaria: genetic clues and copy number variation.* Genome Med, 2009. **1**(9): p. 82.
25. Laishram, D.D., et al., *The complexities of malaria disease manifestations with a focus on asymptomatic malaria.* Malar J, 2012. **11**: p. 29.
26. Marsh, K., et al., *Indicators of life-threatening malaria in African children.* N Engl J Med, 1995. **332**(21): p. 1399-404.
27. *Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster.* Trans R Soc Trop Med Hyg, 2000. **94 Suppl 1**: p. S1-90.
28. Lindblade, K.A., et al., *The silent threat: asymptomatic parasitemia and malaria transmission.* Expert Rev Anti Infect Ther, 2013. **11**(6): p. 623-39.
29. Smith, T., J.A. Schellenberg, and R. Hayes, *Attributable fraction estimates and case definitions for malaria in endemic areas.* Stat Med, 1994. **13**(22): p. 2345-58.
30. Hassanein, T., et al., *Liver failure occurring as a component of exertional heatstroke.* Gastroenterology, 1991. **100**(5 Pt 1): p. 1442-7.
31. Leoratti, F.M., et al., *Pattern of humoral immune response to Plasmodium falciparum blood stages in individuals presenting different clinical expressions of malaria.* Malar J, 2008. **7**: p. 186.
32. Males, S., O. Gaye, and A. Garcia, *Long-term asymptomatic carriage of Plasmodium falciparum protects from malaria attacks: a prospective study among Senegalese children.* Clin Infect Dis, 2008. **46**(4): p. 516-22.
33. Duarte, J., et al., *Total and functional parasite specific IgE responses in Plasmodium falciparum-infected patients exhibiting different clinical status.* Malar J, 2007. **6**: p. 1.
34. Zwetyenga, J., et al., *A cohort study of Plasmodium falciparum diversity during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria.* Trans R Soc Trop Med Hyg, 1999. **93**(4): p. 375-80.
35. Lin, J.T., D.L. Saunders, and S.R. Meshnick, *The role of submicroscopic parasitemia in malaria transmission: what is the evidence?* Trends Parasitol, 2014. **30**(4): p. 183-90.
36. Bottius, E., et al., *Malaria: even more chronic in nature than previously thought; evidence for subpatent parasitaemia detectable by the polymerase chain reaction.* Trans R Soc Trop Med Hyg, 1996. **90**(1): p. 15-9.
37. Smith, T., et al., *Premunition in Plasmodium falciparum infection: insights from the epidemiology of multiple infections.* Trans R Soc Trop Med Hyg, 1999. **93 Suppl 1**: p. 59-64.
38. Magesa, S.M., et al., *Diversity of Plasmodium falciparum clones infecting children living in a holoendemic area in north-eastern Tanzania.* Acta Trop, 2002. **84**(2): p. 83-92.
39. Mombo, L.E., et al., *Human genetic polymorphisms and asymptomatic Plasmodium falciparum malaria in Gabonese schoolchildren.* Am J Trop Med Hyg, 2003. **68**(2): p. 186-90.
40. Boldt, A.B., et al., *Haplotype specific-sequencing reveals MBL2 association with asymptomatic Plasmodium falciparum infection.* Malar J, 2009. **8**: p. 97.
41. WHO, *Guidelines for the treatment of malaria*. 2nd edition ed. 2010, Geneva.
42. Grobusch, M.P. and P.G. Kremsner, *Uncomplicated malaria.* Curr Top Microbiol Immunol, 2005. **295**: p. 83-104.
43. Bell, D. and P. Winstanley, *Current issues in the treatment of uncomplicated malaria in Africa.* Br Med Bull, 2004. **71**: p. 29-43.
44. Miller, L.H., et al., *The pathogenic basis of malaria.* Nature, 2002. **415**(6872): p. 673-9.
45. Migot-Nabias, F., et al., *Red blood cell polymorphisms in relation to Plasmodium falciparum asymptomatic parasite densities and morbidity in Senegal.* Microbes Infect, 2006. **8**(9-10): p. 2352-8.
46. Svenson, J.E., et al., *Imported malaria. Clinical presentation and examination of symptomatic travelers.* Arch Intern Med, 1995. **155**(8): p. 861-8.

47. Dondorp, A.M., et al., *The relationship between age and the manifestations of and mortality associated with severe malaria.* Clin Infect Dis, 2008. **47**(2): p. 151-7.
48. White, N.J., et al., *Malaria.* Lancet, 2014. **383**(9918): p. 723-35.
49. Lalloo, D.G., P. Olukoya, and P. Olliaro, *Malaria in adolescence: burden of disease, consequences, and opportunities for intervention.* Lancet Infect Dis, 2006. **6**(12): p. 780-93.
50. Driss, A., et al., *Genetic polymorphisms linked to susceptibility to malaria.* Malar J, 2011. **10**: p. 271.
51. Malaria Genomic Epidemiology, N., et al., *A novel locus of resistance to severe malaria in a region of ancient balancing selection.* Nature, 2015.
52. Manjurano, A., et al., *African glucose-6-phosphate dehydrogenase alleles associated with protection from severe malaria in heterozygous females in Tanzania.* PLoS Genet, 2015. **11**(2): p. e1004960.
53. Malaria Genomic Epidemiology, N. and N. Malaria Genomic Epidemiology, *Reappraisal of known malaria resistance loci in a large multicenter study.* Nat Genet, 2014. **46**(11): p. 1197-204.
54. Timmann, C., et al., *Genome-wide association study indicates two novel resistance loci for severe malaria.* Nature, 2012. **489**(7416): p. 443-6.
55. Taylor, S.M., C. Cerami, and R.M. Fairhurst, *Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis.* PLoS Pathog, 2013. **9**(5): p. e1003327.
56. Moody, A., *Rapid diagnostic tests for malaria parasites.* Clin Microbiol Rev, 2002. **15**(1): p. 66-78.
57. Tangpukdee, N., et al., *Malaria diagnosis: a brief review.* Korean J Parasitol, 2009. **47**(2): p. 93-102.
58. *A rapid dipstick antigen capture assay for the diagnosis of falciparum malaria. WHO Informal Consultation on Recent Advances in Diagnostic Techniques and Vaccines for Malaria.* Bull World Health Organ, 1996. **74**(1): p. 47-54.
59. S, S.H.a.K., *Treatment and Prevention of Malaria. Antimalarial Drug Chemistry, Action and Use. Milestones in Drug Therapy.* Edition Number 1. ed. 2012: Springer Basel.
60. WHO, *WHO policy recommendation: Seasonal malaria chemoprevention (SMC) for Plasmodium falciparum malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa.* March 2012.
61. Ouattara, A. and M.B. Laurens, *Vaccines against malaria.* Clin Infect Dis, 2015. **60**(6): p. 930-6.
62. Targett, G., *Phase 3 trial with the RTS,S/AS01 malaria vaccine shows protection against clinical and severe malaria in infants and children in Africa.* Evid Based Med, 2015. **20**(1): p. 9.
63. Rts, S.C.T.P., *Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites.* PLoS Med, 2014. **11**(7): p. e1001685.
64. Rts, S.C.T.P., et al., *A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants.* N Engl J Med, 2012. **367**(24): p. 2284-95.
65. Agnandji, S.T., et al., *First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children.* N Engl J Med, 2011. **365**(20): p. 1863-75.
66. WHO, *Chemotherapy of Malaria and Resistance to Antimalarials.* 1973: WHO, Geneva.
67. Bruce-Chwatt LJ, B.R., Canfield CJ, Clyde DF, Peters W and Wernsdorfer WH., *Chemotherapy of malaria*. revised 2nd ed. ed. 1986, Geneva: World Health Organization.
68. White, N.J., *Antimalarial drug resistance.* J Clin Invest, 2004. **113**(8): p. 1084-92.
69. Mwangi, J.M. and L.C. Ranford-Cartwright, *Genetic and genomic approaches for the discovery of parasite genes involved in antimalarial drug resistance.* Parasitology, 2013. **140**(12): p. 1455-67.
70. Kim, Y.S., K. A. *Evolution of Drug Resistance in Malaria Parasite Populations.* 2013. **4(8):6**.
71. Clyde, D.F. and G.T. Shute, *Resistance of Plasmodium falciparum in Tanganyika to pyrimethamine administered at weekly intervals.* Trans R Soc Trop Med Hyg, 1957. **51**(6): p. 505-13.
72. Petersen, I., R. Eastman, and M. Lanzer, *Drug-resistant malaria: molecular mechanisms and implications for public health.* FEBS Lett, 2011. **585**(11): p. 1551-62.

73. Dondorp, A.M., et al., *Artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2009. **361**(5): p. 455-67.
74. Mehlotra, R.K., C.N. Henry-Halldin, and P.A. Zimmerman, *Application of pharmacogenomics to malaria: a holistic approach for successful chemotherapy.* Pharmacogenomics, 2009. **10**(3): p. 435-49.
75. Kerb, R., et al., *Pharmacogenetics of antimalarial drugs: effect on metabolism and transport.* Lancet Infect Dis, 2009. **9**(12): p. 760-74.
76. Ecker, A., et al., *PfCRT and its role in antimalarial drug resistance.* Trends Parasitol, 2012. **28**(11): p. 504-14.
77. Valderramos, S.G. and D.A. Fidock, *Transporters involved in resistance to antimalarial drugs.* Trends Pharmacol Sci, 2006. **27**(11): p. 594-601.
78. Griffing, S., et al., *pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in Plasmodium falciparum in Venezuela.* Antimicrob Agents Chemother, 2010. **54**(4): p. 1572-9.
79. Ecker A, L.A., Fidock DA., *Molecular markers of Plasmodium resistance to antimalarials. In: Staines HM, Krishna S, editors. Treatment and prevention of malaria: antimalarial drug chemistry, action and use.* 2012, Basel, Switzerland:: Birkhauser Verlag.
80. Krishna, S., et al., *Artemisinins and the biological basis for the PfATP6/SERCA hypothesis.* Trends Parasitol, 2010. **26**(11): p. 517-23.
81. Raj, D.K., et al., *Disruption of a Plasmodium falciparum multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione.* J Biol Chem, 2009. **284**(12): p. 7687-96.
82. Ariey, F., et al., *A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.* Nature, 2014. **505**(7481): p. 50-5.
83. Ashley, E.A., et al., *Spread of artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2014. **371**(5): p. 411-23.
84. Taylor, S.M., et al., *Absence of putative artemisinin resistance mutations among Plasmodium falciparum in Sub-Saharan Africa: a molecular epidemiologic study.* J Infect Dis, 2015. **211**(5): p. 680-8.
85. Kamau, E., et al., *K13-propeller polymorphisms in Plasmodium falciparum parasites from sub-Saharan Africa.* J Infect Dis, 2015. **211**(8): p. 1352-5.
86. Artavanis-Tsakonas, K., J.E. Tongren, and E.M. Riley, *The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology.* Clin Exp Immunol, 2003. **133**(2): p. 145-52.
87. Finney, O.C., E.M. Riley, and M. Walther, *Regulatory T cells in malaria--friend or foe?* Trends Immunol, 2010. **31**(2): p. 63-70.
88. Malaguarnera, L. and S. Musumeci, *The immune response to Plasmodium falciparum malaria.* Lancet Infect Dis, 2002. **2**(8): p. 472-8.
89. Langhorne, J., et al., *Immunity to malaria: more questions than answers.* Nat Immunol, 2008. **9**(7): p. 725-32.
90. Scholzen, A., G. Minigo, and M. Plebanski, *Heroes or villains? T regulatory cells in malaria infection.* Trends Parasitol, 2010. **26**(1): p. 16-25.
91. Scholzen, A., et al., *Plasmodium falciparum-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta.* PLoS Pathog, 2009. **5**(8): p. e1000543.
92. Kerdiles, Y.M., et al., *Foxo transcription factors control regulatory T cell development and function.* Immunity, 2010. **33**(6): p. 890-904.
93. Dejean, A.S., et al., *Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells.* Nat Immunol, 2009. **10**(5): p. 504-13.

94. Litvak, V., et al., *A FOXO3-IRF7 gene regulatory circuit limits inflammatory sequelae of antiviral responses.* Nature, 2012. **490**(7420): p. 421-5.
95. Maizels, R.M., *Parasite immunomodulation and polymorphisms of the immune system.* J Biol, 2009. **8**(7): p. 62.
96. Velavan, T.P. and O. Ojurongbe, *Regulatory T cells and parasites.* J Biomed Biotechnol, 2011. **2011**: p. 520940.
97. Hanel, S.A., et al., *Novel and functional regulatory SNPs in the promoter region of FOXP3 gene in a Gabonese population.* Immunogenetics, 2011. **63**(7): p. 409-15.
98. Lee, J.C., et al., *Human SNP links differential outcomes in inflammatory and infectious disease to a FOXO3-regulated pathway.* Cell, 2013. **155**(1): p. 57-69.
99. Notomi, T., et al., *Loop-mediated isothermal amplification of DNA.* Nucleic Acids Res, 2000. **28**(12): p. E63.
100. Hsiang, M.S., B. Greenhouse, and P.J. Rosenthal, *Point of care testing for malaria using LAMP, loop mediated isothermal amplification.* J Infect Dis, 2014. **210**(8): p. 1167-9.
101. Han, E.T., et al., *Detection of four Plasmodium species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis.* J Clin Microbiol, 2007. **45**(8): p. 2521-8.
102. Polley, S.D., et al., *Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification.* J Clin Microbiol, 2010. **48**(8): p. 2866-71.
103. Morris, U., et al., *Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability.* Malar J, 2013. **12**: p. 106.
104. Klein, A., et al., *Comparison of methods for extraction of nucleic acid from hemolytic serum for PCR amplification of hepatitis B virus DNA sequences.* J Clin Microbiol, 1997. **35**(7): p. 1897-9.
105. Cattaneo, C., et al., *Comparison of three DNA extraction methods on bone and blood stains up to 43 years old and amplification of three different gene sequences.* J Forensic Sci, 1997. **42**(6): p. 1126-35.
106. Walsh, P.S., D.A. Metzger, and R. Higuchi, *Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material.* Biotechniques, 1991. **10**(4): p. 506-13.
107. Al-Soud, W.A. and P. Radstrom, *Purification and characterization of PCR-inhibitory components in blood cells.* J Clin Microbiol, 2001. **39**(2): p. 485-93.
108. Jadaon, M.M., et al., *Whole-blood polymerase chain reaction and restriction fragment length polymorphism: a simplified method by microwave irradiation.* Med Princ Pract, 2009. **18**(4): p. 280-3.
109. Sato, Y., et al., *Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues.* Diagn Mol Pathol, 2001. **10**(4): p. 265-71.
110. Bourinbaiar, A.S., V.R. Zacharopoulos, and D.M. Phillips, *Microwave irradiation-accelerated in situ hybridization technique for HIV detection.* J Virol Methods, 1991. **35**(1): p. 49-58.
111. Cotter, C., et al., *The changing epidemiology of malaria elimination: new strategies for new challenges.* Lancet, 2013. **382**(9895): p. 900-11.
112. Sturrock, H.J., et al., *Targeting asymptomatic malaria infections: active surveillance in control and elimination.* PLoS Med, 2013. **10**(6): p. e1001467.
113. Okell, L.C., et al., *Factors determining the occurrence of submicroscopic malaria infections and their relevance for control.* Nat Commun, 2012. **3**: p. 1237.
114. Hopkins, H., et al., *Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda.* J Infect Dis, 2013. **208**(4): p. 645-52.
115. Britton, S., et al., *A simple, high-throughput, colourimetric, field applicable loop-mediated isothermal amplification (HtLAMP) assay for malaria elimination.* Malar J, 2015. **14**(1): p. 335.

116. Novembre, J. and A. Di Rienzo, *Spatial patterns of variation due to natural selection in humans.* Nat Rev Genet, 2009. **10**(11): p. 745-55.
117. Nielsen, R., et al., *Recent and ongoing selection in the human genome.* Nat Rev Genet, 2007. **8**(11): p. 857-68.
118. Sabeti, P.C., et al., *Positive natural selection in the human lineage.* Science, 2006. **312**(5780): p. 1614-20.
119. Vasseur, E. and L. Quintana-Murci, *The impact of natural selection on health and disease: uses of the population genetics approach in humans.* Evol Appl, 2013. **6**(4): p. 596-607.
120. Kwiatkowski, D.P., *How malaria has affected the human genome and what human genetics can teach us about malaria.* Am J Hum Genet, 2005. **77**(2): p. 171-92.
121. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annu Rev Immunol, 2004. **22**: p. 531-62.
122. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.
123. Taylor, A., et al., *Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells.* Immunology, 2006. **117**(4): p. 433-42.
124. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation.* J Exp Med, 2000. **192**(2): p. 295-302.
125. Carter, L., et al., *PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2.* Eur J Immunol, 2002. **32**(3): p. 634-43.
126. Nishimura, H., et al., *Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor.* Immunity, 1999. **11**(2): p. 141-51.
127. Youngson, N.A., et al., *A missense mutation in the transcription factor Foxo3a causes teratomas and oocyte abnormalities in mice.* Mamm Genome, 2011. **22**(3-4): p. 235-48.
128. Bain, C.C., et al., *Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors.* Mucosal Immunol, 2013. **6**(3): p. 498-510.
129. Zaloumis, S.G., et al., *Population pharmacokinetics of intravenous artesunate: a pooled analysis of individual data from patients with severe malaria.* CPT Pharmacometrics Syst Pharmacol, 2014. **3**: p. e145.
130. Dondorp, A., et al., *Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial.* Lancet, 2005. **366**(9487): p. 717-25.
131. Dondorp, A.M., et al., *Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial.* Lancet, 2010. **376**(9753): p. 1647-57.
132. Morris, C.A., et al., *Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration.* Malar J, 2011. **10**: p. 263.
133. Hendriksen, I.C., et al., *Population pharmacokinetics of intramuscular artesunate in African children with severe malaria: implications for a practical dosing regimen.* Clin Pharmacol Ther, 2013. **93**(5): p. 443-50.
134. Kremsner, P.G., et al., *A simplified intravenous artesunate regimen for severe malaria.* J Infect Dis, 2012. **205**(2): p. 312-9.
135. Krishna, S., et al., *Population pharmacokinetics of intramuscular quinine in children with severe malaria.* Antimicrob Agents Chemother, 2001. **45**(6): p. 1803-9.
136. Morris, C.A., et al., *Effects of body size and gender on the population pharmacokinetics of artesunate and its active metabolite dihydroartemisinin in pediatric malaria patients.* Antimicrob Agents Chemother, 2013. **57**(12): p. 5889-900.

137. Nealon, C., et al., *Intramuscular bioavailability and clinical efficacy of artesunate in gabonese children with severe malaria.* Antimicrob Agents Chemother, 2002. **46**(12): p. 3933-9.
138. Staff, P.O., *Correction: In vitro sensitivity of Plasmodium falciparum from China-Myanmar border area to major ACT drugs and polymorphisms in potential target genes.* PLoS One, 2015. **10**(3): p. e0118328.
139. Koleala, T., et al., *Temporal changes in Plasmodium falciparum anti-malarial drug sensitivity in vitro and resistance-associated genetic mutations in isolates from Papua New Guinea.* Malar J, 2015. **14**: p. 37.
140. Bustamante, C., et al., *In vitro-reduced susceptibility to artemether in P. falciparum and its association with polymorphisms on transporter genes.* J Infect Dis, 2012. **206**(3): p. 324-32.
141. Wurtz, N., et al., *Role of Pfmdr1 in in vitro Plasmodium falciparum susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin.* Antimicrob Agents Chemother, 2014. **58**(12): p. 7032-40.
142. Paczkowski, M.M., et al., *Update on cases of delayed hemolysis after parenteral artesunate therapy for malaria - United States, 2008 and 2013.* MMWR Morb Mortal Wkly Rep, 2014. **63**(34): p. 753-5.
143. Centers for Disease, C. and Prevention, *Published reports of delayed hemolytic anemia after treatment with artesunate for severe malaria--worldwide, 2010-2012.* MMWR Morb Mortal Wkly Rep, 2013. **62**(1): p. 5-8.
144. Lee, J., et al., *Severe delayed hemolysis associated with regulated parenteral antimalarial drug.* Emerg Infect Dis, 2015. **21**(1): p. 164-6.
145. Bethell, D., et al., *Dose-dependent risk of neutropenia after 7-day courses of artesunate monotherapy in Cambodian patients with acute Plasmodium falciparum malaria.* Clin Infect Dis, 2010. **51**(12): p. e105-14.
146. Raffray, L., et al., *Severe delayed autoimmune haemolytic anaemia following artesunate administration in severe malaria: a case report.* Malar J, 2014. **13**: p. 398.
147. Rolling, T., et al., *Delayed hemolysis after treatment with parenteral artesunate in African children with severe malaria--a double-center prospective study.* J Infect Dis, 2014. **209**(12): p. 1921-8.
148. Luzzatto, L., C. Nannelli, and R. Notaro, *Glucose-6-Phosphate Dehydrogenase Deficiency.* Hematol Oncol Clin North Am, 2016. **30**(2): p. 373-93.
149. Cappellini, M.D. and G. Fiorelli, *Glucose-6-phosphate dehydrogenase deficiency.* Lancet, 2008. **371**(9606): p. 64-74.
150. Minucci, A., et al., *Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations.* Blood Cells Mol Dis, 2012. **48**(3): p. 154-65.
151. Guindo, A., et al., *X-linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria.* PLoS Med, 2007. **4**(3): p. e66.
152. Clark, T.G., et al., *Allelic heterogeneity of G6PD deficiency in West Africa and severe malaria susceptibility.* Eur J Hum Genet, 2009. **17**(8): p. 1080-5.
153. De Araujo, C., et al., *The role of the G6PD AEth376G/968C allele in glucose-6-phosphate dehydrogenase deficiency in the seerer population of Senegal.* Haematologica, 2006. **91**(2): p. 262-3.
154. Amoah, L.E., et al., *Prevalence of G6PD deficiency and Plasmodium falciparum parasites in asymptomatic school children living in southern Ghana.* Malar J, 2016. **15**(1): p. 388.
155. Suchdev, P.S., et al., *The burden and consequences of inherited blood disorders among young children in western Kenya.* Matern Child Nutr, 2014. **10**(1): p. 135-44.
156. Burchard, G.D., et al., *Spleen size determined by ultrasound in patients with sickle cell trait, HbAC trait and glucose-6-phosphate-dehydrogenase deficiency in a malaria hyperendemic area (Ashanti Region, Ghana).* Acta Trop, 2001. **80**(2): p. 103-9.

157. White, N.J., *Primaquine to prevent transmission of falciparum malaria.* Lancet Infect Dis, 2013. **13**(2): p. 175-81.
158. May, J., et al., *Red cell glucose-6-phosphate dehydrogenase status and pyruvate kinase activity in a Nigerian population.* Trop Med Int Health, 2000. **5**(2): p. 119-23.
159. Uyoga, S., et al., *Glucose-6-phosphate dehydrogenase deficiency and the risk of malaria and other diseases in children in Kenya: a case-control and a cohort study.* Lancet Haematol, 2015. **2**(10): p. e437-44.
160. Ruwende, C. and A. Hill, *Glucose-6-phosphate dehydrogenase deficiency and malaria.* J Mol Med (Berl), 1998. **76**(8): p. 581-8.
161. Min-Oo, G. and P. Gros, *Erythrocyte variants and the nature of their malaria protective effect.* Cell Microbiol, 2005. **7**(6): p. 753-63.

7. ACKNOWLEDGEMENTS

During my six years of PhD, I worked with many people who significantly contributed by diverse means to the brilliant success of my project. I would have liked to name them all here but this might take me several pages and I am especially afraid of forgetting someone. Hence, I want those that the names will not be mentioned here to know that I have really appreciated their contributions and thank them for everything they did with all my heart.

I would like to express my sincere thanks to the following:

My sincere gratitude goes to all my family for the love and support they continuously provided to me. I dedicate this thesis to the memory of my late father Pierre Nguetse, *"Mo'oh Kemgou"* who passed away during my PhD training. I love you "*papa"* and forever you will be in my heart.

I send my very special and deepest thanks to my beloved wife Charlene Nguetse for her unconditional support. I know how difficult these years were for both of us to live far from each other. However, we stayed very strong. Today, it is indeed a privilege for me to publicly thank you for your kindness, moral assistance and support, especially at the time I was really depressed.

I would like to express my sincere thanks to late Prof. Dr. Jürgen Kun, who gave me the opportunity to join his research group in October 2010. I owe him a great debt of gratitude.

Prof. Dr. Peter Kremsner for being both my faculty representative and supervisor, I owe you my deepest gratitude. You gave me the opportunity to work in this highly qualified and reputed research institution. You have brought in me more serenity, confidence and a light of hope. *Vielen Dank*, Peter, for your unconditional support.

Prof. Dr. Andreas Peschel for accepting to be my second faculty representative; I am deeply grateful to you. Through the Interfaculty Graduate School Program (IGIM), I personally appreciated your support. Thank you very much.

I seize this opportunity to express my gratitude to PD. Dr. Thirumalaisamy Velavan, my immediate supervisor, who was always there to help me sail through all the difficulties that a PhD student could face during his scientific investigations. I appreciate your help, contributions and guidance during my doctoral thesis.

I am heartily thankful to Prof. Dr. Leopold Lehman, whose encouragement, supervision and support starting at the University of Douala, Cameroon, and for recommending me to Prof. Dr. Jürgen Kun for this PhD position. *Merci beaucoup* Léo!

My special thanks to all colleagues, especially from the "*Arbeits Gruppe* (AG) Velavan" and the staff of the Institute of Tropical Medicine for the assistance and encouragement offered every time needed. It was a real pleasure to work with you during these long years.

8. CURRICULUM VITAE

Personal data

First Name:	Christian
Last Name:	Ngouadjio Nguetse
Place of birth:	Batouri, Cameroon
Nationality:	Cameroonian

University Education

12/2010 – 12/2016
PhD candidate at the Institute of Tropical Medicine (ITM), Faculty of Medicine, Eberhard Karls University, Tübingen, Germany

10/2007 – 09/2008
M.Sc. in Animal Biology, Faculty of Sciences, University of Yaoundé I, Cameroon

10/2004 – 09/2005
Master I (Maîtrise) in Animal Biology, Faculty of Sciences, University of Douala, Cameroon

10/2001 – 09/2004
B.Sc. in Animal Biology, Faculty of Sciences, University of Douala, Cameroon

Primary, Secondary and High School Education

09/1996 – 07/2001
Baccalaureate of General Education New-Bell, Douala, Cameroon

09/1992 – 07/1996
Certificate of First Cycle Studies of Secondary Level, New-Bell High School, Douala, Cameroon

09/1987 – 07/1992
Certificate of Primary Elementary Studies, Public School of Bepanda, Douala, Cameroon

List of Publications

1. **Nguetse CN,** Meyer CG, Adegnika AA, Agbenyega T, Ogutu BR, Kremsner PG, Velavan TP. Glucose-6-phosphate dehydrogenase deficiency and reduced Haemoglobin levels in African children with severe malaria. *Malaria Journal*. 2016 Jul 7; 15(1):346. PMID: 27388012

2. Kremsner PG, Adegnika AA, Hounkpatin AB, Zinsou JF, Taylor TE, Chimalizeni Y, Liomba A, Kombila M, Bouyou-Akotet MK, Mawili Mboumba DP, Agbenyega T, Ansong D, Sylverken J, Ogutu BR, Otieno GA, Wangwe A, Bojang KA, Okomo U, Sanya-Isijola F, Newton CR, Njuguna P, Kazungu M, Kerb R, Geditz M, Schwab M, Velavan TP, **Nguetse C,** Köhler C, Issifou S, Bolte S, Engleitner T, Mordmüller B, Krishna S. Intramuscular artesunate for severe malaria in African children: A multicenter, randomized controlled trial. *PLOS Medicine*. 2016 Jan 12; 13(1). PMID: 26757276

3. **Nguetse CN,** Kremsner PG, Velavan TP. FOXO3A regulatory polymorphism and susceptibility to severe malaria in Gabonese Children. *Immunogenetics*. 2015 Feb; 67(2):67-71. PMID: 25421486

4. Port JR[#], **Nguetse C[#],** Adupko S, Velavan TP. A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification. *Malaria Journal*. 2014 Nov 24; 13:454. [#]Equal contribution. PMID: 25421401

9. Appendix: PUBLICATIONS I, II, III and IV

MALARIA JOURNAL

METHODOLOGY Open Access

A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification

Julia R Port[1†], Christian Nguetse[1†], Selorme Adukpo[1] and Thirumalaisamy P Velavan[1,2*]

Abstract

Background: Improved living conditions together with appropriate diagnosis can reduce avoidable malarial deaths substantially. Microscopy remains the gold standard in the diagnosis of malaria. However, rapid molecular diagnostic tests (RmDT) are becoming increasingly important and will, most likely, be the diagnostic techniques of choice in the next years.

Methods: In this study, a rapid and reliable nucleic acid extraction procedure from human blood and malarial parasites using microwave irradiation as a promising platform is described. In addition, a tailored loop mediated isothermal amplification (LAMP) methodology that utilizes hydroxynaphthol blue (HNB) and Bst 2.0 DNA polymerases in molecular detection of malarial parasites is described.

Results: Following microwave irradiation for DNA isolation, conventional PCR assays were able to detect up to five malaria parasites/μl. The LAMP methodology described here was capable to detect as low as one *Plasmodium falciparum* parasite/μl after DNA extraction by microwave irradiation. A turnover time of 45 minutes from nucleic acid extraction to final visual read-out was achieved.

Conclusions: The described procedure offers a cheap, simple and fast method of molecular detection of malaria parasites. This test can easily be performed in basic laboratories. The methodology has been validated as a proof of concept and has specifically be developed for use at low-resource settings. Such RmDTs may aid health providers to make timely therapeutic interventions in malaria endemic regions.

Keywords: Malaria, Microwave irradiation, Plasmodium, Isothermal amplification, LAMP, Diagnostics, Point-of-care

Background

Malaria remains a major public health problem in sub-Saharan Africa. Approximately 3.4 billion people are at risk of malaria worldwide with an incidence of 207 million cases in 2012 and 627,000 reported deaths [1]. Microscopy of blood smears is still considered the gold standard for diagnosing malaria infections. Microscopy, however, is frequently unable to detect low-density infections and it requires skilled expertise [2-4]. Consequently, rapid diagnostic tests (RDT), using blood obtained from finger pricks, are now widely used, especially where quick and easy diagnosis of malaria is needed [2,3,5-9]. A major disadvantage of RDTs is that they are relatively expensive and unable to quantify the degree of parasitaemia. In some cases, lack of sufficient sensitivity of RDTs causes, as does microscopy, failure to detect low-density infections [10,11]. Highest detection rates and specificity can currently be achieved only with polymerase chain reaction (PCR) or real-time PCR (qPCR) assays. These techniques use expensive equipment and reagents as well as functional molecular biology laboratories and appropriate training of laboratory staff. For field applications these conditions are often not fulfilled. In addition, PCR reactions require a rather long

* Correspondence: velavan@medizin.uni-tuebingen.de
†Equal contributors
[1]Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, 72074 Tübingen, Germany
[2]Fondation Congolaise pour la Recherche Medicale, Brazzaville, Republic of Congo

time from DNA extraction to visual read-out by electrophoresis or other confirmatory techniques (~5 hours, if performed in a well equipped laboratory) and are as such not appropriate when rapid diagnoses are needed.

In the last 10 years, isothermal amplifications, especially loop-mediated isothermal amplification (LAMP) [12] has revolutionized the field of molecular diagnostics by offering rapid and reliable approaches at highest sensitivity levels. In 2006, a study described a first set of LAMP primers for malaria [13] and LAMP specific primers for four *Plasmodium* species infecting humans were reported in 2007 [14], all targeting the 18 s rRNA region of the *Plasmodium* species. Primers were also designed for the genus *Plasmodium* and for *Plasmodium falciparum*, targeting mitochondrial DNA [15]. A commercial kit for malaria LAMP is currently available from Eiken Chemical Co., Ltd., also targeting this genetic region of plasmodia. This LAMP assay has successfully been tested under field conditions for both the18s rRNA [16] and the mtDNA primers [17,18] and been successful in clinical evaluation of symptomatic and asymptomatic infections [10]. Under field conditions, the easy endpoint visualization of results such as colourimetric change is most advantageous and, thus, desirable. Due to the nature of LAMP reactions, the method offers unique opportunities. LAMP amplification causes production of magnesium pyro-phosphate precipitates, which may be recorded as a rise in turbidity. Such observations are difficult to make for untrained staff and an extra step of visualization would be advantageous. Similar to gel electrophoresis, where LAMP results are visualized by intercalating substances such as SYBR Green© or ethidium-bromide, hydroxynaphtholblue (HNB) can be applied as a potent detection system. As discussed previously [19], SYBR Green© and HNB, usually applied in metal ion titration [20], show higher sensitivity than calcein, which may also be used in LAMP reactions. HNB has been described to be superior to SYBR Green©, as no further equipment is needed for the final read-out and the risk of contamination is minimized as the dye solution is added directly to the reaction mix before amplification [19]. Calcein, a chemical dye quenched with manganese ions may also serve as a fluorescent dye in LAMP reactions. During amplification, the manganese ions are released by pyrophosphate ions generated, resulting in fluorescence. In addition, the free calcein combines with magnesium ions in the LAMP reaction mixture to enhance fluorescent emissions. Compared to SYBR Green© and calcein, HNB is the cheapest dye and successful use of HNB LAMP has recently been demonstrated to be effective in detecting amplified DNA from malaria parasites [21].

The first critical step in standard molecular diagnostics of infectious agents is extraction of DNA. Due to interference with haem, direct DNA amplification from whole blood is difficult to achieve. Therefore, the DNA extraction and purification steps are crucial for subsequent applications. As LAMP is more robust than are standard PCR assays, the extraction step is not that important. Cheap and easy methods have been described using heat denaturation or chemical lysis. In combination with the Eiken LAMP kit, a "boil and spin" method and the "PURE" method, i.e. a simple DNA isolation technique by water bath or thermoblock, are advised for DNA isolation under field applications. An alternative is offered by employing microwave irradiation, which was first described in 1991 [22] and 1995 for hepatitis B virus (HBV) DNA from serum [23], and for extraction of *Toxoplasma gondii* derived DNA in 2010 [24]. Downstream applications may directly be performed without prior purification measures.

Here, a microwave extraction method was established, along with a tailored LAMP approach for diagnosing malaria. The microwave extraction method was tested for parasite and human genes derived from clinical samples. Sensitivity was tested in repeated serial dilutions and the quality was compared to reference extraction methods using a commercial kit.

Methods

Study samples

Whole venous blood samples were collected either into heparinized tubes (5 mls) or by finger prick (5–10 μl) with blood stored on sterile Whatman filter paper at admission to the Albert Schweitzer Hospital, Lambaréné, Gabon, from patients suffering from severe *P. falciparum* infections [25]. Heparinized samples were kept at −80°C until shipment to Germany at −20°C, while blood spots were air-dried and stored in clean, sealed plastic bags at room temperature. Equal volumes of blood were also collected from healthy adult volunteers in Lambaréné.

Parasite cultures and repeated dilution series

Plasmodium falciparum parasite strain 3D7 were kept in culture, synchronized with D-sorbitol in their ring stages. Culture-adapted parasites were then used for extraction procedures and LAMP assays, applying serial dilutions. In brief, following the determination of the parasitaemia by microscopy, the culture was centrifuged to obtain packed cells which contained 10^7 cells/μl. Fresh whole blood was subsequently used to spike the culture and dilution series were repeatedly made, ranging from calculated 500,000 parasites/μl to 1 parasite/μl and eventual negativity. Equal volumes of these dilution series were further used for genomic DNA extraction by microwave irradiation and, subsequently, for standard PCR and tailored LAMP assays.

DNA extraction: microwave irradiation

First, DNA was extracted from whole blood samples as well as from the cultured parasites of the dilution series

using the conventional QIAamp DNA mini blood kit based extraction procedure (Qiagen, Hilden, Germany) following the manufacturer's instructions. Next, three standard operation procedures (SOPs) were established for microwave irradiation based DNA extraction (MDA oven, model number: MW17M70G-AU, 230 V, 50 HZ). The three SOPs were designed to apply in processing (i) fresh whole blood, (ii) archived blood samples in heparinized tubes and for (iii) small amounts of blood stored at ambient temperature and eluted from filter paper in 30 μl PBS. Volumes of 10 μl of blood were transferred into 0.5 ml tubes and treated at 800 W for 2 minutes until precipitated and condensed droplets were visible on and retrievable from the tube walls. One μl of the clear precipitated watery solution containing DNA was taken from the walls or the lid of the tube for further use (Figure 1). Alternatively, for enduring storage, 30 μl of sterile phosphate buffer saline (PBS) were added to the irradiated sample. Notably, smaller tubes than those of 0.5 ml volumes should not be used in the irradiation procedure as they might break and be destroyed due to air expansion and, thus, might bear the danger of contamination hazards.

Amplification using standard PCR

As an example, a nested PCR assay targeting the *pfmdr1* gene of *P. falciparum* was applied as described elsewhere [4]. For each PCR reaction, positive and negative controls were included. Reaction volumes consisted of 20 μl containing 1× Coral load PCR buffer, 0.25 mM of each deoxynucleotide-triphosphate (dNTP), 1 U Taq Polymerase (Qiagen) and 0.2 μM of each primer. The template was 1 μl of the condensed watery solution after DNA extraction by microwave irradiation. Amplification was performed as follows: 35 cycles with an initial denaturation step at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C (outer primers) or 60°C (nested primers) for 30 sec, extension at 65°C for 1 min and final extension at 65°C for 5 min. Amplicons were subjected to electrophoresis on a 1.2% agarose gel stained with SYBR green I in 1× Tris-buffered electrophoresis buffer (90 mM Tris-acetate, pH 8.0, 90 mM boric acid, 2.5 mM EDTA). In addition and for confirmation of the specificity of the amplification process, 1 μl of the purified product was directly used as template for direct sequencing, using the BigDye terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL DNA sequencer. Results were confirmed visually from the sequencing electropherograms. Notably, both the DNA extraction procedure has also be performed to extract human DNA for standard PCR reactions.

Tailored LAMP assays

Primer design and reagents

LAMP primer pairs for *P. falciparum,* as published [14], were provided by Eurofins MWG Synthesis GmbH, Ebersberg, Germany (Table 1). Bst 2.0 WarmStart™ DNA

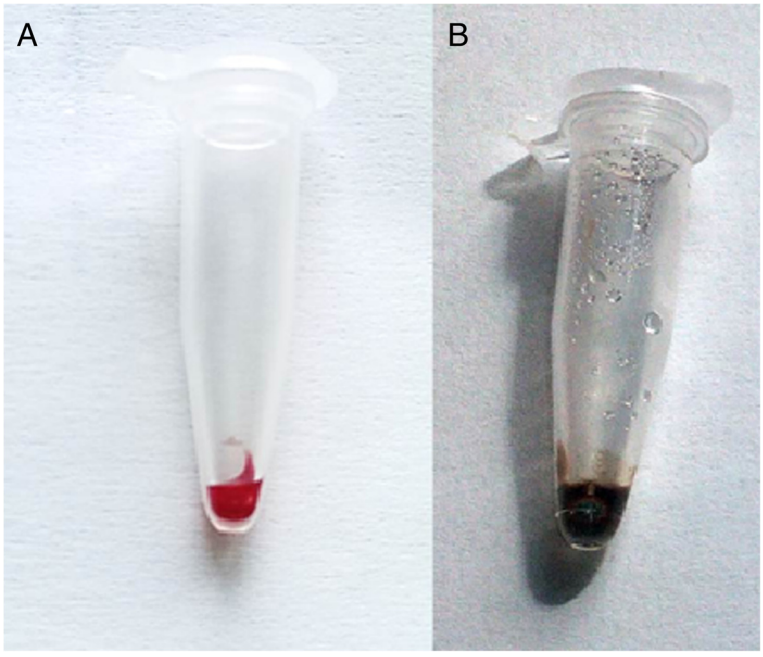

Figure 1 Microwave irradiated finger prick blood sample 10 μl fresh finger prick blood collected by capillary (A) before and (B) after microwave irradiation. Formation of vapour and condensed droplets can be observed on the walls and in the lid.

Table 1 LAMP Primers targeting *Plasmodium* 18 s ribosomal RNA

Plasmodium spp.	Primers	Sequence 5′ - 3′
Core primers	FIP	TCGAACTCTAATTCCCCGTTACCTAT CAGCTTTTGATGTTAGGGT
	BIP	CGGAGAGGGAGCCTGAGAAATAGA ATTGGGTAATTTACGCG
	F3	GTATCAATCGAGTTTCTGACC
	B3c	CTTGTCACTACCTCTCTTCT
Loop primers	LPF	CGTCATAGCCATGTTAGGCC
	LPB	AGCTACCACATCTAAGGAAGGCAG

P. falciparum	Primers	Sequence 5′ - 3′
Core primers	FIP	AGCTGGAATTACCGCGGCTG GGTT CCTAGAGAAACAATTGG
	BIP	TGTTGCAGTTAAAACGTTCGTAGCC CAAACCAGTTTAAATGAAAC
	F3	TGTAATTGGAATGATAGGAATTTA
	B3c	GAAAACCTTATTTTGAACAAAGC
Loop primers	LPF	GCACCAGACTTGCCCT
	LPB	TTGAATATTAAAGAA

Polymerase (New England BioLabs GmbH, Frankfurt am Main, Germany) was used. This cheap polymerase was specifically designed for high throughput assays and field applications. The polymerase may be stored under up to 45°C without requiring refrigeration. HNB (Fluka, Sigma-Aldrich, St. Luis, USA) was used as the visualization dye. In addition, a 3 mM stock solution of HNB was prepared and stored at room temperature [21,26]. Isothermal reaction buffer was prepared as a 2× working solution. After thawing, 2.8 mM of dNTPs were added. Betaine solution for enhancing the polymerase activity and reducing the formation of secondary structures in GC-rich regions was purchased from Sigma Aldrich, Munich, Germany.

LAMP Assays

Reactions were performed at 60°C in volumes of 25 μl containing 1.6 μM FIP, 1 μM BIP, 0.2 μM F3, 0.2 μM B3c, 0,2 μM LPB, 0.4 μM LPF, 120 μM HNB, 2× isothermal reaction buffer, 1.4 mM dNTPs, 0.4 mM Betaine solution, 8U Bst polymerase and 1 μL of watery DNA solution as template, following the STARD guidelines for diagnostics. Two heat blocks (Block Thermostat BT200, Kleinfeld Labortechnik, Gehrden, Germany) were used and preheated to 60°C for DNA amplification for to 45 minutes, and enzyme inactivation for 2 minutes at 80°C.

Visual detection of amplification/endpoint analysis

The amplicon was visualized through a distinct colour change and subsequently confirmed by gel electrophoresis. A change from violet to light sky blue was considered a positive result of amplification. If the reaction remained violet, the sample was assessed as being negative. This assessment was performed at daylight. A positive amplification was determined by a colour change occurring after a minimum of 35 minutes. Absence of colour changes was assigned after 45 minutes. Accordingly, 45 minutes were set as the time needed to achieve a firm result.

Results

DNA extraction by microwave irradiation, standard PCR and LAMP assays

Either condensed vapour droplets or blood remains after microwave irradiation diluted in a 30 μl volume of PBS proved to be appropriate templates for further processing in both standard PCR and LAMP assays. DNA extraction was achieved from various sources, including fresh venous blood, heparinized and archived samples and blood eluted from Whatman filter paper. A 0.5 ml tube containing 10 μl of blood inside a microwave oven led to boiling and partial desiccation of the sample and to the formation of vapour, which condenses and may be retrieved from the walls and lids of the tubes (Figure 1). This haem-free condensed vapour contains the nucleic acid.

A nested PCR targeting the *pfmdr1* gene of *P. falciparum* was applied and successful amplification of DNA extracted from fresh and archived samples as well as from filter papers by microwave irradiation was achieved (Figure 2). This applied to both, DNA containing vapour droplets and blood remains diluted in PBS. For confirmation of specificity of the amplicon, all PCR products were sequenced and aligned with reference sequences. Correct sequences of amplification targets were confirmed in all cases. The amplicons could successfully be applied to SNP analyses of drug resistance markers. The total time of the entire diagnostic procedure was reduced to less than one hour after sample collection. While maintaining high sensitivity levels, expensive equipment or reagents are not required.

Analytical sensitivity

The sensitivity of the PCR assays after microwave-based extraction was established based on repeated dilution series. Heparin blood was spiked with ring stage parasites from 3D7 cultures and serially diluted from 500,000 to one parasite/μl and negativity. DNA was extracted from all samples applying both standard reference methods and microwave irradiation. When using standard DNA isolation methods, one parasite/μl could be reliably detected (Figure 3A). After microwave extraction, five parasites/μl were detected (Figure 3B).

Loop mediated isothermal amplification

No differences in the amplification sensitivity was observed when comparing results obtained by a standard

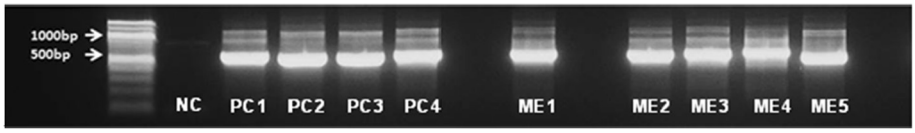

Figure 2 Amplified *pfmdr1* gene products after nested PCR. Standard DNA extraction was carried out using the QIAamp DNA mini blood kit (Qiagen, Hilden, Germany). DNA extraction by microwave irradiation was performed using a microwave oven (MDA, model number: MW17M70G-AU, 230 V, 50 HZ, operated at 800 W). 1 μl of condensed droplets after microwave treatment were utilized for the PCR procedures. First lane: DNA ladder; NC: Negative Control; PC1 and PC2: Standard extraction from archived blood sample and *pfmdr1* amplicons at expected sizes; PC3 and PC4: Standard extraction from 3D7 *P. falciparum* parasites in culture and *pfmdr1* amplicons at expected sizes; ME1 and ME2: Microwave based extraction from archived blood sample and *pfmdr1* amplicons at expected sizes; ME3 and ME4: Microwave based extraction in 3D7 culture parasites and *pfmdr1* amplicons at expected sizes; ME5: Microwave based DNA extraction from fresh blood sample and *pfmdr1* amplicons at expected sizes.

thermocycler or a heating block as proposed here. 3D7 parasites, starting at a dilution of 100,000 parasites/μl to one parasite/μl were subjected to tailored LAMP assays using the DNA extracted by microwave irradiation. The parasite content of the diluted culture was assessed by microscopy, counting 100 oil immersion fields. When direct condensed vapour droplets were used in LAMP, positive amplification was observed with a limit of detection of one parasite/μl (Figure 4A). When blood remains were diluted in PBS, positive amplification was observed at a limit of detection of five parasites/μl (Figure 4B). With HNB used as appropriate dye, negative controls remained violet, while successful LAMP was characterized by a colour change to sky blue. Gel electrophoresis was applied to verify HNB positive results (Figure 5). Repeated tests allowed to set the endpoint of a final decision on test positivity or negativity at a maximum of 45 minutes.

Discussion

Multiple techniques to extract and purify DNA have been described and applied to date. Problems arising from interference with haem, as occurring in extraction of DNA from venous blood, can easily be circumvented in laboratories when using commercially available extraction kits, while, in low resource settings, commercial DNA extraction kits are not always available. Alternatively, intensive heat treatment or shock freezing can also lyse cells for DNA release. Microwave irradiation has been shown in this study to successfully extract DNA from whole blood samples in less than 3 minutes. No further chemicals are required for isolation or purification. DNA isolated by irradiation could be subjected to both standard PCR amplification and to the LAMP procedure. The use of PBS, although causing a certain decrease of sensitivity due to the dilution, was beneficial, as larger volumes of DNA could be stored. Sensitivity levels observed after microwave irradiation were close to those obtained after extraction with commercial kits.

Thus, microwave irradiation offers a cheap, fast and easy technique that can be applied readily for isothermal amplification and that may be applied in field settings. The nucleic acid extraction by microwave irradiation allows to isolate human DNA and nucleic acid materials from pathogens in blood. Our study demonstrates that DNA may be extracted from fresh blood samples and from archived blood samples, either heparinized or stored on filter papers even after more than a decade. LAMP for malaria has been shown to detect low parasite concentrations [17] and to be successfully applicable in asymptomatic *Plasmodium* infections. When aiming at improving malaria control, simple and easy to perform

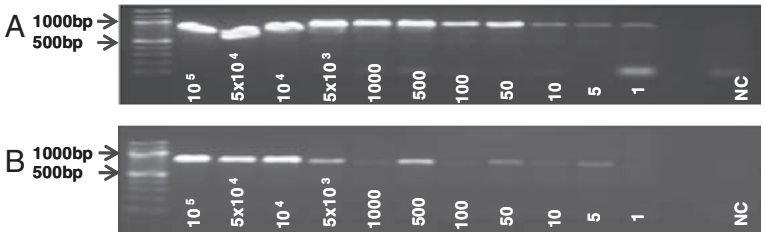

Figure 3 Limit of detection from standard extraction and microwave irradiation procedure. Repeated dilution series were made from 100,000 parasites/μl to one parasite/μl until negativity. Equal volumes of diluted culture were further used for DNA extraction and for standard PCR assays. **A**, Serial dilutions including dilutions from 100,000 parasites to negativitiy. The limit of detection was determined to be 1 parasite/μl culture applying the standard extraction procedure (QIAamp DNA mini blood kit); **B**, After microwave based DNA extraction the limit of detection was 5 parasites/μl.

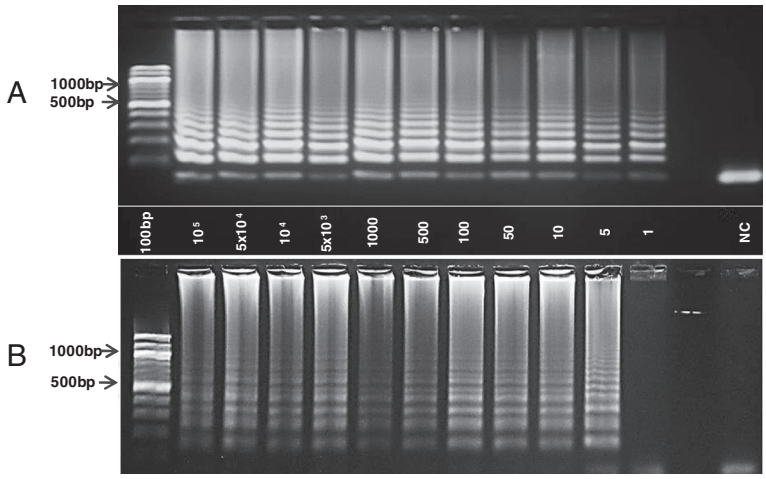

Figure 4 Limit of detection of parasites extracted by microwave irradiation in tailored LAMP assays. Successful amplification was characterized by clear colour changes from violet to sky blue and the negative controls remained violet. NC: Negative control; **A**, Pure condensed vapour droplets were used as DNA templates. LAMP can detect 1 parasite/μl culture fluid. **B**, Vapour droplets diluted in PBS. LAMP detects 5 parasites/μl.

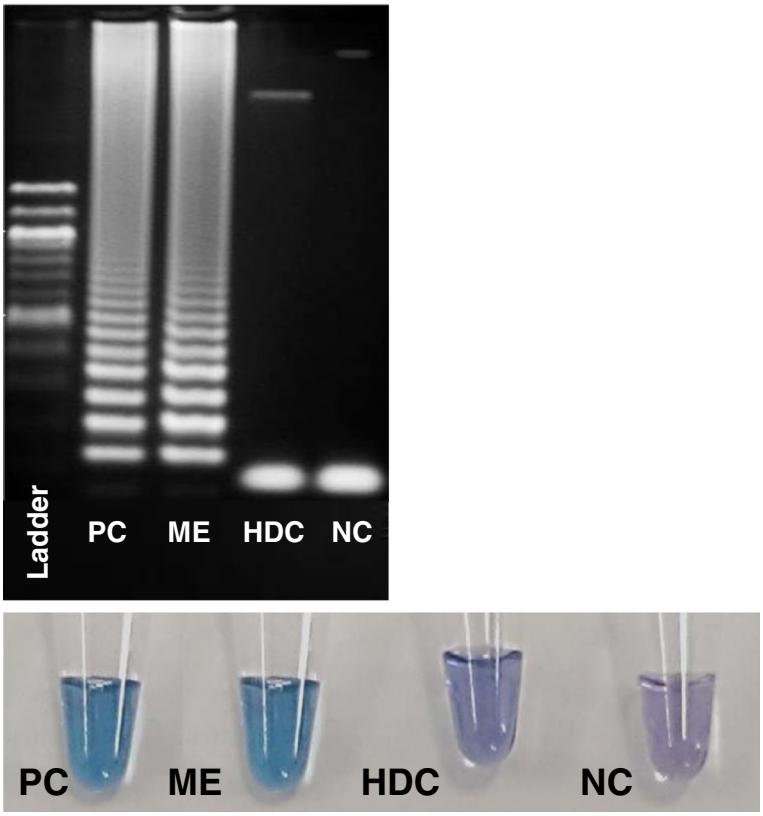

Figure 5 Visual assessment after 35 minutes following the LAMP assays. All amplicons were confirmed by gel electrophoresis. PC, positive control after standard DNA extraction; ME, clinical sample, LAMP assay after microwave DNA extraction; HDC, human DNA control; NC, negative control.

diagnostic measures detecting subclinical infections need to be applied, as asymptomatic carriers play an crucial role in the transmission cycle of plasmodia and in maintaining endemicity [27-29]. Further and ongoing studies will show, whether the microwave extraction/LAMP system is also appropriate for the detection of low parasitaemia and asymptomatic infections.

An apparent advantage of the system we propose here is the avoidance of contamination through aerosolic PCR-generated DNA fragments, as, due to the HNB dye added to the primary reaction mixture tubes do not need to be opened for further result confirmation. Another value of the technique is that the procedure is easy to perform, does not need technicians trained in molecular techniques and that the final result is obtained by means of visual assessment. This has been performed successfully by untrained personnel after providing them one positive and on negative sample result.

In addition to the obvious advantages, the study has limitations. The minimum size of tubes to be used in microwave exposure should not be below a capacity of 0.5 ml, as through damage of the tube, contamination with biological materials might occur. The problem of potential unavailability of electricity used for irradiation and heating of thermoblocks in the field needs to be addressed by identifying alternative power sources for irradiation and heating. Currently, a prototype that utilizes direct current has been designed for LAMP assays. First trials applying this hand-held battery operated thermoblock for LAMP assays are currently carried out and evaluated.

Taken together, the method described offers a cheap, simple and fast technique of molecular detection of malaria parasites. The method has been validated as a proof of concept to be performed in laboratories with limited resources.

Competing interests
The authors declared that they have no competing interests.

Authors' contributions
JP performed the experiments and drafted the manuscript. CN performed LAMP experiments on parasite and human samples. SA performed experiments on parasite cultures and dilution series for LAMP assays. TPV wrote the manuscript. TPV designed, performed, implemented first experimental procedures related to the study and supervised all further experimental procedures related. All authors read and approved the final manuscript.

Acknowledgements
We thank the lab members in the work group AG Velavan, for their consistent help and efforts in setting up the experimental procedures during the proof of concept phase. This study was funded by an internal institutional grant to TPV. The authors acknowledge the support by the Deutsche Forschungsgemeinschaft (DFG) and Open Access Publishing Fund of Tuebingen University

Received: 26 September 2014 Accepted: 17 November 2014
Published: 24 November 2014

References
1. WHO: *World Malaria Report*. Geneva: World Health Organization; 2013.
2. Chinkhumba J, Skarbinski J, Chilima B, Campbell C, Ewing V, San Joaquin M, Ali D, Mathanga D: **Comparative field performance and adherence to test results of four malaria rapid diagnostic tests among febrile patients more than five years of age in Blantyre.** *Malawi Malar J* 2010, **9:**209.
3. Endeshaw T, Graves PM, Ayele B, Mosher AW, Gebre T, Ayalew F, Genet A, Mesfin A, Shargie EB, Tadesse Z, Teferi T, Melak B, Richards FO, Emerson PM: **Performance of local light microscopy and the ParaScreen Pan/Pf rapid diagnostic test to detect malaria in health centers in Northwest Ethiopia.** *PLoS One* 2012, **7:**e33014.
4. Mekonnen SK, Aseffa A, Berhe N, Teklehaymanot T, Clouse RM, Gebru T, Medhin G, Velavan TP: **Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia.** *Malar J* 2014, **13:**244.
5. Chou M, Kim S, Khim N, Chy S, Sum S, Dourng D, Canier L, Nguon C, Ménard D: **Performance of "VIKIA Malaria Ag Pf/Pan" (IMACCESS®), a new malaria rapid diagnostic test for detection of symptomatic malaria infections.** *Malar J* 2012, **11:**295.
6. Dhorda M, Piola P, Nyehangane D, Tumwebaze B, Nalusaji A, Nabasumba C, Turyakira E, McGready R, Ashley E, Guerin PJ, Snounou G: **Performance of a histidine-rich protein 2 rapid diagnostic test, Paracheck Pf®, for detection of malaria infections in Ugandan pregnant women.** *Am J Trop Med Hyg* 2012, **86:**93–95.
7. Mekonnen Z, Ali S, Belay G, Suleman S, Chatterjee S: **Evaluation of the performance of CareStart Malaria Pf/Pv Combo rapid diagnostic test for the diagnosis of malaria in Jimma, southwestern Ethiopia.** *Acta Trop* 2010, **113:**285–288.
8. Wanji S, Kimbi HK, Eyong JE, Tendongfor N, Ndamukong JL: **Performance and usefulness of the Hexagon rapid diagnostic test in children with asymptomatic malaria living in the Mount Cameroon region.** *Malar J* 2008, **7:**89.
9. Mekonnen SK, Aseffa A, Medhin G, Berhe N, Velavan TP: **Re-evaluation of microscopy confirmed *Plasmodium falciparum* and *Plasmodium vivax* malaria by nested PCR detection in southern Ethiopia.** *Malar J* 2014, **13:**48.
10. Aydin-Schmidt B, Xu W, Gonzalez IJ, Polley SD, Bell D, Shakely D, Msellem MI, Björkman A, Mårtensson A: **Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias.** *PLoS One* 2014, **9:**e103905.
11. Foundation for Innovative New Diagnostics (FIND): *Malaria rapid diagnostic test performance. Results of WHO product testing of malaria RDTs: Round 3 (2010–2011)*; 2011:124.
12. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: **Loop-mediated isothermal amplification of DNA.** *Nucleic Acids Res* 2000, **28:**E63.
13. Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS: **Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification.** *Clin Chem* 2006, **52:**303–306.
14. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, Jin L, Takeo S, Tsuboi T: **Detection of four Plasmodium species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis.** *J Clin Microbiol* 2007, **45:**2521–2528.
15. Polley SD, Mori Y, Watson J, Perkins MD, Gonzalez IJ, Notomi T, Chiodini PL, Sutherland CJ: **Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification.** *J Clin Microbiol* 2010, **48:**2866–2871.
16. Sirichaisinthop J, Buates S, Watanabe R, Han ET, Suktawonjaroenpon W, Krasaesub S, Takeo S, Tsuboi T, Sattabongkot J: **Evaluation of loop-mediated isothermal amplification (LAMP) for malaria diagnosis in a field setting.** *Am J Trop Med Hyg* 2011, **85:**594–596.
17. Hopkins H, Gonzalez IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, Agaba B, Kyabayinze DJ, Sutherland CJ, Perkins MD, Bell D: **Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda.** *J Infect Dis* 2013, **208:**645–652.
18. Lee PW, Ji DD, Liu CT: **Application of loop-mediated isothermal amplification for malaria diagnosis during a follow-up study in São Tomé.** *Malar J* 2012, **11:**408.
19. Goto M, Honda E, Ogura A, Nomoto A, Hanaki K: **Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue.** *Biotechniques* 2009, **46:**167–172.

20. Ito A, Ueno K: **Successive chelatometric titration of calcium and magnesium using Hydroxy Naphthol Blue (HNB) indicator.** *Bunseki kagaku* 1970, **19:**393–397.
21. Mohon AN, Elahi R, Khan WA, Haque R, Sullivan DJ Jr, Alam MS: **A new visually improved and sensitive loop mediated isothermal amplification (LAMP) for diagnosis of symptomatic falciparum malaria.** *Acta Trop* 2014, **134:**52–57.
22. Cheyrou A, Guyomarc'h C, Jasserand P, Blouin P: **Improved detection of HBV DNA by PCR after microwave treatment of serum.** *Nucleic Acids Res* 1991, **19:**4006.
23. Costa J, Lopez-Labrador FX, Sanchez-Tapias JM, Mas A, Vilella A, Olmedo E, Vidal J, Jimenez de Anta MT, Rodes J: **Microwave treatment of serum facilitates detection of hepatitis B virus DNA by the polymerase chain reaction. Results of a study in anti-HBe positive chronic hepatitis B.** *J Hepatol* 1995, **22:**35–42.
24. Meganathan P, Singh S, Ling LY, Singh J, Subrayan V, Nissapatorn V: **Detection of Toxoplasma gondii DNA by PCR following microwave treatment of serum and whole blood.** *Southeast Asian J Trop Med Public Health* 2010, **41:**265–273.
25. Velavan TP, Buyukyazici B, Kremsner PG, Kun JF: **Combined promoter haplotypes of the IL10R genes are associated with protection against severe malaria in Gabonese children.** *Immunogenetics* 2012, **64:**87–95.
26. Tanner NA, Evans TC Jr: **Loop-mediated isothermal amplification for detection of nucleic acids.** *Curr Protoc Mol Biol* 2014, **105:**Unit.
27. Cotter C, Sturrock HJ, Hsiang MS, Liu J, Phillips AA, Hwang J, Gueye CS, Fullman N, Gosling RD, Feachem RG: **The changing epidemiology of malaria elimination: new strategies for new challenges.** *Lancet* 2013, **382:**900–911.
28. Okell LC, Bousema T, Griffin JT, Ouedraogo AL, Ghani AC, Drakeley CJ: **Factors determining the occurrence of submicroscopic malaria infections and their relevance for control.** *Nat Commun* 2012, **3:**1237.
29. Sturrock HJ, Hsiang MS, Cohen JM, Smith DL, Greenhouse B, Bousema T, Gosling RD: **Targeting asymptomatic malaria infections: active surveillance in control and elimination.** *PLoS Med* 2013, **10:**e1001467.

doi:10.1186/1475-2875-13-454
Cite this article as: Port *et al.*: A reliable and rapid method for molecular detection of malarial parasites using microwave irradiation and loop mediated isothermal amplification. *Malaria Journal* 2014 **13**:454.

Immunogenetics (2015) 67:67–71
DOI 10.1007/s00251-014-0816-z

ORIGINAL PAPER

FOXO3A regulatory polymorphism and susceptibility to severe malaria in Gabonese children

Christian Ngouadjio Nguetse · Peter G. Kremsner · Thirumalaisamy P. Velavan

Received: 24 October 2014 / Accepted: 14 November 2014 / Published online: 25 November 2014

Abstract The clinical course of malaria varies between affected individuals and host genetic factors have been shown to influence the outcome of malaria. The role of FOXO3-driven pathway in modulating inflammatory responses, including mediation of distinct functions of regulatory T effector cell populations (Tregs) by the transcription factor FOXO3, has recently been recognized. We aimed to study possible associations of a non-coding polymorphism in intron 2 of the *FOXO3A* gene (rs12212067T>G) that was shown earlier to modulate the FOXO3 expression and to be associated with the prognosis of distinct inflammatory and infectious diseases. The *FOXO3A* polymorphism rs12212067T>G was genotyped by direct sequencing in a group of Gabonese children with confirmed *Plasmodium falciparum* malaria. Severe cases of malaria were compared with asymptomatic/mild cases. The *FOXO3A* variant rs12212067T>G was associated with the phenotype of severe malaria, but not with asymptomatic/mild malaria (allelic model: OR=1.54, 95 % CI=1.15–2.05, P=0.0028; dominant model: OR=1.94, 95 % CI=1.36–2.77, P= 0.0002). The *FOXO3A* variant *rs12212067T>G* is associated with increased inflammatory responses to *Plasmodium falciparum* malaria, indicating a role of the FOXO3-dependent pathway in malaria.

Keywords *FOXO3A* · Severe malaria · Tregs · *Plasmodium falciparum* · Gabon

C. N. Nguetse · P. G. Kremsner · T. P. Velavan (✉)
Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, 72074 Tübingen, Germany
e-mail: velavan@medizin.uni-tuebingen.de

C. N. Nguetse
e-mail: christiannguetse@gmail.com

P. G. Kremsner
e-mail: peter.kremsner@uni-tuebingen.de

P. G. Kremsner
Centre de Recherches Médicales de Lambaréné (CERMEL), Lambaréné, Gabon

T. P. Velavan
Fondation Congolaise pour la Recherche Médicale, Brazzaville, Republic of the Congo

Introduction

Malaria is a major public health problem in tropical and subtropical countries and approximately 3.4 billion people are at risk. In 2012, approximately 207 million cases were documented and about 627,000 deaths occurred globally (WHO 2013). Highest malaria incidences and mortality rates are reported from sub-Saharan African (SSA) countries with a mortality of 77 % among children under the age of 5 years, depending on the severity of the disease and access to adequate treatment (WHO 2013). Among the five *Plasmodium* species causing human malaria, *Plasmodium falciparum* is the most virulent agent (WHO 2013). *Plasmodium falciparum*-infected individuals present with different clinical phenotypes, ranging from asymptomatic infections to severe and complicated forms of malaria (Miller et al. 2002). Parasites exert a selective pressure on their hosts and strongly account for shaping genetic diversity in human populations (Maizels 2009). Previous investigations have shown that human gene polymorphisms contribute to the variability of malaria phenotypes (Weatherall and Clegg 2002).

Malaria parasites induce regulatory T cells (Tregs) which in turn suppress anti-parasite effector cells. Tregs are a subset of T cells that function to control immune responses. The primary role of Tregs is active suppression of several pathological and physiological host immune responses, thereby contributing to the maintenance of immune homeostasis. Tregs can reduce injurious host inflammatory and immune responses through mechanisms exerted by cell-to-cell contact, inhibitory

cytokines and by cytokine deprivation (Velavan and Ojurongbe 2011). Several studies have reported on an association between the increased number of Tregs including $CD4^+CD25^+Foxp3^+$ T cells and malaria infection (Brustoski et al. 2006; Scholzen et al. 2009; Vigario et al. 2007). Other studies have demonstrated the association of functional Treg cell polymorphisms with malaria (Hanel et al. 2011; Huang et al. 2012; Koukouikila-Koussounda et al. 2013; Velavan et al. 2011, 2012). Only little information, however, is available regarding the induction of Tregs by the parasite. Malaria parasites trigger the secretion of many pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) by macrophages and monocytes (Malaguarnera and Musumeci 2002). An earlier study has shown that malaria parasites induce soluble immune mediators such as IL-2, a T cell growth factor, the regulatory proteins IL-10, and transforming growth factor-beta (TGF-ß) through induction and expansion of Tregs, thereby down-regulating inflammatory responses (Scholzen et al. 2009). Specialized functions of effector cell populations of the Tregs are mediated by the transcription factor FOXO3 (Kerdiles et al. 2010).

Forkhead box O (FOXO) is a subfamily of transcription factors of the FOX family, which is characterized by a conserved and distinct forkhead domain, a DNA-binding domain of about 100 amino acids (Weigel and Jackle 1990). Based on the sequence similarities in the forkhead box family, more than 100 members of transcription factors ranging from FOXA to FOXR, including subgroups, have been classified in humans (Carter and Brunet 2007). Four of them, FOXO1, FOXO3, FOXO4, and FOXO6, each encoded by distinct genes, have been identified in the FOXO family (Eijkelenboom et al. 2013). FOXO is known to play a role in modulating the immune response during inflammation, oxidative stress, and in response to growth factors (Hedrick et al. 2012). In addition, this subgroup of the forkhead family is believed to regulate the development and differentiation of antigen-responsive lymphocyte populations (Hedrick et al. 2012). A considerable number of transcription factors, including FOXO3 itself, affects the transcriptional activity of the FOXO members in exerting a control mechanism between FOXO and FOXO3 (Essaghir et al. 2009). Regulation of FOXO components is mediated by recruitment and phosphorylation of the protein kinases B of the *AKT* family and the serine/threonine-protein kinase SGK1. This is achieved through the 3-phosphoinositide-dependent kinase 1 (PDK1) following activation of phosphoinositide 3-kinase (PI3K) in response to the growth factor insulin (Alessi et al. 1997; Stokoe et al. 1997). FOXO3 is expressed in many tissues and regulates various cellular activities, including DNA repair mechanisms (Hedrick 2009). In addition, FOXO3 is involved in the regulation of several immune functions in dendritic and T cells (Dejean et al. 2009) as well as in macrophages (Litvak et al. 2012).

Based on previous findings in inflammatory and infectious diseases and the established role of the intronic *FOXO3A* polymorphism rs12212067 (Lee et al. 2013), we studied its possible association with the phenotype of severe malaria in a Gabonese study population.

Materials and methods

Study subjects

Patients were recruited at the Albert Schweitzer Hospital, Lambaréné, Gabon, and the Centre Hospitalier de Libreville, Gabon. The Gabonese individuals belong to different ethnic groups but the majority of the individuals in the current study represent Bantu population. A total of 552 Gabonese children aged 8 to 140 months with confirmed *Plasmodium falciparum* malaria were enrolled. The study group was divided into the two subgroups of mild malaria (n=255) and severe malaria (n=297) as performed in previous studies (Kalmbach et al. 2006; Kun et al. 1998). Mild malaria patients were defined as individuals carrying parasites for days or weeks without developing any clinically relevant symptoms. The subgroup of mild malaria was used as the control group. Cases with severe malaria were children with *Plasmodium falciparum* parasitemia classified based on WHO guidelines (WHO 2010). Blood samples from all participants were collected in heparinized tubes. Storage of specimens was done at −80 °C for subsequent molecular analyses. Written informed consent was obtained from parents/guardians of all participating children. The study was approved by the local Ethics Committee of the International Foundation of the Albert Schweitzer Hospital, Lambaréné, Gabon.

FOXO3A rs12212067 genotyping

Genomic DNA was isolated using the QIAamp DNA mini blood kit (Qiagen, Hilden, Germany) in order to determine the frequency distribution of the SNP rs12212067. The fragment of the *FOXO3A* gene containing the intronic SNP rs12212067T>G was amplified by PCR using the primer pairs 5′-CGCATACGTTGTTGGAGGTT-3′ (forward) and 5′-CCCGAGGCTAAATGGAAATGTG-3′ (reverse). Briefly, 10 ng of genomic DNA were added to a 20 μL reaction mixture containing 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM of $MgCl_2$), 0.125 mM of dNTPs, 0.25 mM of each primer, and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany). The PCR reaction was run on a PTC-200 Thermal cycler (MJ Research, USA). The thermal conditions after the initial denaturation (94 °C, 5 min) were 35 cycles of 94 °C for 45 s, 67 °C for 45 s and 72 °C for 1 min. The PCR was completed with a final extension step of 72 °C for 5 min. PCR products were visualized through

electrophoresis on a 1.2 % agarose gel stained with SYBR green I in 1× Tris-electrophoresis buffer (90 mM Tris-acetate, pH 8.0, 90 mM boric acid, 2.5 mM EDTA).

Subsequently, PCR products were purified (Exo-SAP-IT, USB, Affymetrix, USA) and directly used as templates for DNA sequencing using the BigDye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, USA) on an ABI 3130XL DNA sequencer. The polymorphism was identified by assembling the sequences with the reference sequence of *FOXO3A* gene (NT_025741.15) using the CodonCode Aligner 4.0 software (http://www.codoncode.com/) and visually reconfirmed from their electropherograms.

Statistical analysis

The data were analyzed by using Intercooled Stata software (Stata Corporation, College Station, TX, USA). Two-sided Fisher's exact test was executed to analyze possible associations of SNP rs12212067T>G in the *FOXO3A* gene with the phenotype of severe malaria. The various genetic models (dominant, recessive, allelic) were applied. Hardy-Weinberg equilibria were tested using the random-permutation procedure as implemented in the Arlequin v. 3.5.1.2 software (Excoffier and Lischer 2010). The level of significance was set to a P value of <0.05.

Results

The genotype frequency of SNP rs12212067T>G in the *FOXO3A* gene was in Hardy-Weinberg equilibrium (HWE) in the mild malaria subgroup. The cases groups significantly departed from HWE. Genotype frequencies of cases and controls are shown in Table 1. The genotype distribution between cases and controls were also tested using a 2×3 test for heterogeneity. A difference between the cases and controls were observed (chi-square=15.89, df=2, $P= 0.0003$). This test establishes a difference between the cases and controls. The frequency of the minor allele *G* was 29 and 21 % in cases and controls, respectively. This was also reflected when looking at the genotypes, where *TG* occurred more frequently among cases compared to controls (50 vs. 32 %). The association of SNP rs12212067*T*>*G* with the severity of malaria was reproducible in different genetic models. The allelic model provided an association of the minor allele *G* with an increased risk to severe malaria (OR=1.54, 95 % CI=1.15–2.05, P=0.0028). The dominant model also showed a significant contribution of the minor allele *G* to severe malaria (OR=1.94, 95 % CI=1.36–2.77, P=0.0002).

Discussion

The strong pressure of malaria in endemic areas has led to selection of human genetic factors that are associated with protection against severe malaria. However, little is known about the prognosis of malaria severity. The distribution of the genotype frequency of SNP rs12212067T>G in the *FOXO3A* in severe malaria cases deviated from HWE. Nevertheless, based on the genotyping technique of direct sequencing, sampling procedure and characteristics of the study population, we can exclude typing error and sampling bias. As the study population has been recruited in a narrow area in Gabon, population admixture is very unlikely. A diseased population is expected not to conform with HWE when a true association

Table 1 Association of *FOXO3A* rs12212067T>G with severe malaria in Gabonese patients

FOXO3A rs12212067T>G	Severe malaria n=297 (%)	Mild malaria n=255 (%)	OR (95 % CI)	P value
Genotype				
TT	138 (0.46)	160 (0.63)	1	Reference
TG	146 (0.5)	83 (0.32)	2.04 (1.4–2.95)	0.0001
GG	13 (0.04)	12 (0.05)	1.3 (0.5–3.1)	NS
Allele				
T	422 (0.71)	403 (0.79)	1	Reference
G	172 (0.29)	107 (0.21)	1.54 (1.15–2.05)	0.0028
Dominant				
TT	138 (0.46)	160 (0.63)	1	Reference
GG + *TG*	159 (0.54)	95 (0.37)	1.94 (1.36–2.77)	0.0002
Recessive				
TG + *TT*	284 (0.96)	243 (0.95)	1	
GG	13 (0.04)	12 (0.05)	0.93 (0.38–2.27)	NS

The P values were calculated using the two-sided Fisher's exact test

NS not significant

with the examined polymorphism exists. However, distribution of alleles according to HWE is expected, and present in our study, in the control group (Zintzaras 2010). Another study (Esser and Tomluk 2005) supported the fact that if genotype distribution of the patient group shows deviation from HWE, this may provide additional support for an association of the marker locus with the disease. Therefore, we believe that the investigated SNP rs12212067T>G in the *FOXO3A* might be one of the signatures of negative selection for severe malaria in the Gabonese population, while the natural selection had been documented to play an important role during malarial infection and in other infectious diseases (Karlsson et al. 2014). Notably, our study may be seen as a replication study, as it confirms the association described in Kenyan and Vietnamese study groups (Lee et al. 2013).

During infection, Tregs play an important role in regulating the clinical outcome of infectious diseases through their control of immune responses and their contribution to immune homeostasis (Sakaguchi 2000, 2004). The maintenance of this immune homeostasis is mediated by immunosuppressive cytokines, including IL-10 and TGF-ß (Levings et al. 2002; Taylor et al. 2006). This mediation appears to be modulated strongly by the transcription factor FOXO3 (Dejean et al. 2009). Therefore, it is reasonable that any polymorphism in the *FOXO3A* gene might modify immune responses and, consequently, the prognosis of the disease.

With regard to *FOXO3A* variability, information on the clinical outcome of inflammatory or infectious diseases is scarce (Lee et al. 2013; Wang et al. 2014). In the present study, we assessed the contribution of the intronic variant *rs12212067T>G* of *FOXO3A* to the outcome of *Plasmodium falciparum* malaria among Gabonese children. Our finding suggests that *FOXO3A* is involved in the regulation of the immune response during malaria. The minor allele *G* was significantly associated with severe malaria, the complicated form of the disease. This is in agreement with a previous study where identical associations were observed in sizable study groups from Vietnam and Kenya (Lee et al. 2013).

Many studies have indicated a role of FOXO3 in the regulation of inflammatory cytokines (Dejean et al. 2009; Litvak et al. 2012), although the relevance of such functions in humans still remains unclear (Lee et al. 2013). The association of the *FOXO3A* allele *G* with susceptibility to severe malaria suggests an interference of this variant with human inflammatory responses. In fact, it was shown that FOXO3 restricts the production of inflammatory cytokines in human monocytes through the TGFß1 mechanism (Lee et al. 2013). Thus, the explanation of how the minor allele *G* increases the risk of severe malaria is based on the FOXO3-driven pathway that confines the inflammatory responses in monocytes, which through TGFß1, reduces the production of pro-inflammatory cytokines including TNFα and increases the production of anti-inflammatory cytokines such as IL-10 (Lee et al. 2013).

Our study provides information on the FOXO3 transcription factor, in particular, regarding the contribution of rs12212067T>G polymorphism in the regulation of the immune response during *Plasmodium falciparum* malaria. With allele frequencies of 25 % in the present study, 19 % in African Americans, 25 % in the Luhya ethnic group from Kenya, and 21 % in Yoruba from Nigeria; the minor *FOXO3A* allele rs12212067*G* appears to have reached stability in the African populations studied or to be subjected to negative selection, further reducing the occurrence of this allele. Whether *FOXO3A* rs12212067*G* confers any advantage with regard to other phenotypes needs to be studied.

Several limitations apply to our study. Although sufficient statistical significance of our result was achieved and the results are in agreement with previous findings in Kenyan and Vietnamese studies, the study group is rather small. Furthermore, as this finding of the rs12212067*G* association was not confirmed in a recent genome-wide association study on malaria (Timmann et al. 2012), the usefulness and validity of candidate gene studies in general and in this study in particular may be questioned. We believe, however, that in polygenic diseases, all genetic contributions identified should be considered in order to add to and eventually complete our understanding of the genetic basis of infectious diseases.

Acknowledgments We would like to thank the staff and technicians of Lambaréné, Gabon, for their assistance in collecting the blood samples of patients during the study. We also express our gratitude to all the study participants involved in this study.

Conflicts of interest The authors have no conflicts of interest to declare.

References

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261–269

Brustoski K, Moller U, Kramer M, Hartgers FC, Kremsner PG, Krzych U, Luty AJ (2006) Reduced cord blood immune effector-cell responsiveness mediated by CD4+ cells induced in utero as a consequence of placental *Plasmodium falciparum* infection. J Infect Dis 193:146–154

Carter ME, Brunet A (2007) FOXO transcription factors. Curr Biol 17: R113–R114

Dejean AS, Beisner DR, Ch'en IL, Kerdiles YM, Babour A, Arden KC, Castrillon DH, DePinho RA, Hedrick SM (2009) Transcription factor FOXO3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. Nat Immunol 10:504–513

Eijkelenboom A, Mokry M, de Wit E, Smits LM, Polderman PE, van Triest MH, van Boxtel R, Schulze A, de Laat W, Cuppen E, Burgering BM (2013) Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Mol Syst Biol 9:638

Essaghir A, Dif N, Marbehant CY, Coffer PJ, Demoulin JB (2009) The transcription of FOXO genes is stimulated by FOXO3 and repressed by growth factors. J Biol Chem 284:10334–10342

Esser C, Tomluk J (2005) Reporting Hardy-Weinberg tests in case-control studies: reasons for caution but not for panic reactions. J Investig Dermatol 124:1082–1083

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567

Hanel SA, Velavan TP, Kremsner PG, Kun JF (2011) Novel and functional regulatory SNPs in the promoter region of FOXP3 gene in a Gabonese population. Immunogenetics 63:409–415

Hedrick SM (2009) The cunning little vixen: FOXO and the cycle of life and death. Nat Immunol 10:1057–1063

Hedrick SM, Hess MR, Doedens AL, Goldrath AW, Stone EL (2012) FOXO transcription factors throughout T cell biology. Nat Rev Immunol 12:649–661

Huang X, Kuhne V, Kun JF, Soboslay PT, Lell B, Tp V (2012) In-vitro characterization of novel and functional regulatory SNPs in the promoter region of IL2 and IL2R alpha in a Gabonese population. BMC Med Genet 13:117

Kalmbach Y, Boldt AB, Fendel R, Mordmuller B, Kremsner PG, Kun JF (2006) Increase in annexin V-positive B cells expressing LILRB1/ILT2/CD85j in malaria. Eur Cytokine Netw 17:175–180

Karlsson EK, Kwiatkowski DP, Sabeti PC (2014) Natural selection and infectious disease in human populations. Nat Rev Genet 15:379–393

Kerdiles YM, Stone EL, Beisner DR, McGargill MA, Ch'en IL, Stockmann C, Katayama CD, Hedrick SM (2010) FOXO transcription factors control regulatory T cell development and function. Immunity 33:890–904

Koukouikila-Koussounda F, Ntoumi F, Ndounga M, Tong HV, Abena AA, Velavan TP (2013) Genetic evidence of regulatory gene variants of the STAT6, IL10R and FOXP3 locus as a susceptibility factor in uncomplicated malaria and parasitaemia in Congolese children. Malar J 12:9

Kun JF, Mordmuller B, Lell B, Lehman LG, Luckner D, Kremsner PG (1998) Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria. Lancet 351:265–266

Lee JC, Espeli M, Anderson CA, Linterman MA, Pocock JM, Williams NJ, Roberts R, Viatte S, Fu B, Peshu N, Hien TT, Phu NH, Wesley E, Edwards C, Ahmad T, Mansfield JC, Gearry R, Dunstan S, Williams TN, Barton A, Vinuesa CG, Parkes M, Lyons PA, Smith KG (2013) Human SNP links differential outcomes in inflammatory and infectious disease to a FOXO3-regulated pathway. Cell 155:57–69

Levings MK, Bacchetta R, Schulz U, Roncarolo MG (2002) The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. Int Arch Allergy Immunol 129:263–276

Litvak V, Ratushny AV, Lampano AE, Schmitz F, Huang AC, Raman A, Rust AG, Bergthaler A, Aitchison JD, Aderem A (2012) A FOXO3-IRF7 gene regulatory circuit limits inflammatory sequelae of antiviral responses. Nature 490:421–425

Maizels RM (2009) Parasite immunomodulation and polymorphisms of the immune system. J Biol 8:62

Malaguarnera L, Musumeci S (2002) The immune response to *Plasmodium falciparum* malaria. Lancet Infect Dis 2:472–478

Miller LH, Baruch DI, Marsh K, Doumbo OK (2002) The pathogenic basis of malaria. Nature 415:673–679

Sakaguchi S (2000) Regulatory T cells: key controllers of immunologic self-tolerance. Cell 101:455–458

Sakaguchi S (2004) Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 22:531–562

Scholzen A, Mittag D, Rogerson SJ, Cooke BM, Plebanski M (2009) *Plasmodium falciparum*-mediated induction of human CD25FOXP3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. PLoS Pathog 5:e1000543

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 277:567–570

Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA (2006) Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 117:433–442

Timmann C, Thye T, Vens M, Evans J, May J, Ehmen C, Sievertsen J, Muntau B, Ruge G, Loag W, Ansong D, Antwi S, Asafo-Adjei E, Nguah SB, Kwakye KO, Akoto AO, Sylverken J, Brendel M, Schuldt K, Loley C, Franke A, Meyer CG, Agbenyega T, Ziegler A, Horstmann RD (2012) Genome-wide association study indicates two novel resistance loci for severe malaria. Nature 489:443–446

Velavan TP, Ojurongbe O (2011) Regulatory T cells and parasites. J Biomed Biotechnol 2011:520940

Velavan TP, Bechlars S, Tomiuk J, Kremsner PG, Kun JF (2011) Molecular characterization of regulatory polymorphisms in the promoter region of the STAT6 gene in a Gabonese population. Mem Inst Oswaldo Cruz 106:65–69

Velavan TP, Buyukyazici B, Kremsner PG, Kun JF (2012) Combined promoter haplotypes of the IL10R genes are associated with protection against severe malaria in Gabonese children. Immunogenetics 64:87–95

Vigario AM, Gorgette O, Dujardin HC, Cruz T, Cazenave PA, Six A, Bandeira A, Pied S (2007) Regulatory CD4+ CD25+ FOXP3+ T cells expand during experimental *Plasmodium* infection but do not prevent cerebral malaria. Int J Parasitol 37:963–973

Wang Y, Zhou L, Chen J, Li J, He L, Wu P, Wang M, Tong N, Zhang Z, Fang Y (2014) Association of the 3′UTR FOXO3a polymorphism rs4946936 with an increased risk of childhood acute lymphoblastic leukemia in a Chinese population. Cell Physiol Biochem 34:325–332

Weatherall DJ, Clegg JB (2002) Genetic variability in response to infection: malaria and after. Genes Immun 3:331–337

Weigel D, Jackle H (1990) The fork head domain: a novel DNA binding motif of eukaryotic transcription factors? Cell 63:455–456

WHO (2010) Guidelines for the treatment of malaria

WHO (2013) World malaria report: 2013

Zintzaras E (2010) Impact of Hardy-Weinberg equilibrium deviation on allele-based risk effect of genetic association studies and meta-analysis. Eur J Epidemiol 25:553–560

PLOS | MEDICINE

RESEARCH ARTICLE

Intramuscular Artesunate for Severe Malaria in African Children: A Multicenter Randomized Controlled Trial

Peter G. Kremsner[1,2]*, Akim A. Adegnika[1,2], Aurore B. Hounkpatin[1,2], Jeannot F. Zinsou[1,2], Terrie E. Taylor[3], Yamikani Chimalizeni[3], Alice Liomba[3], Maryvonne Kombila[4], Marielle K. Bouyou-Akotet[4], Denise P. Mawili Mboumba[4], Tsiri Agbenyega[5,6], Daniel Ansong[5,6], Justice Sylverken[5,6], Bernhards R. Ogutu[7], Godfrey A. Otieno[7], Anne Wangwe[7], Kalifa A. Bojang[8], Uduak Okomo[8], Frank Sanya-Isijola[8], Charles R. Newton[9], Patricia Njuguna[9], Michael Kazungu[9], Reinhold Kerb[10,11], Mirjam Geditz[10,11], Matthias Schwab[10,12], Thirumalaisamy P. Velavan[1], Christian Nguetse[1], Carsten Köhler[1], Saadou Issifou[1,2], Stefanie Bolte[1], Thomas Engleitner[1], Benjamin Mordmüller[1,2], Sanjeev Krishna[1,2,13]*

1 Institut für Tropenmedizin, Eberhard Karls Universität Tübingen, Tübingen, Germany, **2** Centre de Recherches Médicales de Lambaréné, Hôpital Albert Schweitzer, Lambaréné, Gabon, **3** Blantyre Malaria Project, University of Malawi College of Medicine, Blantyre, Malawi, **4** Department of Parasitology Mycology, Faculty of Medicine, Université des Sciences de la Santé, Libreville, Gabon, **5** Department of Physiology, University of Science and Technology, School of Medical Sciences, Kumasi, Ghana, **6** Departments of Child Health and Medicine, Komfo Anokye Teaching Hospital, Kumasi, Ghana, **7** Centre for Clinical Research, Kenya Medical Research Institute, Kisumu, Kenya, **8** Medical Research Council Laboratories, Fajara, The Gambia, **9** Centre for Geographic Medicine Research–Coast, Kenya Medical Research Institute, Kilifi, Kenya, **10** Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, Germany, **11** Eberhard Karls Universität Tübingen, Tübingen, Germany, **12** Abteilung Klinische Pharmakologie, Universitätsklinikum Tübingen, Tübingen, Germany, **13** Institute for Infection and Immunity, St George's, University of London, London, United Kingdom

* peter.kremsner@uni-tuebingen.de (PGK); s.krishna@sgul.ac.uk (SK)

OPEN ACCESS

Citation: Kremsner PG, Adegnika AA, Hounkpatin AB, Zinsou JF, Taylor TE, Chimalizeni Y, et al. (2016) Intramuscular Artesunate for Severe Malaria in African Children: A Multicenter Randomized Controlled Trial. PLoS Med 13(1): e1001938. doi:10.1371/journal.pmed.1001938

Academic Editor: Abdisalan Mohamed Noor, Kenya Medical Research Institute - Wellcome Trust Research Programme, KENYA

Received: January 16, 2015

Accepted: December 2, 2015

Published: January 12, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The study was funded by European and Developing Countries Clinical Trials Partnership (http://www.edctp.org; CT.2004.31070.001) and Federal Ministry of Education and Research (http://www.bmbf.de/en/; BMBF grant 01KA1011). Additional support was received by Central African Network on Tuberculosis, HIV/AIDS and Malaria; http://www.cantam.org/content/about-cantam; CANTAM, German Center for Infection Research (http://www.

Abstract

Background

Current artesunate (ARS) regimens for severe malaria are complex. Once daily intramuscular (i.m.) injection for 3 d would be simpler and more appropriate for remote health facilities than the current WHO-recommended regimen of five intravenous (i.v.) or i.m. injections over 4 d. We compared both a three-dose i.m. and a three-dose i.v. parenteral ARS regimen with the standard five-dose regimen using a non-inferiority design (with non-inferiority margins of 10%).

Methods and Findings

This randomized controlled trial included children (0.5–10 y) with severe malaria at seven sites in five African countries to assess whether the efficacy of simplified three-dose regimens is non-inferior to a five-dose regimen. We randomly allocated 1,047 children to receive a total dose of 12 mg/kg ARS as either a control regimen of five i.m. injections of 2.4 mg/kg (at 0, 12, 24, 48, and 72 h) (n = 348) or three injections of 4 mg/kg (at 0, 24, and 48 h)

dzif.de/en/; DZIF), Deutsche Forschungsgemeinschaft (DFG; http://www.dfg.degrant; KE 1629/1-1) and Robert Bosch Stiftung (Stuttgart, Germany). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: GK, BM, CK, SB, and TE report grants from European and Developing Countries Clinical Trials Partnership and Bundesministerium für Building und Forschung Deutschland for the conduct of the study. SK reports grants from European and Developing Countries Clinical Trials Partnership for the conduct of the study, personal fees from Merck Serono and shareholder at QuantuMDx. AAA, JFZ, ABH, TET, YC, AL, MK, MKBA, DPMM, TA, DA, JS, BRO, GAO, AW, KAB, UO, FSI, CRN, PN, MK, and SI report grant from European and Developing Countries Clinical Trials Partnership for the conduct of the study. MS, MG, and RK report grants from DFG and Robert Bosch Stiftung for the conduct of the study. TPV and CN report grant from Deutsche Forschungsgemieinschaft for the conduct of the study. SK is a member of the Editorial Board of *PLOS Medicine*.

Abbreviations: AE, adverse event; ARS, artesunate; DHA, dihydroartemisinin; DHAG, dihydroartemisinin glucuronide; DMB, data monitoring board; G6PDH, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; HR, hazard ratio; ITT, intention-to-treat; i.m., intramuscular; i.v., intravenous; PK, pharmacokinetics; PP, per-protocol; SAE, serious adverse event; SMAC, Severe Malaria in African Children.

either i.m. (n = 348) or i.v. (n = 351), both of which were the intervention arms. The primary endpoint was the proportion of children with ≥99% reduction in parasitemia at 24 h from admission values, measured by microscopists who were blinded to the group allocations. Primary analysis was performed on the per-protocol population, which was 96% of the intention-to-treat population. Secondary analyses included an analysis of host and parasite genotypes as risks for prolongation of parasite clearance kinetics, measured every 6 h, and a Kaplan–Meier analysis to compare parasite clearance kinetics between treatment groups. A post hoc analysis was performed for delayed anemia, defined as hemoglobin ≤ 7g/dl 7 d or more after admission.

The per-protocol population was 1,002 children (five-dose i.m.: n = 331; three-dose i.m.: n = 338; three-dose i.v.: n = 333); 139 participants were lost to follow-up. In the three-dose i.m. arm, 265/338 (78%) children had a ≥99% reduction in parasitemia at 24 h compared to 263/331 (79%) receiving the five-dose i.m. regimen, showing non-inferiority of the simplified three-dose regimen to the conventional five-dose regimen (95% CI −7, 5; p = 0.02). In the three-dose i.v. arm, 246/333 (74%) children had ≥99% reduction in parasitemia at 24 h; hence, non-inferiority of this regimen to the five-dose control regimen was not shown (95% CI −12, 1; p = 0.24). Delayed parasite clearance was associated with the ^{N86Y}Pfmdr1 genotype. In a post hoc analysis, 192/885 (22%) children developed delayed anemia, an adverse event associated with increased leukocyte counts. There was no observed difference in delayed anemia between treatment arms.

A potential limitation of the study is its open-label design, although the primary outcome measures were assessed in a blinded manner.

Conclusions

A simplified three-dose i.m. regimen for severe malaria in African children is non-inferior to the more complex WHO-recommended regimen. Parenteral ARS is associated with a risk of delayed anemia in African children.

Trial registration

Pan African Clinical Trials Registry PACTR201102000277177

Introduction

Studies to optimize artesunate (ARS) treatment regimens in malaria have been surprisingly sparse, given that ARS is now established as the treatment of choice for severe malaria in both adults and children [1,2]. WHO recommends ARS (2.4 mg/kg) administered by intravenous (i.v.) or intramuscular (i.m.) routes at 0, 12, 24, 48, and 72 h after admission [2], although simpler regimens would be preferable, assuming that safety and efficacy were not compromised [2]. The advantages of a simpler regimen are obvious to health care workers in under-resourced settings, where finding and maintaining i.v. access in small, sick children to ensure that correct doses are given on time is a challenge [1,3,4].

In an earlier study, a simplified three-dose ARS i.v. regimen was found to be non-inferior in pharmacodynamic efficacy to the conventional WHO regimen [1], and its pharmacokinetics (PK) were defined with a formulation that conformed to standards of good manufacturing

practice. Since then, WHO has prequalified another formulation of ARS (Guilin Pharmaceutical, Shanghai, China), making it more widely used. We have also compared the i.v. and i.m. routes of this product for severe malaria in African children and have described the PK of a WHO-recommended dose regimen using ARS [4]. This regimen of one dose of 2.4 mg/kg followed by four doses of 1.2 mg/kg has been superseded by a regimen of five doses of 2.4 mg/kg, and there has since been debate about the simplified (once daily) i.v. regimen for severe malaria [5,6]. The i.m. route has not yet been studied in adequately powered dose optimization trials.

We examined i.m. ARS in severe malaria in seven hospitals of the Severe Malaria in African Children (SMAC) network [7,8]. We assessed whether splitting the total dose of 12 mg/kg into a simplified once daily i.m. or i.v. three-dose regimen (4 mg/kg per dose) is non-inferior to the WHO-recommended five-dose regimen (2.4 mg/kg per dose). We also examined associations of genetic polymorphisms of *pfmdr1* and *kelch-13* with parasite clearance kinetics and, in post hoc analysis, the occurrence of delayed anemia.

Our primary study objective was to assess the non-inferiority of i.v. ARS and i.m. ARS simplified dosing regimens (4 mg/kg ARS at 0, 24, and 48 h; 12 mg/kg total dose) to the standard i.m. treatment dosing regimen (2.4 mg/kg ARS at 0, 12, 24, 48, and 72 h; 12 mg/kg total dose) in clearing parasitemia in African children with severe malaria. Our secondary study objectives were to compare the tolerability and safety of the three ARS dosing regimens, to analyze host and parasite genotypes as risks for prolongation of parasite clearance kinetics, measured every 6 h, and to compare parasite clearance kinetics between treatment groups. An exploratory objective was to analyze genetic polymorphisms in humans and parasites linked to disease and treatment, and a post hoc objective was to assess the occurrence of delayed anemia.

Methods

The trial was performed according to the principles of the Declaration of Helsinki and Good Clinical Practice. Ethics committees and competent authorities for each study site approved the study. A data monitoring board (DMB) provided oversight for the study with respect to safety and efficacy as well as appropriate implementation of the defined stopping rules.

Study Design

This was an open-label, randomized, multicenter, parallel-group, three-arm study to compare the antimalarial activity and safety of three ARS dosing regimens in children with severe *Plasmodium falciparum* malaria. Patients (as shown in Fig 1) were randomly assigned to one of three dosing regimens consisting of a total of 12 mg/kg parenteral ARS: (i) 2.4 mg/kg i.m. on admission and at 12, 24, 48, and 72 h, (ii) 4 mg/kg i.m. on admission and at 24 and 48 h, and (iii) 4 mg/kg i.v. on admission and at 24 and 48 h. Time points of administration of ARS for each group are represented in Table 1.

Parasitemia was measured by thick blood smears at 6-h intervals and prior to the each dose of treatment for at least 48 h following the first dose of study drug, or until three consecutive negative smears were recorded within the last 24-h period. Thick blood films were also examined on days 7, 14, and 28.

The primary efficacy endpoint was the proportion of patients with $\geq$99% parasite reduction from the baseline asexual parasite count at 24 ± 1 h. Parasitemia was always quantitated before the 24-h dose was administered, i.e., after either one (intervention arms) or two (control arm) doses of ARS had been administered. This endpoint was derived from discussions in the SMAC network and from our own studies [1,4] and is based on the following reasoning. First, WHO guidelines for the treatment of severe malaria [9] are based on studies that have used multiple outcomes. For efficacy, these are death, parasite clearance time, fever clearance time,

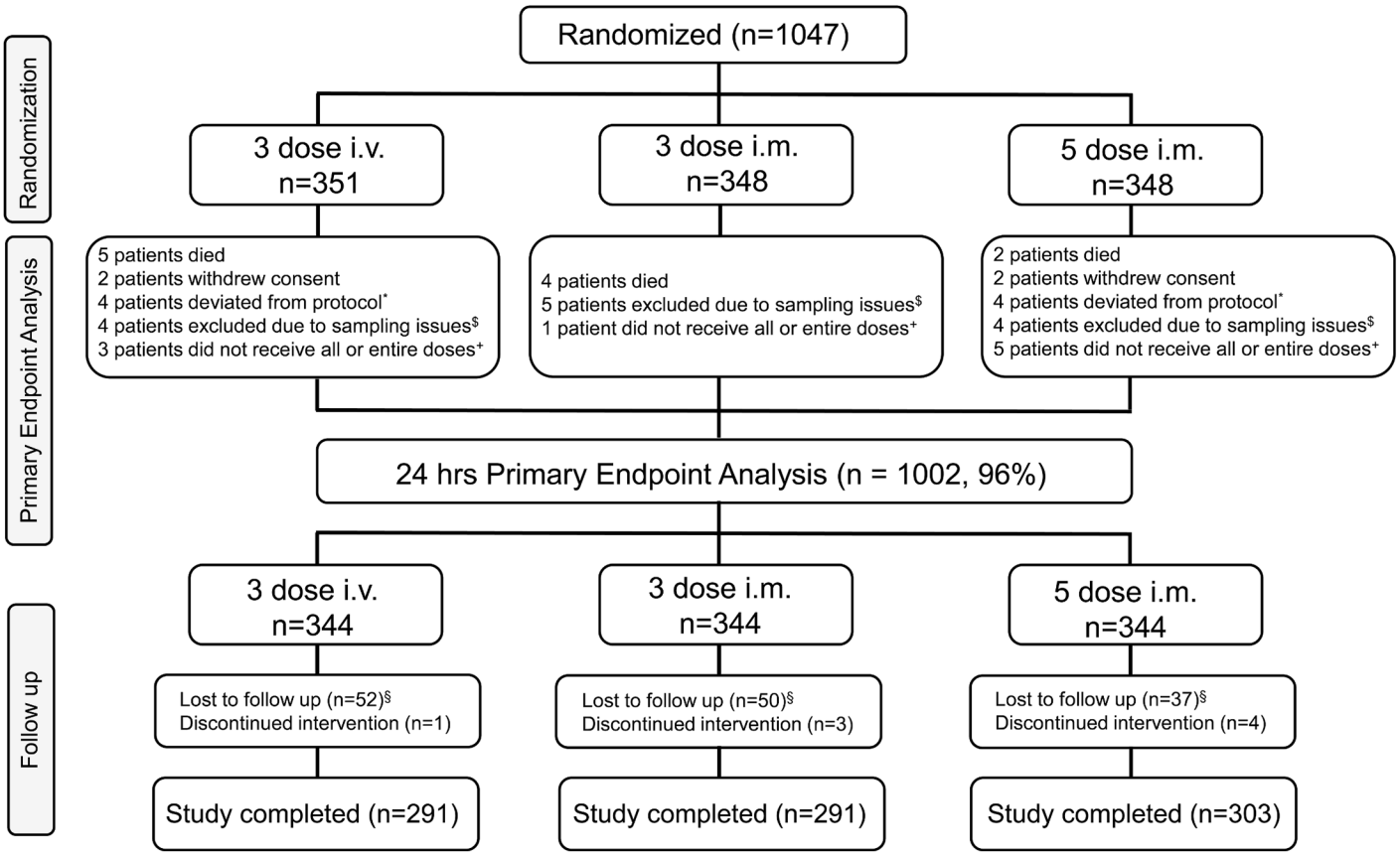

Fig 1. Trial profile. *These patients completed the study but were not included for the primary endpoint analysis because of protocol deviations. $These patients completed the study but were not included for the primary endpoint analysis because of sampling issues. +These patients completed the study but were not included for the primary endpoint analysis because of dosing issues. §Lost to follow-up includes patients who (i) withdrew consent (n = 8), (ii) moved from the study area (n = 9), and (iii) were discharged from the study due to malaria infection on day 28 (n = 1), amongst a variety of other reasons.

doi:10.1371/journal.pmed.1001938.g001

time to discharge from hospital (days), and coma resolution time. As studies using mortality as an endpoint are impracticable in seasoned centers where overall mortality from severe malaria is <5%—requiring sample sizes that are too large—we have chosen a parasitemia clearance parameter as an endpoint. Parasite clearance kinetics was also used when a quinine loading dose (20 mg/kg salt, if no pretreatment) was being developed and compared with the then standard dose (10 mg/kg salt), when a mortality study comparing the two regimens was ruled out on sample size grounds. Parasite clearance time was a crucial determinant of efficacy for comparing the same drug in two dosing regimens [10]. In severe malaria, clinically advantageous benefits of more rapid clearance of parasites by ARS (even when given by suppository) have been reported in comparison with i.m. artemether [11]. The 24-h time point was chosen as an

Table 1. Dosing regimen of artesunate.

Group	Route	Dose	0 h	12 h	24 h	48 h	72 h
Five-dose i.m.	i.m.	2.4 mg/kg	X	X	X	X	X
Three-dose i.m.	i.m.	4.0 mg/kg	X		X	X	
Three-dose i.v.	i.v.	4.0 mg/kg	X		X	X	

doi:10.1371/journal.pmed.1001938.t001

endpoint because most deaths (>60%) from severe malaria take place within 24 h of admission, and accurate assessment of parasitemia becomes more difficult at later time points.

Evaluation of the whole regimens is included in the secondary outcome measures (and there is no discrepancy between the results for this and the 24-h end point). Further endpoints were time to total clearance of asexual parasites, time to 99% reduction of asexual parasites, time to 90% reduction of asexual parasites, time to 50% reduction of asexual parasites, proportion of patients with genotype-uncorrected adequate clinical and parasitological response on day 28, percent reduction in asexual parasites from baseline at 24 h after initiation of randomized study drug, and percent reduction in asexual parasites from baseline at 48 h after initiation of randomized study drug.

During the conduct of this study, several patients in the case series developed delayed hemolytic anemia following ARS therapy, mostly in the second and third week from the start of therapy [12–15]. Motivated by these events, we amended our trial protocol and undertook two exploratory post hoc analyses of delayed anemia. In the first, all trial participants were screened and treated for delayed anemia, defined as hemoglobin (Hb) $\leq$ 7 g/dl seven or more days after admission. In the second, a subgroup of 72 patients in Kumasi and Lambaréné who underwent detailed hematological monitoring for 28 d following discharge from the hospital were assessed for hemoglobin and laboratory markers of hemolysis (such as lactate dehydrogenase) during follow-up [16]. For the patients in the subgroup, we were able to assess laboratory markers of hemolysis (such as lactate dehydrogenase) in more intensive follow-up, whereas the last day of scheduled sampling for these particular markers for all other patients in the study was day 7.

Participants

Children were aged 6 mo to 10 y, with a diagnosis of *P. falciparum* infection (parasitemia $\geq$ 5,000 parasites/μl on initial blood smear) and clinical signs and symptoms severe enough to require hospitalization, according to the SMAC definition of severe malaria that best reflects the policies of African hospitals [7,8]. Most (87%) of these children also fulfilled one or more criteria of the WHO definition of severe malaria [17], which include severe anemia (hematocrit of <15% or Hb < 5 g/dl with a parasite density of >10,000/μl), hyperlactatemia ($\geq$5 mmol/l), hyperparasitemia (>250,000 parasites/μl), hypoglycemia (whole blood or plasma glucose $\leq$ 2.2 mmol/l), and hemoglobinuria (urine that is dark red or black, with a dipstick that is positive for Hb/myoglobin).

In addition, children were required to be willing and able to comply with the study protocol for the duration of the study, be willing to remain in the hospital for at least 3 d, and have had written informed consent provided by their parents or guardians. Exclusion criteria included known serious adverse reaction or hypersensitivity to artemisinins, any underlying disease that might compromise the diagnosis and evaluation of the response to the study medication, participation in any investigational drug study during the 30 d prior to screening, and adequate (according to WHO and country-specific guidelines) antimalarial treatment within 24 h prior to admission.

Patients were recruited at the Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon (n = 245); Queen Elizabeth Central Hospital, Blantyre, Malawi (n = 211); the Université des Sciences de la Santé, Libreville, Gabon (n = 150); the School of Medical Sciences at the University of Science and Technology (Komfo Anokye Teaching Hospital), Kumasi, Ghana (n = 149), Kenya Medical Research Institute Kondele Children's Hospital, Kisumu, Kenya (n = 129); Edward Francis Small Teaching Hospital (former Royal Victoria Teaching Hospital), Medical Research Council Laboratories, Banjul, The Gambia (n = 90); and the Kenya Medical Research Institute Centre for Geographic Medicine–Coast, Kilifi, Kenya (n = 73).

A total of 45 participants were lost to follow-up between the time of randomization and the primary endpoint analysis time point at 24 h. Loss to follow-up was due to death, withdrawal of consent, protocol deviations, not receiving all doses, or sampling issues. Sampling issues were defined as physical difficulties in obtaining blood due to the small size of participants and difficulties with venipuncture. A total of 139 participants were lost to follow-up after the 24-h primary endpoint analysis time point. Participants were considered lost to follow-up after the 24-h time point if they were unable to be reached when research staff tried to contact them at least five times at two different times of day via telephone, and with at least two house visits, within a 2-wk period.

Patient participation included hospitalization for at least 3 d and follow-up for at least 28 d following the first dose of study drug. Participants had scheduled follow-up visits in the clinic on days 7, 14, and 28, during which vital sign evaluation, physical examination, adverse event (AE) review, and blood sampling for hematology, biochemistry, parasitological assessments, PK analysis, and exploratory analyses were conducted

Randomization and Masking

Randomization was balanced at each study site in a 1:1:1 ratio for each regimen. Randomization cards were supplied in numbered, sealed envelopes. The envelope for each participant was opened after inclusion in the trial, directly before treatment initiation. Microscopists were not informed about group allocations.

Procedures

ARS for injection (Artesun; Guilin Pharmaceutical, Shanghai, China) was supplied as powder and reconstituted before injection. Artemether-lumefantrine was given at discharge in a weight-normalized dosing regimen [18].

Other concomitant therapies were given according to published guidelines [2] and the standard operating procedures of the sites. Malarial infection recurring within 28 d was treated with artemether-lumefantrine.

Malaria smears were done every 6 h until three consecutive negative smears were recorded [19] and were read independently by two microscopists. Vital signs were recorded at least twice daily, and physical examination was done repeatedly, over the period of hospitalization.

Population PK studies were performed on a subset (n = 288) of patients for the parent compound ARS, the primary metabolite dihydroartemisinin (DHA) and the secondary metabolite, the primary DHA glucuronide (DHAG), using established population PK techniques that were refined using the results of the first dose optimization study [1]. PK data were available for 39 of the patients in the anemia analysis. Venous samples (400 μl) were collected 30, 60, 120, 240, or 360 min after each of three dosings. Allocation to one of the five predefined sampling time points was random within each treatment arm. Samples were stored at −80°C until use. ARS, DHA, and DHAG concentrations were assayed using liquid chromatography/mass spectrometry [20]. In total, 851 samples were analyzed from three study centers (from 116 patients from Lambaréné, 84 from Kisumu, and 88 from Kumasi). Parasite and host polymorphisms were examined in the following genes using published methods and primers for PCR: *pfmdr1*, *kelch-13*, the sickle cell gene, and the gene for glucose-6-phosphate dehydrogenase (G6PDH) [20,21]. This analysis was carried out on the subset of patients included in the PK analysis.

Statistical Analysis

Fisher's exact test for one-sided equivalence [22] was used to assess treatment group differences in parasite clearance for the per-protocol (PP) and intention-to-treat (ITT) populations, and

the 95% CI of the difference in proportion is given. Primary analysis was on the PP population, since it is more conservative in non-inferiority models. Testing was done hierarchically, with comparisons of the two experimental arms (three-dose i.m. and three-dose i.v.) against the control (five-dose i.m.), corrected for multiple testing using the Bonferroni method. Only when both tests rejected the null hypothesis was a further test comparing the two experimental arms planned. As secondary endpoints, parasite clearance times were calculated using Kaplan–Meier estimates and a Cox model with treatment arm and study center as covariates, when not otherwise described. Alpha < 0.05 was considered significant.

The needed sample size was calculated based on the results of our prior study [1,6]. We assumed that 82% of patients would have a $\geq$99% reduction in parasitemia at 24 h (primary endpoint) and set power to 0.8, alpha to 0.05, and delta to 0.1. Using the Farrington and Manning procedure [23] as implemented in the Design package of R v. 2.10.1, the calculated sample size needed was 316 per arm when multiple comparisons between the groups were included. The total estimated sample size needed, with 10% headroom for loss to follow-up, was therefore 1,044 participants.

The non-inferiority margin delta was pre-specified as an absolute difference of 10% for the primary endpoint on the basis of our previous study [1] and published methods for analyzing time-to-event outcomes [24]. For the Cox model, the non-inferiority margin was translated into a hazard ratio (HR) assuming a 82% cure rate ($\geq$99% reduction in parasitemia at 24 h) with the control (five-dose i.m.) regimen and an at least 72% cure rate with the experimental (three-dose i.m. and three-dose i.v.) regimens.

All patients who received at least one dose of the study drug were included in the safety analysis. Delayed anemia was analyzed using a logistic regression model for non-hematological variables, a two-way ANOVA for genotype analysis, and an ANCOVA for hematological variables.

Descriptive statistics for the drug concentration data were calculated for the set of all patients in this study who received the full dose of ARS and who had plasma concentration data available. A population PK model, assuming 100% conversion of ARS to DHA, was developed using the nonlinear mixed-effects modeling software Phoenix NLME 1.2 (Pharsight, St. Louis, Missouri, US). The final population PK models for ARS, DHA, and DHAG were evaluated using visual predictive checks. Plasma concentration over time data were described by a one-compartment PK model with additive residual error and an exponential term for interpatient variability. Initial PK parameter estimates were from our previous SMAC trial [1]. Route of administration, study center, weight, age, height, delayed anemia, parasitemia, and host and parasite genotypes were added as covariates in the model by stepwise forward inclusion. Model improvement by covariates was statistically tested by the decrease in −2 log likelihood. The final population PK model included all covariates associated with a significant increase in log likelihood (5% significance).

Role of the Funding Source

The clinical sponsor of the trial was Universitätsklinikum Tübingen, and the corresponding author P. G. K. acted as the sponsor's representative. The corresponding authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

Results

In all, 1,047 patients were randomized and received at least one dose of study drug, as shown in Fig 1. This is the safety population and also defines the ITT population as all patients had

P. falciparum infection. The PP population is defined as all patients from the ITT population who received all doses of randomized study drug and for whom the primary endpoint could be calculated. This is the primary analysis population for efficacy and is 96% of the ITT population (n = 1,002).

Recruitment was from 4 July 2011 until 25 September 2012. As pre-specified in the protocol, after 50 patients completed the trial procedures up to 72 h, all serious AEs (SAEs) including deaths were reviewed by the DMB, with no findings that required stopping the study. After 100 patients completed the trial up to 24 h in each cohort, parasitemia evaluations were reviewed by the DMB to confirm that in all cohorts, 99% reduction in parasitemia was achieved in at least 60% of patients after 24 ± 1 h (stopping rule). After DMB reviews, there was no finding to stop recruitment. There were to be ad hoc reviews if SAEs and/or deaths in one cohort increased significantly compared with any other. For deaths in any cohort, the threshold to trigger review by the DMB was 4% (stopping rule), but it was not invoked. Anemia was studied in patients as shown in Fig 2. Table 2 summarizes the baseline demographic, clinical, and laboratory variables of patients in the ITT population.

Figs 3 and 4 present evidence that the three-dose i.m. route for ARS is non-inferior to the WHO-recommended five-dose i.m. regimen. Both i.m. routes had a higher proportion of patients with ≥99% reduction in parasitemia at 24 h (78%, or 265/338, for the three-dose i.m. group and 79%, or 263/331, for the five-dose i.m. group) (by about 5%) than the three-dose i.v. route (74%, or 246/333). This three-dose i.v. regimen had previously been found to be comparable in efficacy to the WHO-recommended five-dose i.v. regimen [1]. Time to 99% parasite clearance, specified as a secondary analysis, was comparable between treatment groups (Fig 5; S1 Table). Since the Fisher's exact test for one-sided equivalence does not account for stratifying covariates, which may be anti-conservative, the robustness of the results was tested with Cox proportional hazards models adjusted for study center, which showed non-inferiority (lower confidence interval limit of the HR > 0.74) [24,25] for all comparisons (three-dose i.m. versus five-dose i.m., HR 1.04 [97.5% CI 0.88–1.24]; three-dose i.v. versus five-dose i.m., HR 0.89 [97.5% CI 0.75–1.06]; three-dose i.m. versus three-dose i.v., HR 1.18 [95% CI 1.00–1.37]). For the three treatment groups, the estimates of time to 90% parasite clearance, adjusted for center and initial parasitemia, were significantly different (Fig 6). This difference can be attributed to a more rapid clearance in the three-dose i.m. group compared with the five-dose i.m. group (HR 1.21 [95% CI 1.04–1.41]). In addition, no difference was seen in fever clearance time between groups (S3–S5 Tables). No case required rescue treatment before discharge from hospital, and 16 patients died, with no group differences. There were 41/885 (5%) patients who were parasitemic at 28 d: 13 in the three-dose i.v. group, 11 in the three-dose i.m. group, and 17 in the five-dose i.m. group.

^{N86Y}Pfmdr1 was found in 107/287 (37%) parasites and was associated with delayed time to 99% and 100% parasite clearance estimates of 2.8 h (95% CI 0.9–4.8 h; $p = 0.005$) and 4.8 h (95% CI 1.9–7.6 h; $p < 0.001$), respectively. No other ^{N86Y}Pfmdr1 polymorphisms (Y184F, S1034C, N1042D, and D1246Y), including increased gene copy number of *pfmdr1* (found in 13 samples, 5%), were associated with time to parasite clearance. There were no previously reported polymorphisms in *kelch-13* sequences (M476I, Y493H, R539T, I543T, and C580Y). Tests of associations between parasite genotypes and clearance time estimates were corrected for center and treatment group.

Drug detection was linear, with ranges of 1–2,500 nM, 165–16,500 nM, and 4–10,000 nM for ARS, DHA, and DHAG, respectively. In total, 851 samples from 288 patients (153 male and 135 female, aged 0.5 to 10 y, mean 3.8 y) were analyzed: 92, 99, and 97 patients received the five-dose i.m., three-dose i.m., and three-dose i.v. regimens, respectively. The population estimates of PK parameters of the base models are presented in Table 3. These data confirm that

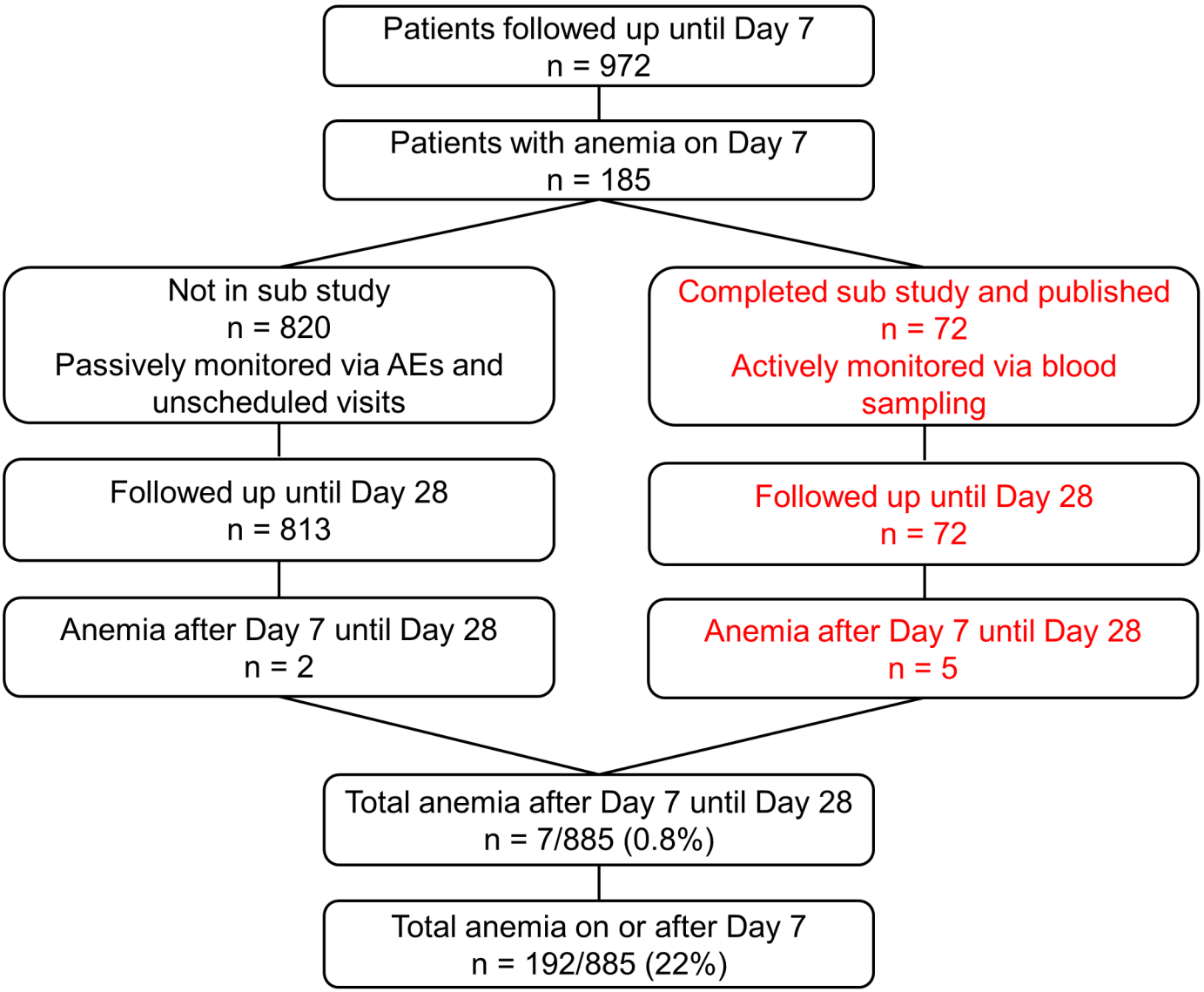

Fig 2. Post hoc analyses of patients with anemia. The right hand side of the diagram (in red) shows patients included in the substudy.

doi:10.1371/journal.pmed.1001938.g002

the three regimens studied are comparable in their PK parameters, with the exception of a larger volume of distribution of DHA following i.m. injection. In particular, estimates of time to clearance were comparable between groups. Fig 7 presents plots of observed concentration–time profiles for ARS, DHA, and DHAG according to treatment regimen. Estimated population mean PK profiles are shown by the red lines. Interestingly, ARS plasma concentrations varied more after i.v. than after i.m. administration. Study center, age, sex, weight, parasitemia, and delayed anemia were considered as covariates. Of these covariates, parasitemia influenced volume of distribution after i.v. ARS only, while the strongest influence was seen for study center on volume of distribution/bioavailability and clearance of ARS (i.m.), DHA, and DHAG.

The occurrence of laboratory and clinical AEs and SAEs (Table 4) was similar in the three groups. Out of 75 SAEs, 14 (five severe anemia, six persistent fever, two vomiting, and one cough) were judged as possibly related to the study drug.

Table 2. Patient clinical and laboratory findings on admission in intention-to-treat population.

Variable	Arm			Total (*n* = 1,047)
	Three-Dose i.v. (*n* = 351)	Three-Dose i.m. (*n* = 348)	Five-Dose i.m. (*n* = 348)	
Clinical findings				
Female	162 (46%)	166 (48%)	168 (48%)	496 (47%)
Male	189 (54%)	182 (52%)	180 (52%)	551 (53%)
Age, y	4.0 (2.4)	4.1 (2.5)	4.2 (2.5)	4.1 (2.5)
Aged 0–3 y	201 (57%)	185 (53%)	185 (53%)	571 (54%)
Aged 4–7 y	122 (35%)	132 (38%)	130 (37%)	384 (37%)
Aged >8 y	28 (8%)	31 (9%)	33 (10%)	92 (9%)
Pulse, beats/minute	134 (26)	133 (25)	133 (25)	133 (25)
Respirations/minute	40 (13)	39 (11)	40 (13)	40 (12)
Temperature, °C	38.2 (1.2)	38.1 (1.2)	38.1 (1.2)	38.1 (1.2)
Weight, kg	14.3 (5.3)	14.3 (5.0)	14.8 (5.1)	14.4 (5.5)
Laboratory findings				
Parasitemia per microliter × 10^{-3}*	129.0 (4.6–2,965.0)	114.0 (5.0–1,439.0)	119.0 (4.1–2,675.0)	121.0 (4.1–2,965.0)
Hb, g/dl	8.5 (2.4)	8.7 (2.3)	8.7 (2.4)	8.6 (2.4)
White blood cell count, 10^3/μl	10.3 (5.5)	10.1 (5.2)	9.7 (4.6)	10.0 (5.1)
Neutrophils, 10^3/μl	5.5 (3.2)	5.3 (3.0)	5.4 (3.3)	5.4 (3.2)
Platelet count, 10^3/mm^3	115 (117)	107 (108)	123 (130)	115 (119)
Clinical signs of severe malaria				
Severe anemia	34 (10%)	42 (12%)	40 (11%)	116 (11%)
Hyperlactatemia	21 (6%)	18 (5%)	23 (7%)	62 (6%)
Hyperparasitemia	103 (29%)	91 (26%)	103 (30%)	297 (28%)
Hypoglycemia	13 (4%)	7 (2%)	18 (5%)	38 (4%)
Jaundice	28 (8%)	29 (8%)	25 (7%)	82 (8%)
Hemoglobinuria	6 (2%)	6 (2%)	7 (2%)	19 (2%)
Respiratory distress	30 (9%)	35 (10%)	28 (8%)	93 (9%)
Severe vomiting	25 (7%)	36 (10%)	26 (7%)	87 (8%)
Prostration	169 (48%)	160 (46%)	140 (40%)	469 (45%)
Cerebral malaria	32 (9%)	28 (8%)	23 (7%)	83 (8%)
Generalized seizures	39 (11%)	44 (13%)	28 (8%)	111 (11%)

Data are given as mean (standard deviation) or *n* (percent), except for parasitemia per milliliter, which is given as geometric mean (range). Clinical classification was according to the following definitions: severe anemia (hematocrit of <15% or Hb < 5 g/dl with a parasite density of >10,000/μl), hyperlactatemia (≥5 mmol/l), hyperparasitemia (>250,000 parasites/μl), hypoglycemia (whole blood or plasma glucose ≤ 2.2 mmol/l), and hemoglobinuria (urine that is dark red or black, with a dipstick that is positive for Hb/myoglobin).

*PP population.

doi:10.1371/journal.pmed.1001938.t002

During the conduct of this trial, a series of case reports described delayed hemolytic anemia in travelers after receiving artemisinins [26] and prompted an urgent evaluation of the risk of delayed hemolysis in a subset of our patients in Kumasi and Lambaréné, in whom detailed hematological monitoring after discharge was possible, as an amendment to the protocol [16]. For these patients, we defined delayed hemolysis as the coexistence of (i) low haptoglobin (<0.30 mg/dl) on day 14, (ii) any decrease in hemoglobin between days 7 and 14, and (iii) any increase in lactate dehydrogenase between days 7 and 14 leading to a lactate dehydrogenase level of over 350 U/l on day 14.

This substudy identified five out of 72 evaluable patients with anemia between days 7 and 28 [16]. In a post hoc analysis, we also included anemia detected by hemoglobin measurement

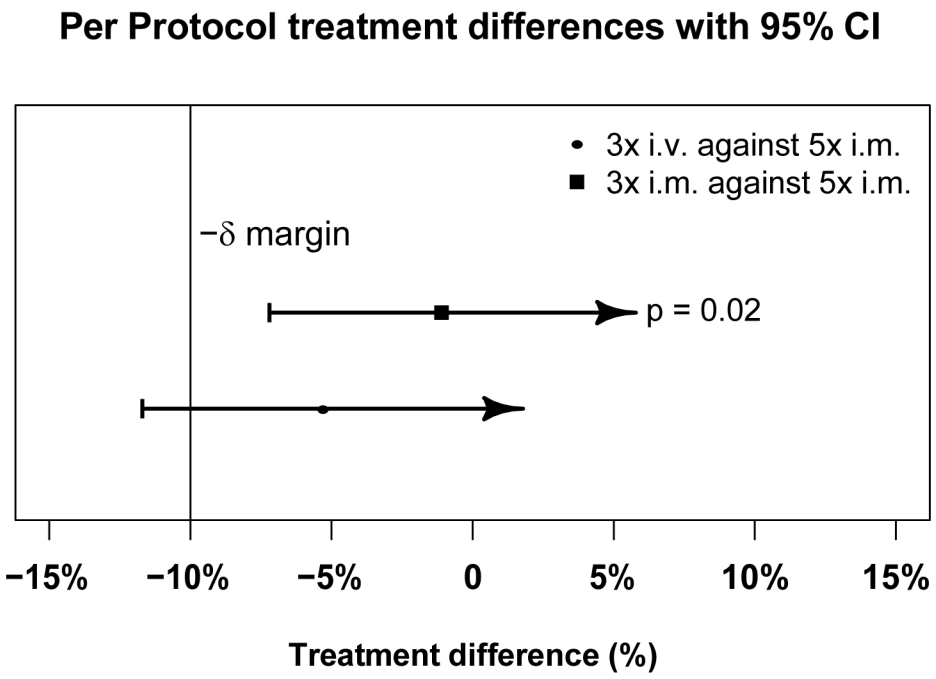

Fig 3. Per-protocol population primary endpoint analysis. PP treatment difference in proportions of patients with ≥99% parasite reduction, with corresponding 95% confidence intervals. The vertical line indicates the non-inferiority margin (δ). The three-dose i.m. treatment group is non-inferior to the five-dose i.m. treatment group ($p = 0.02$), whereas the three-dose i.v. group is not non-inferior ($p = 0.24$). Note that the p-value is calculated using Fisher's exact test for one-sided equivalence under the assumption that both regimens are equally efficacious.

doi:10.1371/journal.pmed.1001938.g003

on day 7 or later. This increased the total number of patients with anemia (Hb ≤ 7 g/dl) a week or more after the start of therapy to 192, although most were detected on day 7 due to sampling bias, as the last scheduled blood sampling per protocol in our study was day 7. We also investigated the AEs in all our patients (including those reported earlier [16]) by assessing anemia at passive follow-up after day 7, and detailed information is given in Table 5. This analysis includes the 72 patients from our earlier reported substudy [16].

We examined the relationships between other hematological variables, parasitemia, and delayed anemia, and allowed for admission values by including these in covariance analysis of the full dataset. There was no significant association between delayed anemia and admission parasitemia ($p = 0.30$) or platelet counts on day 7 ($p = 0.11$). A significant association emerged with leukocyte count ($p < 0.001$) and neutrophil count ($p < 0.001$) on day 7. Those with delayed anemia had higher leukocyte (Fig 8) and neutrophil counts than those without delayed anemia, regardless of the definition applied for delayed anemia.

G6PDH-deficient participants (A−) had more anemia on admission (odds ratio 4.3 [95% CI 2.1–9.0], $p < 0.001$) than those with G6PDH non-deficient genotypes, but this relationship did not hold for delayed anemia (odds ratio 1.4 [95% CI 0.6–3.2]). HbAC or HbAS genotype was not associated with delayed anemia, and there was no relationship between delayed anemia and PK parameters.

Discussion

This study consolidates previous work aiming to optimize dosage regimens using parenteral ARS for severe malaria [1]. Here, simpler three-dose i.m. and i.v. regimens have been compared

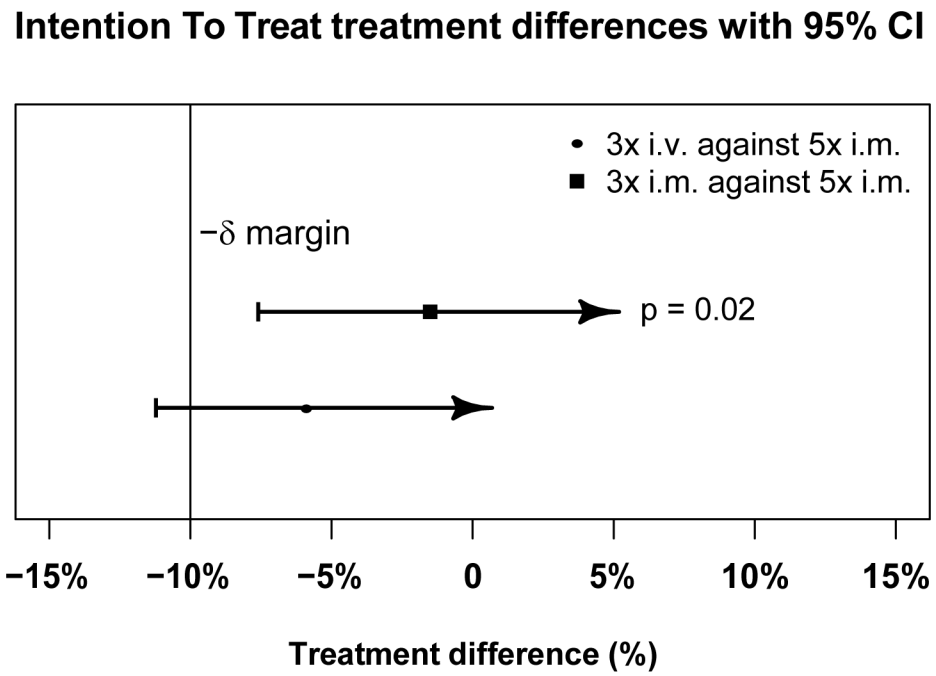

Fig 4. Intention-to-treat population primary endpoint analysis. ITT treatment difference in proportions of patients with ≥99% parasite reduction, with corresponding 95% confidence intervals. The vertical line indicates the non-inferiority margin (δ). The three-dose i.m. treatment group is non-inferior to the five-dose i.m. treatment group ($p = 0.02$), whereas the three-dose i.v. group is not non-inferior ($p = 0.24$). Note that the *p*-value is calculated using Fisher's exact test for one-sided equivalence under the assumption that both regimens are equally efficacious.

doi:10.1371/journal.pmed.1001938.g004

to the WHO-recommended five-dose i.m. regimen. This study provides new insights into ARS PK, delayed anemia, and genetic markers of delayed parasite clearance. Our studies used the pharmacodynamic endpoint of parasite clearance rather than other clinical outcome measures such as mortality because the latter requires impracticably large studies (as debated [5,6]). In comparing the same drug given in different doses and by different routes, parasite clearance kinetics should accurately reflect differences in in vivo antiparasitic activity because the mechanism of action is the same. It follows that if there are no differences in parasite clearance kinetics between treatments, then they can be considered to be of comparable efficacy. For uncomplicated malaria, for example, inadequate oral dosing with ARS is associated with prolonged parasite clearance kinetics, and in severe malaria a loading dose of quinine significantly shortened parasite clearance times compared with a non-loading-dose regimen [10,26].

From our previous study, we concluded that a simplified once daily regimen was non-inferior in efficacy to the conventional i.v. ARS dosing regimen [1]. This study establishes that the once daily i.v. is not consistently non-inferior to the three-dose and five-dose i.m. regimens. There are several corollaries to this observation. First, clearance kinetics with i.v. once daily ARS in this study (74% achieved ≥99% clearance from baseline at 24 h) is very similar to the previous result of 76% with i.v. once daily ARS. This latter result was also comparable to the five-dose WHO-recommended i.v. regimen in that smaller study and points to a preference for the i.m. route because it is associated with faster clearance kinetics (Figs 5–7). Second, these results confirm that the endpoint chosen is both robust and pharmacodynamically sensitive as a measure of ARS antimalarial efficacy. Also, a once daily simplified i.m. regimen is of comparable efficacy to the five-dose WHO-recommended i.m. regimen, a finding that was robust

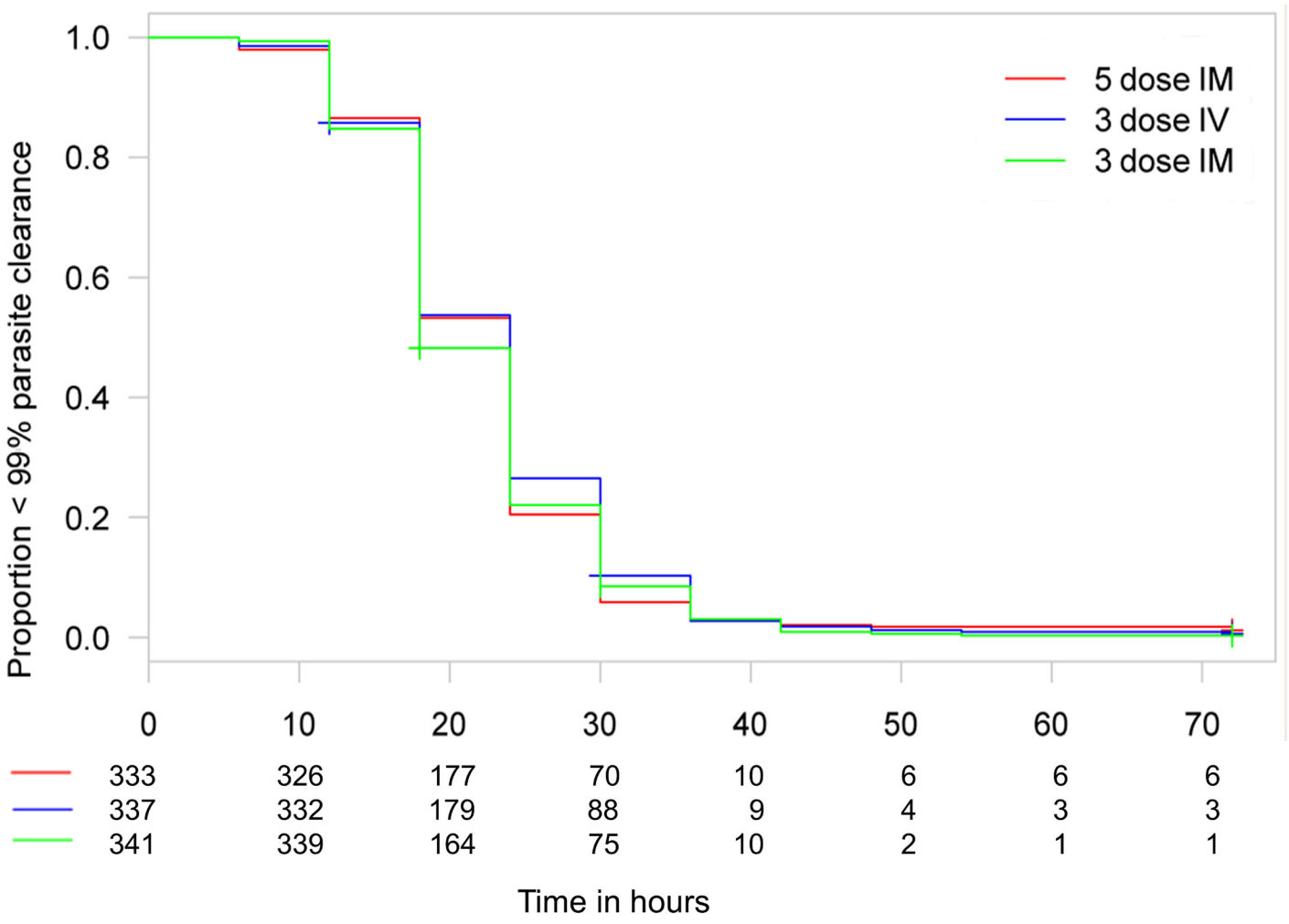

Fig 5. Kaplan–Meier plot for time to 99% parasite clearance in the per-protocol population. Time to 99% parasite clearance under parenteral ARS treatment is shown. Using the 10% delta at 24 h, both three-dose regimens are non-inferior to the five-dose regimen in a Cox proportional hazards model. The PP population has been used for the secondary endpoints.

doi:10.1371/journal.pmed.1001938.g005

when analyzed using different statistical methodologies. This finding has important implications for practice. The i.m. route for administration of antimalarials is preferable to the i.v. route in small children [27]. Analysis of secondary endpoints of parasite clearance supports the results of the primary endpoint analysis, and suggests that the once daily i.m. regimen has an even faster time to 90% parasite clearance than the conventional i.m. regimen (median 12 versus 18 h; Figs 5 and 6; S1 Table).

The frequently observed ^{N86Y}Pfmdr1 polymorphism has previously been associated with increased sensitivity to artemisinin in in vitro assays in Senegal [28] and decreased sensitivity to artemether in Nigeria [29], with no effect in Thai isolates [30]. Our results provide in vivo evidence for decreased sensitivity to ARS (DHA) of parasites with ^{N86Y}Pfmdr1. Changes in the frequencies of polymorphisms in *pfmdr1* are clearly worth monitoring in future epidemiological studies. High unadjusted cure rates (95%) in our patients may reflect the large artemisinin (24 mg/kg total dose of ARS and artemether) component of the treatment course and efficacy of the combination partner (lumefantrine).

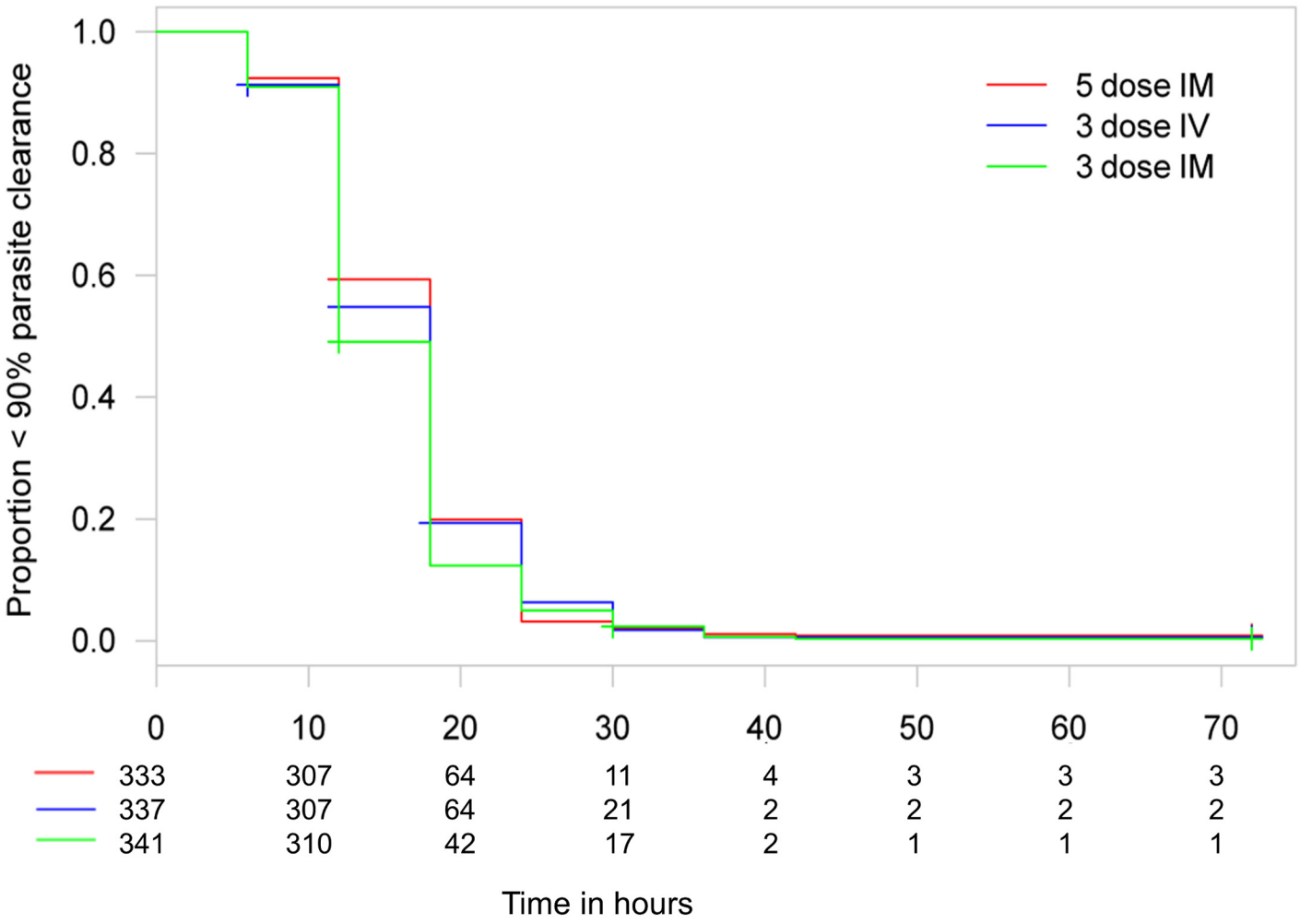

Fig 6. Kaplan–Meier plot for time to 90% parasite clearance in the per-protocol population. Time to 90% parasite clearance under parenteral ARS treatment is shown. Using the 10% delta at 24 h, both three-dose regimens are non-inferior to the five-dose regimen in a Cox proportional hazards model. The PP population has been used for the secondary endpoints.

doi:10.1371/journal.pmed.1001938.g006

The results of our population PK analysis are consistent with classical and previous population PK studies on parenteral ARS [4,6], including in Tanzania [31]. A suggestion, based on modeling, that doses higher (~3 mg/kg per dose for children <10 kg) [31] than currently recommended by WHO may be needed for smaller children is obviated if our higher dose (4 mg/kg) and simpler regimen is implemented. As noted previously, there was no relationship

Table 3. Population pharmacokinetic analysis of parenteral artesunate in severe malaria.

Arm	Drug Metabolite	Volume of Distribution (Liters) (95% CI)	Clearance (Liters/Hour) (95% CI)
Three-dose i.v.	ARS	32.0 (22.3–41.6)	42.0 (28.9–55.1)
	DHA	12.0 (10.2–13.8)	9.9 (8.4–11.3)
	DHAG	36.6 (32.0–41.3)	10.8 (9.0–12.7)
Three-dose or five-dose i.m	ARS	21.1 (18.2–24.0)	33.3 (29.5–37.1)
	DHA	25.3 (22.4–28.2)	8.5 (7.4–9.5)
	DHAG	66.5 (58.2–74.8)	10.1 (8.3–11.9)

doi:10.1371/journal.pmed.1001938.t003

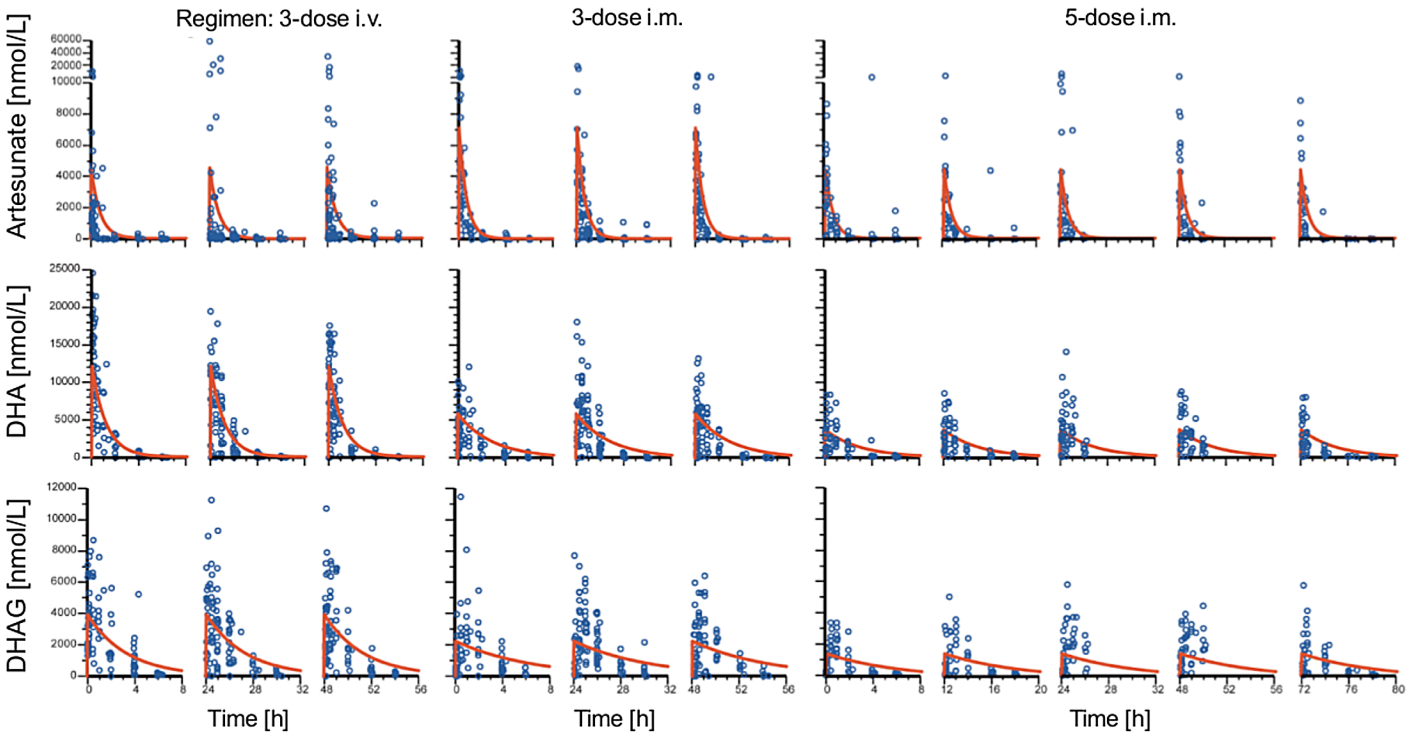

Fig 7. Population pharmacokinetic profiles of artesunate, dihydroartemisinin, and dihydroartemisinin glucuronide. Plots of observed concentration–time profiles for ARS and its major metabolites, DHA and DHAG, are presented according to treatment regimen. Estimated population mean PK profiles are shown by red lines. The three columns of results for each regimen represent the findings after each dose.

doi:10.1371/journal.pmed.1001938.g007

between PK parameters and efficacy. We also present, to our knowledge for the first time, an analysis of the glucuronide derivative of DHA (Fig 7; Table 3). This is quantitatively the most important primary metabolite of DHA. Although it has poor antimalarial activity (IC_{50} of DHAG = 5.7 μM, mean of two experiments), DHAG plasma concentrations commonly peaked above 5 μM (Fig 7), rendering a contribution to parasite clearance possible. Repeated dosing with ARS, in any regimen, does not show evidence of accumulation (Fig 7). This analysis also allows us to examine other potential mechanisms for toxicity, which hitherto has not been possible.

During the conduct of our trial a series of case reports appeared about delayed anemia in travelers who had received parenteral ARS. Therefore, we amended the study protocol to address

Table 4. Serious adverse events in the intention-to-treat population.

SAE Outcome	Arm			Total
	Three-Dose i.v.	Three-Dose i.m.	Five-Dose i.m.	
SAEs	26	28	21	75
SAEs with a possible relationship to study drug	5 (19%)	3 (11%)	6 (29%)	14
SAEs with no relationship to study drug	21 (81%)	25 (89%)	15 (71%)	51
Deaths	6	6	4	16
Neurological sequelae after study completion (day 28)	3	1		4

Data are given as *n* or *n* (percent).

doi:10.1371/journal.pmed.1001938.t004

Table 5. Anemia occurrences from day 7 until day 28.

Anemia Outcome	Number of Patients
Total patients with at least one late anemia episode	192 out of 885 followed up until day 28
Hb $\leq$ 7 g/dl on day 7	185 out of 972 followed up until day 7
Hb $\leq$ 7 g/dl on day 28	7
Late transfusion (> day 7)	4
Anemia on active follow-up (substudy)	5

doi:10.1371/journal.pmed.1001938.t005

the occurrence of delayed hemolytic anemia in our ongoing study. We identified delayed anemia after ARS treatment using a small subgroup of patients from the current study [16] in whom it was possible to study anemia defined by several criteria (low haptoglobin, elevated lactate dehydrogenase level, decrease inHb, exclusion of sickle cell disease and G6PDH deficiency). Secondly, in a post hoc analysis that included the whole study population, we defined delayed anemia as Hb $\leq$ 7 g/dl 7 d or more after admission. We confirmed the occurrence of delayed anemia in a significant proportion (22%) of African children by using this simplified definition that may therefore have greater practical utility. This definition of anemia does not reveal any

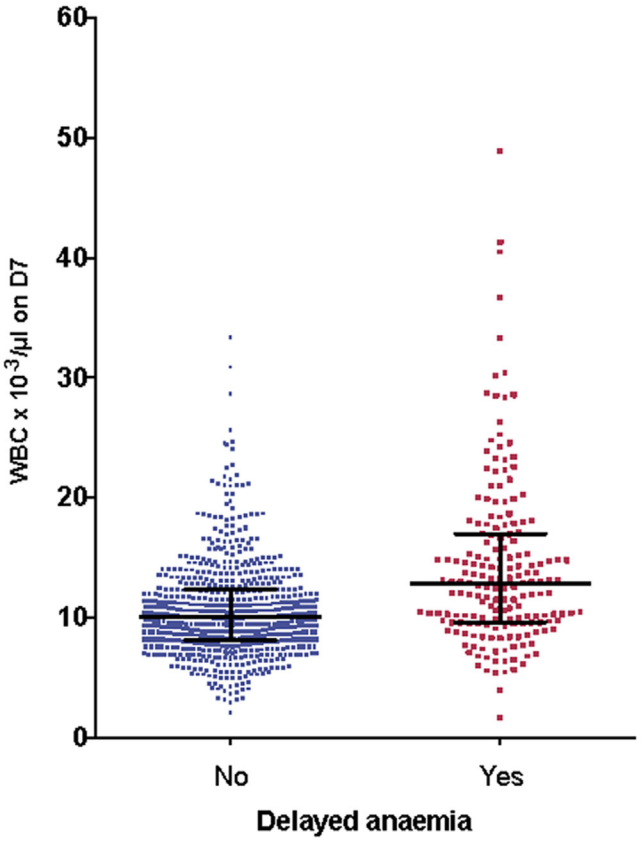

Fig 8. Association between delayed anemia and increased white blood cell count at day 7. Individual white cell counts (WBC) on day 7 (D7) are presented as medians and interquartile ranges, divided into those who developed delayed anemia (12.8×10^3/µl, interquartile range 9.6–17, $n = 186$) and those who did not (10.1×10^3/µl, interquartile range 8.1–12.4, $n = 689$). Patients with delayed anemia had a significantly higher white blood cell count at day 7 ($p < 0.001$).

doi:10.1371/journal.pmed.1001938.g008

relationships with PK parameters for ARS, DHA, or indeed DHAG. Instead, a higher leucocyte (Fig 8) and neutrophil count at day 7 is associated with delayed anemia, suggesting that if ARS is the cause of anemia, the mechanism does not involve bone marrow toxicity, because ARS can cause dose-dependent neutropenia [25]. In a recent study of 60 travelers treated with ARS for severe malaria, 13/66 (22%) had delayed anemia, which compares well with our findings. In this study, pitting was significantly associated with delayed anemia [32]. Pitting is a process whereby dead early-stage parasites are removed from erythrocytes by the spleen. Pitting may contribute to the pathophysiology of delayed anemia, which is associated with markers of delayed hemolysis, although available findings from AQUAMAT do not support this [33]. In the AQUAMAT study, the incidence of post-admission severe anemia (Hb $<$ 50 g/l) was comparable in the quinine (5.7%) and ARS (4.6%) groups [34], and both groups had identical proportions of patients (55%) receiving blood transfusions. It is unfortunate that only neurological sequelae were monitored after discharge in the AQUAMAT study, as the risks of delayed anemia with ARS compared to quinine could have been quantified in this cohort.

Weaknesses of this study are its open-label design, which may introduce biases in outcome variables, although allocation bias was minimized and the primary outcome measures of parasite clearance were assessed in a blinded way. The primary analysis at 24 h was before the full treatment regimens had been completed, which may appear as a study limitation unless all parasite clearance estimates are also considered. Additionally, delayed anemia was first described in travelers—and could therefore be addressed—only after the study was mostly complete. It was studied in detail in 72 patients [16], with the remaining analysis being performed post hoc. As there was no comparator arm using a drug other than ARS, further studies will be needed to clarify the impact of artemisinins on delayed anemia. Some colleagues outside the SMAC network may argue that mortality needs to be an endpoint in a study with severe malaria. However, our studies show that case fatality rates in severe malaria trials performed following principles of good clinical practice should not exceed 5%, but rather be between 1% and 2%, regardless of whether the WHO definition or our SMAC definition of cases is used [35]. Thus, death as an endpoint is precluded by sample size requirements.

Simplifying ARS usage with a once daily i.m. regimen in severe malaria is supported by our results, but because delayed anemia is common, patients should be monitored for this complication.

Supporting Information

S1 Data. Dataset used for the analysis.
(XLSX)

S1 Table. ≥99% parasite clearance 24 h after treatment initiation for the per-protocol population.
(DOCX)

S2 Table. Time to parasite clearance (hours) for the intention-to-treat population.
(DOCX)

S3 Table. Fever clearance times, 37.5°C threshold, for the per-protocol population.
(DOCX)

S4 Table. Fever clearance times, 37.7°C threshold, for the per-protocol population.
(DOCX)

S5 Table. Fever clearance times, 38.0°C threshold, for the per-protocol population.
(DOCX)

S6 Table. Biochemical measurements for the intention-to-treat population.
(DOCX)

S7 Table. Study design.
(DOCX)

S1 Text. Protocol.
(PDF)

S2 Text. CONSORT statement.
(DOC)

Acknowledgments

We thank Guilin Pharmaceutical for supplying ARS. We thank Ute Hofmann from the Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie for PK sample analysis.

Author Contributions

Conceived and designed the experiments: PGK SK. Performed the experiments: AAA JFZ ABH TET YC AL MK MKBA DPMM TA DA JS BRO GAO AW KAB UO FSI CRN PN MK SI TPV CN MS MG RK. Analyzed the data: PGK SK BM TE MS MG RK TPV CN. Wrote the first draft of the manuscript: SK PGK BM. Contributed to the writing of the manuscript: AAA JFZ ABH TET YC AL MK MKBA DPMM TA DA JS BRO GAO AW KAB UO FSI CRN PN MK SI. Enrolled patients: AAA JFZ ABH TET YC AL MK MKBA DPMM TA DA JS BRO GAO AW KAB UO FSI CRN PN MK SI. Agree with the manuscript's results and conclusions: AAA JFZ ABH TET YC AL MK MKBA DPMM TA DA JS BRO GAO AW KAB UO FSI CRN PN MK SI PGK SK BM MS MG RK TPV CN CK SB TE. Overall project managers: CK SB. All authors have read, and confirm that they meet, ICMJE criteria for authorship.

References

1. Kremsner PG, Taylor T, Issifou S, Kombila M, Chimalizeni Y, Kawaza K, et al. A simplified intravenous artesunate regimen for severe malaria. J Infect Dis. 2012; 205:312–319. doi: 10.1093/infdis/jir724 PMID: 22180622
2. World Health Organization. Guidelines for the treatment of malaria. Geneva: World Health Organization; 2015.
3. Krishna S, Planche T, Agbenyega T, Woodrow C, Agranoff D, Bedu-Addo G, et al. Bioavailability and preliminary clinical efficacy of intrarectal artesunate in Ghanaian children with moderate malaria. Antimicrob Agents Chemother. 2001; 45:509–516. PMID: 11158748
4. Nealon C, Dzeing A, Muller-Romer U, Planche T, Sinou V, Kombila M, et al. Intramuscular bioavailability and clinical efficacy of artesunate in gabonese children with severe malaria. Antimicrob Agents Chemother. 2002; 46:3933–3939. PMID: 12435698
5. Dondorp AM, Maude RJ, Hendriksen IC, Day NP, White NJ. Artesunate dosing in severe falciparum malaria. J Infect Dis. 2012; 206:618–619. doi: 10.1093/infdis/jis389 PMID: 22732917
6. Kremsner PG, Taylor T, Issifou S, Kombila M, Chimalizeni Y, Kawaza K, et al. A simplified intravenous artesunate regimen for severe malaria—reply. J Infect Dis. 2012; 206:621–622.
7. Kremsner PG, Valim C, Missinou MA, Olola C, Krishna S, Issifou S, et al. Prognostic value of circulating pigmented cells in African children with malaria. J Infect Dis. 2009; 199:142–150. doi: 10.1086/595295 PMID: 19086817
8. Taylor T, Olola C, Valim C, Agbenyega T, Kremsner P, Krishna S, et al. Standardized data collection for multi-center clinical studies of severe malaria in African children: establishing the SMAC network. Trans R Soc Trop Med Hyg. 2006; 100:615–622. PMID: 16551469
9. World Health Organization. Management of severe malaria: a practical handbook— 3rd ed. Available: http://apps.who.int/iris/bitstream/10665/79317/1/9789241548526_eng.pdf. Accessed 3 December 2015.

10. White NJ, Looareesuwan S, Warrell DA, Warrell MJ, Chanthavanich P, Bunnag D, et al. Quinine loading dose in cerebral malaria. Am J Trop Med Hyg. 1983; 32:1–5.
11. Karunajeewa HA, Reeder J, Lorry K, Dabod E, Hamzah J, Page-Sharp M, et al. Artesunate suppositories versus intramuscular artemether for treatment of severe malaria in children in Papua New Guinea. Antimicrob Agents Chemother. 2006; 50:968–974. PMID: 16495259
12. Kreeftmeijer-Vegter AR, van Genderen PJ, Visser LG, Bierman WF, Clerinx J, van Veldhuizen CK, et al. Treatment outcome of intravenous artesunate in patients with severe malaria in the Netherlands and Belgium. Malar J. 2012; 11:102. doi: 10.1186/1475-2875-11-102 PMID: 22462806
13. Rolling T, Schmiedel S, Wichmann D, Wittkopf D, Burchard GD, Cramer JP. Post-treatment haemolysis in severe imported malaria after intravenous artesunate: case report of three patients with hyperparasitaemia. Malar J. 2012; 11:169. doi: 10.1186/1475-2875-11-169 PMID: 22594446
14. Rolling T, Wichmann D, Schmiedel S, Burchard GD, Kluge S, Cramer JP. Artesunate versus quinine in the treatment of severe imported malaria: comparative analysis of adverse events focussing on delayed haemolysis. Malar J. 2013; 12:241. doi: 10.1186/1475-2875-12-241 PMID: 23855745
15. Centers for Disease Control and Prevention. Published reports of delayed hemolytic anemia after treatment with artesunate for severe malaria—worldwide, 2010–2012. MMWR Morb Mortal Wkly Rep. 2013; 62:5–8. PMID: 23302816
16. Rolling T, Agbenyega T, Issifou S, Adegnika AA, Sylverken J, Spahlinger D, et al. Delayed hemolysis after treatment with parenteral artesunate in African children with severe malaria—a double-center prospective study. J Infect Dis. 2014; 209:1921–1928. doi: 10.1093/infdis/jit841 PMID: 24376273
17. World Health Organization Communicable Diseases Cluster. Severe falciparum malaria. Trans R Soc Trop Med Hyg. 2000; 94 (Suppl 1):S1–S90. PMID: 11103309
18. Four Artemisinin-Based Combinations (4ABC) Study Group. A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial. PLoS Med. 2011; 8:e1001119. doi: 10.1371/journal.pmed.1001119 PMID: 22087077
19. Planche T, Krishna S, Kombila M, Engel K, Faucher JF, Ngou-Milama E, et al. Comparison of methods for the rapid laboratory assessment of children with malaria. Am J Trop Med Hyg. 2001; 65:599–602. PMID: 11716121
20. Geditz MC, Heinkele G, Ahmed A, Kremsner PG, Kerb R, Schwab M, et al. LC-MS/MS method for the simultaneous quantification of artesunate and its metabolites dihydroartemisinin and dihydroartemisinin glucuronide in human plasma. Anal Bioanal Chem. 2014; 406:4299–4308. doi: 10.1007/s00216-014-7820-x PMID: 24760398
21. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature. 2014; 505:50–55. doi: 10.1038/nature12876 PMID: 24352242
22. Wellek S. Testing statistical hypotheses of equivalence and noninferiority. 2nd ed. London: Chapman and Hall/CRC; 2010.
23. Farrington CP, Manning G. Test statistics and sample size formulae for comparative binomial trials with null hypothesis of non-zero risk difference or non-unity relative risk. Stat Med. 1990; 9:1447–1454. PMID: 2281232
24. Parpia S, Thabane L, Julian JA, Whelan TJ, Levine MN. Empirical comparison of methods for analyzing multiple time-to-event outcomes in a non-inferiority trial: a breast cancer study. BMC Med Res Methodol. 2013; 13:44. doi: 10.1186/1471-2288-13-44 PMID: 23517401
25. Tunes da SG, Logan BR, Klein JP. Methods for equivalence and noninferiority testing. Biol Blood Marrow Transplant. 2009; 15(1 Suppl):120–127. doi: 10.1016/j.bbmt.2008.10.004 PMID: 19147090
26. Angus BJ, Thaiaporn I, Chanthapadith K, Suputtamongkol Y, White NJ. Oral artesunate dose-response relationship in acute falciparum malaria. Antimicrob Agents Chemother. 2002; 46:778–782. PMID: 11850261
27. Krishna S, Nagaraja NV, Planche T, Agbenyega T, Bedo-Addo G, Ansong D, et al. Population pharmacokinetics of intramuscular quinine in children with severe malaria. Antimicrob Agents Chemother. 2001; 45:1803–1809. PMID: 11353629
28. Van TD, Dieye B, Valim C, Daniels RF, Sene PD, Lukens AK, et al. Changes in drug sensitivity and anti-malarial drug resistance mutations over time among Plasmodium falciparum parasites in Senegal. Malar J. 2013; 12:441. doi: 10.1186/1475-2875-12-441 PMID: 24314037
29. Bustamante C, Folarin OA, Gbotosho GO, Batista CN, Mesquita EA, Brindeiro RM, et al. In vitro-reduced susceptibility to artemether in P. falciparum and its association with polymorphisms on transporter genes. J Infect Dis. 2012; 206:324–332. doi: 10.1093/infdis/jis359 PMID: 22615315

30. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet. 2004; 364:438–447. PMID: 15288742

31. Hendriksen IC, Mtove G, Kent A, Gesase S, Reyburn H, Lemnge MM, et al. Population pharmacokinetics of intramuscular artesunate in African children with severe malaria: implications for a practical dosing regimen. Clin Pharmacol Ther. 2013; 93:443–450. doi: 10.1038/clpt.2013.26 PMID: 23511715

32. Jaureguiberry S, Ndour PA, Roussel C, Ader F, Safeukui I, Nguyen M, et al. Postartesunate delayed hemolysis is a predictable event related to the lifesaving effect of artemisinins. Blood. 2014; 124:167–175. doi: 10.1182/blood-2014-02-555953 PMID: 24859359

33. Bethell D, Se Y, Lon C, Socheat D, Saunders D, Teja-Isavadharm P, et al. Dose-dependent risk of neutropenia after 7-day courses of artesunate monotherapy in Cambodian patients with acute Plasmodium falciparum malaria. Clin Infect Dis. 2010; 51:e105–e114. doi: 10.1086/657402 PMID: 21070142

34. Dondorp AM, Fanello CI, Hendriksen IC, Gomes E, Seni A, Chhaganlal KD, et al. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet. 2010; 376:1647–1657. doi: 10.1016/S0140-6736(10)61924-1 PMID: 21062666

35. Helbok R, Kendjo E, Issifou S, Lackner P, Newton CR, Kombila M, et al. The Lambarene Organ Dysfunction Score (LODS) is a simple clinical predictor of fatal malaria in African children. J Infect Dis. 2009; 200:1834–1841. doi: 10.1086/648409 PMID: 19911989

Editors' Summary

Background

Globally, about 200 million cases of malaria—a mosquito-borne parasitic disease—occur every year. Malaria infections, which can be caused by several parasites, can be "uncomplicated" or "severe." Prompt treatment of uncomplicated malaria, which presents with flu-like symptoms, is essential to prevent the development of severe malaria. The World Health Organization (WHO) recommends artemisinin combination therapy (ACT) for the first-line treatment of uncomplicated malaria in countries where the disease is always present. In ACT, artemisinin derivatives (fast-acting antimalarial drugs that are cleared rapidly from the body) are combined with a slower-acting, more slowly eliminated partner drug to prevent the original infection recurring and to reduce the risk of the malaria parasites becoming resistant to either drug. Severe malaria, which is usually caused by *Plasmodium falciparum*, is characterized by anemia and by damage to the brain and other organs. Severe malaria kills more than 400,000 people (mainly young children living in sub-Saharan Africa) every year.

Why Was This Study Done?

WHO recommends that severe malaria be treated with intravenous or intramuscular injections of artesunate, a parenteral (injectable) form of artemisinin; patients with severe malaria cannot take pills reliably or safely. Specifically, WHO recommends that patients be given 2.4 mg of artesunate per kilogram of body weight intravenously or intramuscularly at the time of admission (0 hours) and at 12, 24, 48, and 72 hours (followed by ACT to ensure full parasite clearance). But this five-dose regimen is complex. A simpler regimen would be easier to administer in resource-limited settings, where giving the correct doses on time to small, sick children can be challenging. In this open-label, non-inferiority randomized controlled trial (RCT), the researchers investigate the efficacy of a three-dose artesunate regimen for the treatment of severe malaria in African children. RCTs compare outcomes in people randomly chosen to receive different interventions; in an open-label RCT, both the researchers and the participants know which treatment is being given; a non-inferiority trial investigates whether one treatment is not worse than another treatment.

What Did the Researchers Do and Find?

The researchers randomly allocated 1,047 children aged six months to ten years with severe malaria attending seven clinical centers in five African countries to receive a total dose of 12 mg of artesunate per kilogram of body weight as five intramuscular injections of 2.4 mg/kg given at 0, 12, 24, 48, and 72 hours (the control regimen) or as three intramuscular or intravenous injections of 4 mg/kg given at 0, 12, and 24 hours (three-dose intramuscular and intravenous regimens, respectively). The trial's primary endpoint was the proportion of children whose parasitemia (parasite count in the blood) at 24 hours was $\leq$1% of that at admission (in other words, $\geq$99% parasite clearance). Among the 1,002 children who received the planned drug doses (the per-protocol population), 78% in the three-dose intramuscular group had $\geq$99% parasite clearance compared to 79% in the five-dose intramuscular group, a result that met a preset criterion for non-inferiority at 24 hours of the three-dose intramuscular regimen to the control regimen. However, because only 74% of the children in the three-dose intravenous group had $\geq$99% parasite clearance, this regimen was not shown to be non-inferior to the conventional five-dose regimen.

What Do These Findings Mean?

These findings when combined with the findings of several secondary analyses suggest that, in African children, a three-dose intramuscular artesunate regimen is non-inferior to the WHO-recommended regimen for the treatment of severe malaria. The study's open-label design may limit the accuracy of its findings, as may its use of a primary endpoint midway through drug treatment rather than at the end (the researchers note that 60% of deaths from severe malaria occur during the first 24 hours of illness and that parasitemia is harder to measure later during treatment) and its use of parasite clearance rather than death as the primary endpoint (case fatality rates in severe malaria treatment trials are very low, so a much larger study would be needed if death were used as the primary endpoint). Overall, these findings support the use of the three-dose intramuscular artesunate regimen for the treatment of severe malaria. Importantly, however, 22% of the children in the study developed delayed anemia, irrespective of treatment regimen. Thus, although further studies are needed to clarify whether treatment with artesunate or the malaria infection itself was responsible for the delayed anemia, patients treated with artesunate for severe malaria should be routinely monitored for this complication.

Additional Information

This list of resources contains links that can be accessed when viewing the PDF on a device or via the online version of the article at http://dx.doi.org/10.1371/journal.pmed.1001938.

- Information is available from the World Health Organization on malaria (in several languages); the *World Malaria Report 2014* provides details of the current global malaria situation, including information on malaria in individual African countries; WHO's *Guidelines for the Treatment of Malaria* and its *Management of Severe Malaria: A Practical Handbook* are available
- The US Centers for Disease Control and Prevention provides information on malaria (in English and Spanish), including personal stories about malaria
- The UK National Health Service Choices website also provides information about malaria, including a personal story
- Information is available from the Roll Back Malaria Partnership on the global control of malaria
- The Scientists Against Malaria collaboration applies modern drug design and modeling techniques to develop new treatments against malaria; its website includes information about many aspects of malaria
- Public Health England provides a collection of guidance and research and analysis on malaria
- MedlinePlus provides links to additional information on malaria (in English and Spanish)
- More information about this trial is available

Nguetse *et al. Malar J (2016) 15:346*
DOI 10.1186/s12936-016-1396-1

Malaria Journal

RESEARCH Open Access

Glucose-6-phosphate dehydrogenase deficiency and reduced haemoglobin levels in African children with severe malaria

Christian N. Nguetse[1], Christian G. Meyer[1,2], Ayola Akim Adegnika[1,3], Tsiri Agbenyega[4,5], Bernhards R. Ogutu[6], Peter G. Kremsner[1,3] and Thirumalaisamy P. Velavan[1,2,7*]

Abstract

Background: Extensive studies investigating the role of host genetic factors during malaria associate glucose-6-phosphate dehydrogenase deficiency with relative protection. G6PD deficiency had been reported to associate with anti-malarial drug induced with haemolytic anaemia.

Methods: A total of 301 Gabonese, Ghanaian, and Kenyan children aged 6–120 months with severe malaria recruited in a multicentre trial on artesunate were included in this sub-study. *G6PD* normal (type B), heterozygous (type A^+) and deficient (type A^-) genotypes were determined by direct sequencing of the common African mutations G202A and A376G. Furthermore, multivariate analyses were executed to associate possible contributions of G6PD deficiency with baseline haemoglobin levels, parasitaemia and with severe malarial anaemia.

Results: Two hundred and seventy-eight children (132 females and 146 males) were successfully genotyped for *G6PD* variants. The overall prevalence of G6PD deficiency was 13 % [36/278; 3 % (4/132) female homozygous and 22 % (32/146) male hemizygous], 14 % (40/278) children were female heterozygous while 73 % (202/278) were G6PD normal [67 % (88/132) females and 78 % (114/146) males] individuals. Multivariate regression revealed a significant association of moderately and severely deficient *G6PD* genotypes with haemoglobin levels according to the baseline data ($p < 0.0001$; G6PD heterozygous: $p < 0.0001$; G6PD deficient: $p = 0.009$), but not with severe malarial anaemia ($p = 0.66$). No association of *G6PD* genotypes with baseline parasitaemia.

Conclusions: In this study, moderately (type A^+) and severely (type A^-) G6PD deficiency showed significant association with lower haemoglobin concentrations at baseline in African children with severe malaria without leading to severe malarial anaemia. In addition, there was no association of *G6PD* variant types with parasite densities on admission.

Keywords: Glucose-6-phosphate dehydrogenase deficiency, African children, Severe malaria

Background

Malaria remains a major health problem, with approximately 3.2 billion people at risk. In 2015, WHO reported approximately 214 million cases and about 438,000 deaths occurring in the world with the highest morbidity and mortality rates observed in Africa, especially among children under 5 years of age [1]. The control of the disease particularly in low transmission settings is the key target for malaria elimination [2] but such success is still a great challenge [3]. One approach to reduce the disease incidence is to block the transmission. Primaquine, a 8-aminoquinoline effective for both transmission blocking of *Plasmodium falciparum* and anti-relapse treatment against *Plasmodium vivax* has been recommended for many years by the World Health Organization (WHO) [4, 5]. However, primaquine has a major drawback which limits its widely use. The drug is known to cause acute

*Correspondence: velavan@medizin.uni-tuebingen.de
[7] Fondation Congolaise pour la Recherche Médicale, Brazzaville, Republic of Congo
Full list of author information is available at the end of the article

haemolytic anaemia in individuals with glucose-6-phosphate-dehydrogenase (G6PD) deficiency [6, 7].

G6PD is a key enzyme catalysing the first reaction in the pentose phosphate pathway and provides a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to help cells to counterbalance oxidative stress [8]. G6PD deficiency is a common, X-linked hereditary enzyme deficiency affecting approximately 400 million people worldwide [9], mainly in malaria-endemic regions [10]. Among the 186 mutations identified until 2012 in the *G6PD* gene [11], the variants 376A (G6PD type B), 376G (G6PD deficiency type A^+ and 202A (severe G6PD deficiency type A^-) are the most common ones. The deficiency types A^+ (moderately deficient) and A^- (severely deficient) constitute up to 90 % of reported *G6PD* variants [8, 12, 13]. Other mutations such as A542T, G680T or T968C have also been identified in parts of Africa and been suggested to contribute to G6PD deficiency. However, information available on these mutations is very scarce. For example, the T968C mutation has been reported to be common only in The Gambia [14] and Senegal [15]. Although, G6PD diagnostic enzyme tests are available, they are currently not widely used in most clinical studies, particularly because primaquine, is not regularly used in Africa. Any attempts to control malaria need to take into account also tertian malaria, which, although occurring rarely only in many parts of Africa, contributes to the world-wide malaria burden.

Previous case–control studies have reported an association of G6PD deficiency, in particular of the 202A variant, with an increased risk of severe malarial anaemia and a protection or reduced risk against cerebral malaria [16, 17]. Therefore, it is interesting to assess the influence of this allele on the clinical presentation of severe malaria among the African children recruited into this study.

This study was a sub-study of a multicentre trial of artesunate conducted by the "Severe Malaria in African Children" (SMAC) consortium which assessed the non-inferiority of a simplified 3-dose regimen of intramuscular and intravenous artesunate [18]. The aim was to determine using direct sequencing the frequency distribution of G6PD deficiency in malaria children from Africa. Some exploratory analyses have also been performed to investigate the effect of *G6PD* genotypes on asexual parasitaemia, haemoglobin concentrations and severe malarial anaemia during admission.

Methods

Study design and participants

This study was a sub-study of the SMAC follow-up study. The SMAC study was an open-label, randomized, multicentre (Gabon, Malawi, Ghana, Kenya, and The Gambia), parallel-group, three-arm study to compare the anti-malarial activity and safety of three artesunate (ARS) dosing regimens in children with severe *P. falciparum* malaria. Patients were randomly assigned to one of three dosing regimens consisting of a total of 12 mg/kg parenteral ARS: (i) 2.4 mg/kg intramuscular on admission and at 12, 24, 48 and 72 h, (ii) 4 mg/kg intramuscular on admission and at 24 and 48 h, and (iii) 4 mg/kg intravenous on admission and at 24 and 48 h post admission. Parasitaemia was assessed by thick blood smears at 6 h intervals and prior to the each dose of treatment for at least 48 h following the first dose of study drug. Malaria occurs holoendemically and transmission rates in all study countries are high and perennial.

The present study involved participants of the SMAC study from Gabon, Ghana and Kenya. Three hundred and one children aged 6–120 months with a diagnosis of *P. falciparum* infection (parasitaemia ≥5000 parasites/μL on initial blood smear) made using an alternative to conventional thick film examination (Lambaréné method) [19] were randomly selected from the SMAC follow-up study [18]. Blood samples (400 μL) from all participants were collected in heparinized tubes. Specimens were stored at −80 °C for subsequent molecular analyses.

Ethics statement

The study was conducted in accordance with Good Clinical Practices, and approved by authorities for each study site (the Regional Ethics Committee in Lambaréné (CERIL) for Gabon, Committee on Human Research, Publication and Ethics, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, for Ghana and the National Ethics Research Committee, Kenya Medical Research Institute (KEMRI) for Kenya). Children were enrolled into this study if a parent or guardian was willing to provide written informed consent in accordance with local practice.

G6PD genotyping

Genomic DNA was isolated using QIAamp DNA mini blood kit (Qiagen, Hilden, Germany). A 968 bp fragment of the *G6PD* gene containing the polymorphisms 202G > A and 376A > G was amplified by PCR using primers 5′-GCCCCTGTGACCTCCCGCCA-3′ (forward) and 5′-GCAACGGCAAGCCTTACATCTGG-3′ (reverse). The main focus was directed only to these two variants although some other deficient genetic mutations such as A542T (Senegal 1 %, The Gambia 2.2 %), G680T (The Gambia 0 %, Senegal 0 %) and T968C (The Gambia 7.8 %, Senegal 10 %) have been reported at a substantially lower prevalences only [14, 15], and might have been present in this study population. However, they seem not to be responsible for the prevalence of G6PD deficiency in all parts of Africa [20]. Briefly, 10 ng of genomic

DNA were added to a 20 µL reaction mixture containing 1 × PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM of $MgCl_2$), 0.125 mM of dNTPs, 0.25 mM of each primer and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany). The PCR was run on a PTC-200 Thermal cycler (MJ Research, Waltham, USA). Thermal conditions after initial denaturation (94 °C, 5 min) were 35 cycles of 94 °C for 45 s, 65 °C for 1 min, and 72 °C for 1 min. PCR reactions were completed with a final extension step of 72 °C for 5 min. PCR products were visualized through electrophoresis on a 1.2 % agarose gel stained with SYBR green I in 1x Tris-electrophoresis buffer (90 mM Tris–acetate, pH 8.0, 90 mM boric acid, 2.5 mM EDTA).

Subsequently, PCR products were purified (Exo-SAP-IT, USB, Affymetrix, USA) and directly used as templates for DNA sequencing using the BigDye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) on an ABI 3130XL DNA sequencer. *G6PD* polymorphisms were identified by assembling the sequences with the reference sequence of *G6PD* (NG_009015.2) gene using the Codoncode Aligner 4.0 software (http://www.codoncode.com) and visually reconfirmed from their electropherograms.

Statistical analysis

Data were analysed by using GraphPad Prism v. 5.0 for windows (GraphPad software, San Diego, CA). The effect of *G6PD* genotypes was determined on initial parasitaemia and haemoglobin values using a multivariate regression model. The children were classified in groups of normal, intermediate (female heterozygous) and deficient (hemizygous males and homozygous females) individuals. To evaluate the effect of *G6PD* genotypes on haemoglobin concentrations, the model included adjustment for age, gender, centre, weight, temperature and parasitaemia. To investigate the *G6PD* effect on parasitaemia, parasite densities were log-transformed and the model included adjustment for age, gender, centre, weight, haemoglobin levels and temperature. For the construction of the multivariate regression model, a subjective model-building approach that excludes possible confounders such as gender, age and origin of study participants was applied. Kruskal–Wallis of One-way ANOVA with Dunn's Multiple Comparison and Mann–Whitney tests were used to determine the differences among categories. The level of significance was set to a *P* value of 0.05.

Results

Patients

According to the SMAC definition of severe malaria which perfectly reflects the policies of most African hospitals [21, 22], the frequency of severe malaria syndromes at presentation were substantially different across the three study sites (Table 1). The majority of children fulfilled one or more criteria of the WHO definition of severe malaria [23, 24], which include severe anaemia (haematocrit of <15 % or Hb <5 g/dL with a parasitaemia of >10,000/µL), hyperlactataemia (≥5 mmol/L), hyperparasitaemia (>250,000 parasites/µL), hypoglycaemia (whole blood or plasma glucose ≤2.2 mmol/L), and haemoglobinuria (urine that is dark red or black, with a dipstick that is positive for Hb/myoglobin). A description of children screened, recruited and genotyped for their G6PD status is shown in Fig. 1. From the 287 malaria children enrolled, the *G6PD* genotypes were available for only 278 children. One hundred and forty-six children (53 %) were males. The median age was 2 (IQR: 1–4) years ranging from 6 months to 10 years with a mean haemoglobin value of 8.5 (± 2.4) g/dL.

Prevalence of *G6PD* genotypes and associations with baseline variables

Overall, 202 (73 %) children were classified as G6PD normal [type B; 114 (78 %) males, 88 (67 %) females], while 40 (14 %) were female heterozygous (type A^+) and 36 (13 %) were G6PD deficient [type A^-; 32 (22 %) males hemizygous and 4 (3 %) female homozygous]. In Table 2, the genotype frequencies of *G6PD* for males and females

Table 1 Distribution of severe malaria syndromes by study centre

	All (%)	Lambaréné, Gabon (%)	Kumasi, Ghana (%)	Kisumu, Kenya (%)
Severe malaria syndromes at admission[a]				
Respiratory distress	10/278 (4)	1/108 (1)	9/87 (10)	0/83 (0)
Prostration	55/278 (20)	4/108 (4)	43/87 (49)	8/83 (10)
Cerebral malaria	13/278 (5)	0/108 (0)	12/87 (14)	1/83 (1)
General seizure	20/278 (7)	4/108 (4)	13/87 (15)	3/83 (4)
Severe anaemia	26/278 (9)	5/108 (5)	20/87 (23)	1/83 (1)
Jaundice	21/278 (8)	0/108 (0)	16/87 (18)	5/83 (6)

Children can appear in more than one category

[a] Missing data for some syndromes

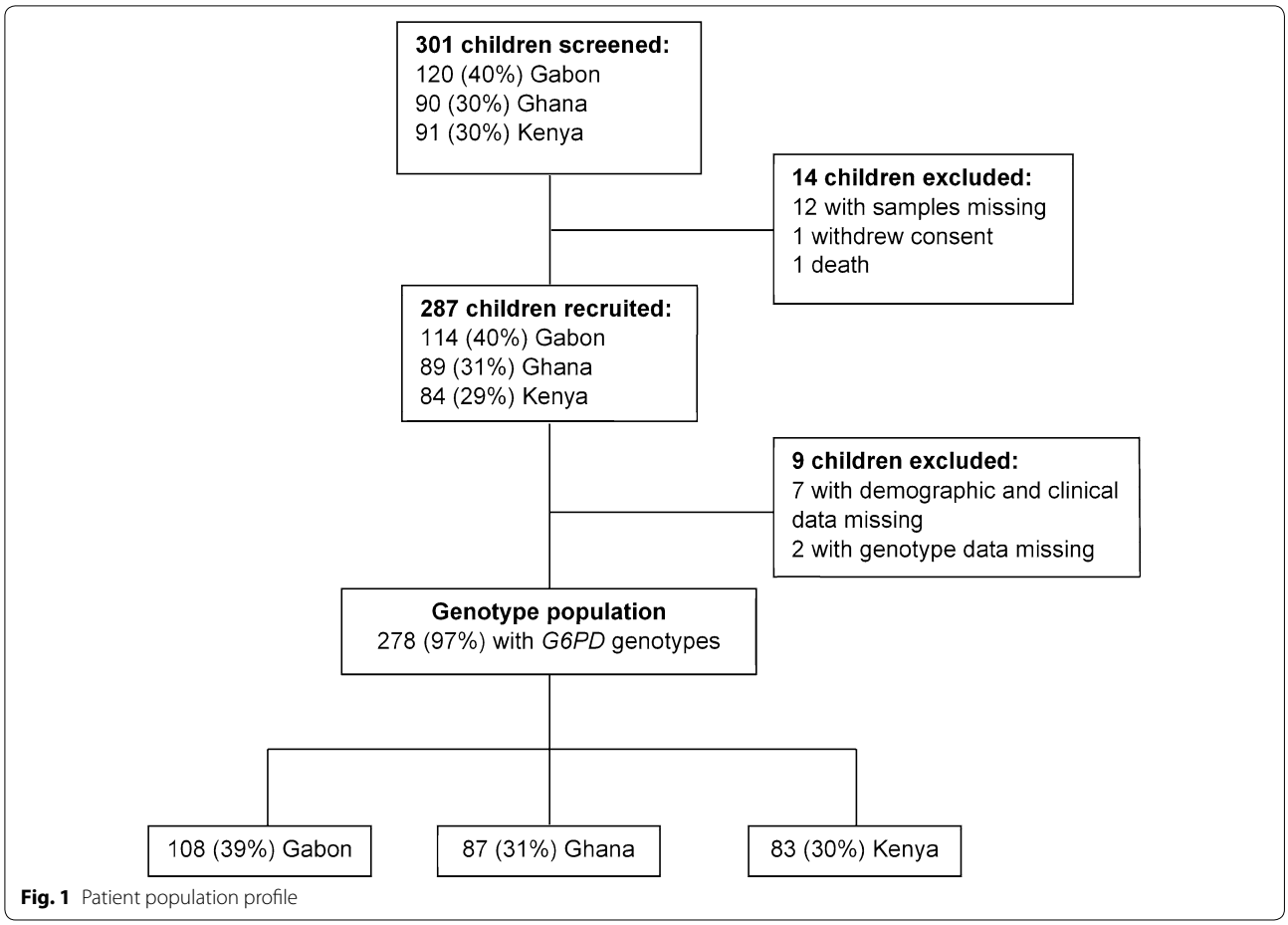

Fig. 1 Patient population profile

Table 2 Frequency of *G6PD* genotypes in malaria children from the three study centres

Country	Centre	Male, N	B	A+	A−	Female, N	BB	BA+	A+A+	BA−	A+A−	A−A−
Gabon	Lambaréné	52	28 (54)	9 (17)	15 (29)	56	20 (35)	14 (25)	2 (4)	1 (2)	17 (30)	2 (4)
Ghana	Kumasi	46	25 (54)	8 (18)	13 (28)	41	10 (24)	11 (27)	2 (5)	1 (2)	15 (37)	2 (5)
Kenya	Kisumu	48	31 (65)	13 (27)	4 (8)	35	22 (63)	7 (20)	0 (0)	0 (0)	6 (17)	0 (0)
Total		146	84 (58)	30 (20)	32 (22)	132	52 (39)	32 (24)	4 (3)	2 (2)	38 (29)	4 (3)

Data are shown as N (%). G6PD genotype: male normal = A+ or B; male hemizygous = A−; female normal = BB or BA+ or A+A+; female heterozygous = BA− or A+A−; female homozygous = A−A−

by study site are shown. Between the study centres, there was a significant difference ($p < 0.0001$) in the prevalence of A^- G6PD deficiency among males. Among females, the frequency of A^- G6PD deficiency was lower compared to males. The prevalence was 4 % in Lambaréné (Gabon), 5 % in Kumasi (Ghana) and 0 % in Kisumu (Kenya).

To investigate the association of *G6PD* genotypes with baseline variables, the children were grouped based on gender and *G6PD* genotype. Baseline demographic and clinical data are given in Table 3. Using a multivariate regression analysis adjusted for age, gender, centre, weight, temperature and parasitaemia, there was a significant association of the *G6PD* genotypes with the adjusted mean baseline of haemoglobin concentrations ($p < 0.0001$). Furthermore, a comparison between children G6PD normal and heterozygous ($p < 0.0001$), and between individuals G6PD normal and G6PD deficient ($p = 0.009$) showed a significant difference in adjusted mean baseline haemoglobin. The *G6PD* mutant

Table 3 Baseline demographic and clinical data following *G6PD* genotypes

Characteristic	Male normal (n = 114)	Male hemizygous (n = 32)	Female normal (n = 88)	Female heterozygous (n = 40)	Female homozygous (n = 4)	All children (n = 278)
Age, years [range]	3.3 (2.8) [0–10]	2.6 (2.2) [0–8]	2.2 (1.9) [0–8]	2.3 (2.6) [0–10]	2.5 (3) [0–6]	2.7 (2.5) [0–10]
0–3, n (%)	65 (57)	23 (72)	69 (78)	30 (75)	2 (50)	189 (68)
4–7, n (%)	39 (34)	8 (25)	18 (21)	7 (18)	2 (50)	74 (27)
≥8, n (%)	10 (9)	1 (3)	1 (1)	3 (7)	0 (0)	15 (5)
Weight, kg	15.6 (5.2)	12.6 (4.7)	13.2 (4)	13.2 (5.9)	16.3 (2.7)	14.1 (5)
Parasitemia per µL, geometric mean [range]	150,464 [6240–1,270,080]	95,369 [8153–709,400]	138,296 [5377–1,677,780]	105,520 [6380–745,100]	172,340 [32,176–397,440]	132,349 [5377–1,677,780]
Temperature, °C	37.9 (1.1)	38.4 (1)	38.2 (1.1)	38 (1.2)	37.7 (1.3)	38.1 (1.1)
Haemoglobin, g/dL (years)	8.9 (2.4)	7.8 (2.3)	8.9 (2.3)	7.1 (2.3)	8.2 (1.4)	8.5 (2.4)
0–3	8.2 (2.4)	7.6 (2.1)	8.6 (2.3)	6.7 (2.2)	8.1 (2.1)	8.1 (2.4)
4–7	9.9 (2)	8.2 (2.8)	9.7 (1.8)	6.9 (2)	8.3 (1.3)	9.3 (2.2)
≥8	9.8 (1.8)	NA	NA	10.6 (0.6)	NA	10.2 (1.7)
Haematocrit, %	27.4 (7.3)	23.8 (7.1)	27.3 (7)	21.7 (7.6)	25.5 (4)	26.1 (7.5)
Red blood cell count, 10^12/L	3.8 (1)	3.3 (1.2)	3.6 (1)	3 (1)	3.1 (0.4)	3.6 (1.1)
Platelet count, 10^9/L	151.6 (230.4)	98 (82)	117.3 (118.8)	111 (77.7)	63.3 (30)	127.5 (167.9)
White blood cell count, 10^9/L	9.4 (4.2)	11.6 (7.9)	9.8 (4.3)	11.6 (4.4)	7.7 (3.4)	10.1 (4.9)

Values are mean (SD) [range] unless otherwise indicated. G6PD genotype: male normal = A or B; male hemizygous = A−; female normal = B/B or B/A or A/A; female homozygous = A−/A−; female heterozygous = B/A− or A/A−

NA not applicable; *SD* standard deviation

genotypes were associated with a 2.7 g/dL and 1 g/dL decrease in haemoglobin levels in heterozygous and deficient children, respectively. The multivariate regression analysis adjusted for age, gender, centre, weight, haemoglobin levels and temperature did not result in any association ($p = 0.29$) of the *G6PD* genotypes on adjusted mean baseline parasite densities. In addition, there was no difference in adjusted log mean parasite densities between G6PD normal and heterozygous individuals ($p = 0.18$), and between G6PD normal and deficient children ($p = 0.07$).

Severe malarial anaemia and cerebral malaria

Following the WHO guidelines [23] which defines severe malarial anaemia (SMA) as Hb <5 g/dL, 26 children (9 %) were affected by SMA in this study. The mean haemoglobin concentrations was 4 g/dL (range 1.9–4.9 g/dL). There was no difference ($p = 0.66$) of the haemoglobin levels between SMA children with different *G6PD* genotypes. However, SMA occurred more frequently among G6PD normal 15/26 (58 %) compared to G6PD heterozygous 8/26 (31 %) and G6PD deficient 3/26 (12 %) children.

Only 13 patients (5 %) had cerebral malaria. Nine of them were G6PD normal and four were female G6PD heterozygous.

Discussion

Glucose-6-phosphate dehydrogenase deficiency has raised in frequencies in malaria-endemic settings as a consequence of the evolutionary pressure exerted by malaria on the human genome [16]. A plethora of previous studies have indicated and suggested a correlation between malaria endemicity and the occurrence of G6PD deficiency (reviewed in [25]).

The main objective of this study was to assess the distribution of *G6PD* genotypes among African children from three geographically countries presenting with severe malaria and participating in the SMAC clinical trial on different artesunate treatment regimens. While meanwhile many *G6PD* variants have been described [8, 11], the main focus was on the most relevant three variants (376A (G6PD type B, no deficiency), 376G (moderate G6PD deficiency type A^+) and 202A severe G6PD deficiency type A^-) in Africa [26].

The prevalence of severe G6PD deficiency as determined genetically was higher compared to previous findings from Gabon with 17 % in the present study *versus* 14 % reported earlier among males and 7 vs 2 % among females [27]. In Ghana 28 % were observed in this study, compared to 9 % among males and 5 vs 3 % among females indicated previously [28]. However, in Kenya the prevalence of severe G6PD deficiency among

females was lower compared to previous reports with 0 vs 5 % and equal among males 8 vs 8 % [29]. Regardless of the site, G6PD deficiency was considerably higher among males (22 %) compared to females (3 %), with an overall prevalence of 13 % across sites. This finding confirms that males are affected by this blood disorder rather than females and that G6PD deficient females are rather uncommon [26].

A significant association of moderately and severely deficient *G6PD* genotypes and haemoglobin levels according to the baseline data was observed. In fact, compared to children with the normal *G6PD* genotype (haemoglobin median: 9.3 g/dL), *G6PD* heterozygous (haemoglobin median: 6.6 g/dL) and deficient (haemoglobin median: 8.3 g/dL) children had 2.7 and 1 g/dL lower haemoglobin concentrations, respectively. The results contradict previous findings, which did not observe any association between *G6PD* genotypes and haemoglobin levels [30–32]. While in their study, May et al. [32] found lower levels of haemoglobin in *G6DP* deficient individuals, the association was not significant. An explanation could be the different designs of that and the present study. Here, patients with severe malaria were included, which implies high parasitaemia and haemoglobin concentrations <5 g/dL, whereas in the above cited study, the authors excluded patients with haemoglobin concentrations $\leq$7 g/L [30] and recruited individuals only with uncomplicated malaria [31] or who were asymptomatically infected [32].

In the case of SMA, there was no difference of haemoglobin levels among children with different *G6PD* genotypes. This may suggest that G6PD deficiency is not associated with this complication. However, this is in fact surprising especially as a significant difference of baseline haemoglobin values between G6PD normal and deficient individuals was observed. Moreover, in a large case–control study in Kenyan individuals, a significant increased risk to severe malarial anaemia associated with lower haemoglobin levels in G6PD deficient children with severe malaria at the time of hospital admission was found [33]. The likely explanation could be due to the sample size which was rather small.

Although not significant, parasite densities were lower in G6PD deficient children than in G6PD heterozygous, compared to G6PD normal individuals. However, this trend is in agreement with previous works [34, 35]. Conflicting results have been reported regarding parasitaemia and the various *G6PD* genotypes. Other studies have indicated a significantly lower parasitaemia associated with female G6PD heterozygous individuals, suggesting a protective advantage by this genotype [36–39].

In comparison to individuals with uncomplicated malaria, this study population was hyperparasitemic according to the criteria of severe malaria provided by WHO with lower parasitaemia in G6PD deficient individuals. However, either in uncomplicated or in severe/complicated malaria, G6PD deficiency was always associated with lower parasitaemia [30, 33]. Although the underlying genetic mechanisms are not completely clear, mechanisms suggested are impaired growth of *P. falciparum* parasites in G6PD deficient red blood cells [40] and slow rates of parasite replication [41, 42], more efficient clearance of infected red blood cells [43], and lower abundance of *P. falciparum* 6-phosphogluconolactonase mRNA in parasites from G6PD deficient individuals [44] both uncomplicated and severe malaria.

Several limitations apply to this study. Although some of the observations are in agreement with previous findings, the study group is rather small. The focus was only on the *G6PD* mutations 202G > A and 376A > G, although other variants such as A542T, G680T or T968C have also been reported to contribute to G6PD deficiency, albeit at far lower frequencies. The associations observed between *G6PD* genotypes and the baseline clinical parameters at admission could in fact also be the result of different circumstances. First, only patients with severe malaria were included, which could explain the findings, especially as the *G6PD* deficient allele 202A appears to confer protection against cerebral malaria and increases the risk of severe malarial anaemia [16, 17]. Second, depending on the mechanism of protection, G6PD deficiency might be associated with delayed presentation to the hospital and be a plausible explanation of the differences observed in haemoglobin concentrations at first admission. Third, alpha-thalassaemia, another haemoglobinopathy which has been associated with lower haemoglobin levels in alpha-thalassaemic Nigerian children and adults [45] cannot be ruled out as a confounding variable as it was not investigated in this study. The statistical analysis using multivariable regression model is in part subjective due to the adjusted variables added.

Conclusions

G6PD deficiency is more common among children from Gabon and Ghana than in Kenya. A significant association of the *G6PD* genotypes studied to lower haemoglobin levels was observed, suggesting a possible contributions of G6PD deficiency to the reduced production of erythrocytes in affected individuals. This was, however, not related to severe malarial anaemia experienced by some children. There was no evidence of a significant association between lower parasitaemia observed in G6PD deficient individuals compared to normal ones.

Abbreviations
G6PD: glucose-6-phosphate dehydrogenase; ARS: artesunate; SMAC: severe malaria in African children; WHO: World health organization; NADPH: nicotinamide adenine dinucleotide phosphate; SMA: severe malarial anaemia.

Authors' contributions
TPV designed the experiments, supervised the experiments and data analysis. AAA, TA, BRO are involved in patient recruitment from respective study sites and are principal investigators for SMAC study. PGK contributed to study design and materials. CNN performed the experiments and drafted the manuscript. TPV and CGM contributed in writing the manuscript. All authors read and approved the final manuscript.

Author details
[1] Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, 72074 Tübingen, Germany. [2] Vietnamese-German Center for Medical Research, Hanoi, Vietnam. [3] Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon. [4] Department of Physiology, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. [5] Departments of Child Health and Medicine, Komfo Anokye Teaching Hospital, Kumasi, Ghana. [6] Centre for Clinical Research, Kenya Medical Research Institute, Kisumu, Kenya. [7] Fondation Congolaise pour la Recherche Médicale, Brazzaville, Republic of Congo.

Acknowledgements
The authors acknowledge Velia Grummes for technical help during sequencing procedures.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All relevant data are within the paper.

Ethics approval and consent to participate
The study was conducted in accordance with Good Clinical Practices, and approved by authorities for each study site (the Regional Ethics Committee in Lambaréné (CERIL) for Gabon, Committee on Human Research, Publication and Ethics, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, for Ghana and the National Ethics Research Committee, Kenya Medical Research Institute (KEMRI) for Kenya). Children were enrolled into this study if a parent or guardian was willing to provide written informed consent in accordance with local practice.

Funding
The authors acknowledge the financial support from fortüne Grant (2270-0) from University Klinikum Tübingen and DFG Grant (DFG Ku 775/17-1) for German—African Cooperation project in Infectiology.

Received: 11 March 2016 Accepted: 15 June 2016
Published online: 07 July 2016

References

1. WHO. World malaria report. Geneva: World Health Organization; 2015.
2. WHO. Global plan for artemisinin resistance containment (GPARC). Geneva: World Health Organization; 2011.
3. Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, et al. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet. 2012;379:413–31.
4. White NJ. The role of anti-malarial drugs in eliminating malaria. Malar J. 2008;7(Suppl 1):S8.
5. Wells TN, Burrows JN, Baird JK. Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination. Trends Parasitol. 2010;26:145–51.
6. Howes RE, Battle KE, Satyagraha AW, Baird JK, Hay SI. G6PD deficiency: global distribution, genetic variants and primaquine therapy. Adv Parasitol. 2013;81:133–201.
7. Khim N, Benedet C, Kim S, Kheng S, Siv S, Leang R, et al. G6PD deficiency in *Plasmodium falciparum* and *Plasmodium vivax* malaria-infected Cambodian patients. Malar J. 2013;12:171.
8. Cappellini MD, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. Lancet. 2008;371:64–74.
9. WHO Working Group. Glucose-6-phosphate dehydrogenase deficiency. Bull World Health Organ. 1989;67:601–11.
10. Beutler E. Glucose-6-phosphate dehydrogenase deficiency: a historical perspective. Blood. 2008;111:16–24.
11. Minucci A, Moradkhani K, Hwang MJ, Zuppi C, Giardina B, Capoluongo E. Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations. Blood Cells Mol Dis. 2012;48:154–65.
12. Ruwende C, Khoo SC, Snow RW, Yates SN, Kwiatkowski D, Gupta S, et al. Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. Nature. 1995;376:246–9.
13. Bwayo D, Kaddumukasa M, Ddungu H, Kironde F. Prevalence of glucose-6-phosphate dehydrogenase deficiency and its association with *Plasmodium falciparum* infection among children in Iganga distric in Uganda. BMC Res Notes. 2014;7:372.
14. Clark TG, Fry AE, Auburn S, Campino S, Diakite M, Green A, et al. Allelic heterogeneity of G6PD deficiency in West Africa and severe malaria susceptibility. Eur J Hum Genet. 2009;17:1080–5.
15. De AC, Migot-Nabias F, Guitard J, Pelleau S, Vulliamy T, Ducrocq R. The role of the G6PD AEth376G/968C allele in glucose-6-phosphate dehydrogenase deficiency in the seerer population of Senegal. Haematologica. 2006;91:262–3.
16. Malaria Genomic Epidemiology Network. Reappraisal of known malaria resistance loci in a large multicenter study. Nat Genet. 2014;46:1197–204.
17. Shah SS, Rockett KA, Jallow M, Sisay-Joof F, Bojang KA, Pinder M, et al. Heterogeneous alleles comprising G6PD deficiency trait in West Africa exert contrasting effects on two major clinical presentations of severe malaria. Malar J. 2016;15:13.
18. Kremsner PG, Adegnika AA, Hounkpatin AB, Zinsou JF, Taylor TE, Chimalizeni Y, et al. Intramuscular artesunate for severe malaria in African children: a multicenter randomized controlled trial. PLoS Med. 2016;13:e1001938.
19. Planche T, Krishna S, Kombila M, Engel K, Faucher JF, Ngou-Milama E, et al. Comparison of methods for the rapid laboratory assessment of children with malaria. Am J Trop Med Hyg. 2001;65:599–602.
20. Shah SS, Macharia A, Makale J, Uyoga S, Kivinen K, Craik R, et al. Genetic determinants of glucose-6-phosphate dehydrogenase activity in Kenya. BMC Med Genet. 2014;15:93.
21. Kremsner PG, Valim C, Missinou MA, Olola C, Krishna S, Issifou S, et al. Prognostic value of circulating pigmented cells in African children with malaria. J Infect Dis. 2009;199:142–50.
22. Taylor T, Olola C, Valim C, Agbenyega T, Kremsner P, Krishna S, et al. Standardized data collection for multi-center clinical studies of severe malaria in African children: establishing the SMAC network. Trans R Soc Trop Med Hyg. 2006;100:615–22.
23. World Health Organization. Communicable diseases cluster. severe *Falciparum malaria*. Trans R Soc Trop Med Hyg. 2000;94(Suppl 1):S1–90.
24. WHO. Management of severe malaria—a practical handbook. 3rd ed. Geneva: World Health Organization; 2013.
25. Luzzatto L, Nannelli C, Notaro R. Glucose-6-Phosphate Dehydrogenase Deficiency. Hematol Oncol Clin North Am. 2016;30:373–93.
26. Guindo A, Fairhurst RM, Doumbo OK, Wellems TE, Diallo DA. X-linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. PLoS Med. 2007;4:e66.
27. Mombo LE, Ntoumi F, Bisseye C, Ossari S, Lu CY, Nagel RL, et al. Human genetic polymorphisms and asymptomatic *Plasmodium falciparum* malaria in Gabonese schoolchildren. Am J Trop Med Hyg. 2003;68:186–90.
28. Burchard GD, Browne EN, Sievertsen J, May J, Meyer CG. Spleen size determined by ultrasound in patients with sickle cell trait, HbAC trait and glucose-6-phosphate-dehydrogenase deficiency in a malaria hyperendemic area (Ashanti Region, Ghana). Acta Trop. 2001;80:103–9.
29. Suchdev PS, Ruth LJ, Earley M, Macharia A, Williams TN. The burden and consequences of inherited blood disorders among young children in western Kenya. Matern Child Nutr. 2014;10:135–44.

30. Carter N, Pamba A, Duparc S, Waitumbi JN. Frequency of glucose-6-phosphate dehydrogenase deficiency in malaria patients from six African countries enrolled in two randomized anti-malarial clinical trials. Malar J. 2011;10:241.
31. Enevold A, Lusingu JP, Mmbando B, Alifrangis M, Lemnge MM, Bygbjerg IC, et al. Reduced risk of uncomplicated malaria episodes in children with alpha+-thalassemia in northeastern Tanzania. Am J Trop Med Hyg. 2008;78:714–20.
32. May J, Meyer CG, Grossterlinden L, Ademowo OG, Mockenhaupt FP, Olumese PE, et al. Red cell glucose-6-phosphate dehydrogenase status and pyruvate kinase activity in a Nigerian population. Trop Med Int Health. 2000;5:119–23.
33. Uyoga S, Ndila CM, Macharia AW, Nyutu G, Shah S, Peshu N, et al. Glucose-6-phosphate dehydrogenase deficiency and the risk of malaria and other diseases in children in Kenya: a case-control and a cohort study. Lancet Haematol. 2015;2:e437–44.
34. Martin SK, Miller LH, Alling D, Okoye VC, Esan GJ, Osunkoya BO, et al. Severe malaria and glucose-6-phosphate-dehydrogenase deficiency: a reappraisal of the malaria/G-6-PD hypothesis. Lancet. 1979;1:524–6.
35. Nkuo-Akenji TK, Wepngong P, Akoachere JF. Effects of ABO/Rh blood groups, G-6-PD enzyme activity and haemoglobin genotypes on malaria parasitaemia and parasite density. Afr J Health Sci. 2004;11:93–7.
36. Luzzatto L, Bienzle U. The malaria/G-6-PD hypothesis. Lancet. 1979;1:1183–4.
37. Bienzle U, Ayeni O, Lucas AO, Luzzatto L. Glucose-6-phosphate dehydrogenase and malaria. Greater resistance of females heterozygous for enzyme deficiency and of males with non-deficient variant. Lancet. 1972;1:107–10.
38. Gilles HM, Fletcher KA, Hendrickse RG, Lindner R, Reddy S, Allan N. Glucose-6-phosphate-dehydrogenase deficiency, sickling, and malaria in African children in South Western Nigeria. Lancet. 1967;1:138–40.
39. Allison AC, Clyde DF. Malaria in African children with deficient erythrocyte glucose-6-phosphate dehydrogenase. BMJ. 1961;1:1346–9.
40. Luzzatto L, Usanga FA, Reddy S. Glucose-6-phosphate dehydrogenase deficient red cells: resistance to infection by malarial parasites. Science. 1969;164:839–42.
41. Manjurano A, Sepulveda N, Nadjm B, Mtove G, Wangai H, Maxwell C, et al. African glucose-6-phosphate dehydrogenase alleles associated with protection from severe malaria in heterozygous females in Tanzania. PLoS Genet. 2015;11:e1004960.
42. Miller J, Golenser J, Spira DT, Kosower NS. *Plasmodium falciparum*: thiol status and growth in normal and glucose-6-phosphate dehydrogenase deficient human erythrocytes. Exp Parasitol. 1984;57:239–47.
43. Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, et al. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. Blood. 1998;92:2527–34.
44. Sodeinde O, Clarke JL, Vulliamy TJ, Luzzatto L, Mason PJ. Expression of *Plasmodium falciparum* G6PD-6PGL in laboratory parasites and in patient isolates in G6PD-deficient and normal Nigerian children. Br J Haematol. 2003;122:662–8.
45. Mockenhaupt FP, Falusi AG, May J, Ademowo OG, Olumese PE, Meyer CG, et al. The contribution of alpha+-thalassaemia to anaemia in a Nigerian population exposed to intense malaria transmission. Trop Med Int Health. 1999;4:302–7.