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El Dr Quique Bassat, investigador del Instituto de Salud Global de Barcelona y del Centro de Investigação em Saúde de Manhiça,

hace constar

que la tesis titulada
SEVERE MALARIA DISEASE IN A RURAL DISTRICT HOSPITAL IN MOZAMBIQUE. FROM EVIDENCE GENERATION TO PREVENTION AND TREATMENT

presentada por Rosauro Varo Cobos ha sido realizada bajo su dirección, y cumple todos los requisitos que dicta la normativa vigente para la presentación de tesis doctorales como un compendio de artículos en la Facultad de Medicina de la Universitat de Barcelona,

y considera,

que la memoria resultante es apta para optar al grado de Doctor en Medicina con mención Internacional por la Universidad de Barcelona

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Y para que quede constancia, firma el presente documento
Quique Bassat
Barcelona, 15 de Junio de 2020

A mis abuelos Maruja, Pepe, Rosauro y Valle

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ABBREVIATIONS

ACT: Artemisinin-based combination therapies
ADHD: attention-deficit hyperactivity disorder
AE: Adverse events
ALT: alanine aminotransferase
Ang-1: Angiopoietin-1
Ang-2: Angiopoietin-2
ARD: acute respiratory distress
AST: aspartate aminotransferase
BBB: blood-brain-barrier
BDNF: brain-derived neurotrophic factor
BMS: β-methasone hemisuccinate
CFR: case fatality rate
CI: confidence interval
CISM: Centro de Investigação em Saúde de Manhiça (Manhiça Health Research Centre)
CNBS: Mozambican National Bioethics Committee
CM: cerebral malaria
CMD: common mental disorders
CMAR: Children-month at risk
CSF: cerebro-spinal fluid
CS: curdlan sulphate
CT: computed tomography
Cys C: Cystatine C
DCA: dichloroacetate
DDT: dichloro-diphenyl-trichloroethane
DFO: desferrioxamine
DHA-PPQ: Dihydroartemisinin-piperaquine
DRC: The Democratic Republic of the Congo
DSS: demographic surveillance system
EBT: Exchange blood transfusions
ECM: experimental cerebral malaria
EPCR: endothelial protein C receptor
FDA: Food and Drug Administration
Hib: Haemophilus Influenzae b
GAG: Sulfated glycosaminoglycans
GMEP: Global Malaria Eradication Program
GMS: Greater Mekong Sub-regiong
GSK3β: glycogen synthase kinase 3
GTS: Global Technica Strategy
HIV: Human Immunodeficiency Virus
HO-1: Heme oxygenase-1
HR: Hazard ratio
HRP-2: histidine-rich protein 2
IL-6: interleukin 6
IL-8: interleukin 8
iNO: inhaled nitric oxid
IP-10: 10 kDa interferon γ-induced protein
IPTi: Intermittent preventive treatment in infants
IPTp: Intermittent preventive treatment in pregnancy
iRBC: infected red blood cells

IRS: Indoor residual spraying
LDH: lactate dehydrogenase
LLINs: long-lasting insecticide treated bednets
LODS: Lambaréné Organ Dysfunction Score
MDH: Manhiça's District Hospital
MMV: Medicines for malaria venture
MRI: magnetic resonance imaging
MS: milliseconds
MSS: morbidity surveillance system
NAC: N-acetylcysteine
NO: nitric oxide
nRBCs: nucleated red blood cells
oAc: oral activated charcoal
OR: odds ratio
PAR: partial artemisinin resistance
PCV: packed cell volume
PEs: parasitized erythrocytes
PfEMP1: Plasmodium falciparum erythrocyte membrane protein 1
P. falciparum: Plasmodium falciparum
PPAR-γ: peroxisome proliferator-activated receptor-γ
PPQ: piperaquine
qPCR: real-time quantitative PCR
RCT: randomized control trials
rHuEPO: Recombinant Human Erythropoietin
RXR: retinoic X receptor
SAE: Serious adverse event
SSA: sub-Saharan Africa
SM: severe malaria
SMA: severe malarial anaemia
sFt-1: soluble FMS-like tyrosine kinase-1
sTNFR-1: soluble tumor necrosis factor receptor 1
sTREM-1: soluble triggering receptor expressed on myeloid cells 1
TNF: Tumor Necrosis Factor
TZD: thiazolidinedione
oAC: Oral activated charcoal
OR: odds ratio
PADH: post–artesunate delayed haemolysis
PAR: partial artemisinin resistance
PCV: packed cell volume
PTX: Pentoxifylline
RBCs: red blood cells
RDT: Rapid diagnostic test
SD: Standard deviation
SEA: South-East Asia
SP: sulfadoxine-pyrimethamine
UDCA: Ursodeoxycholic acid
WHO: World Health Organization
WBCC: White blood cell count

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SUMMARY (English)

Malaria is the most important parasitic disease worldwide and, in 2018 alone, caused around 228 million clinical episodes, between two and four million cases of severe disease and an estimated number of 405,000 deaths. These data show that, despite renewed efforts to eradicate malaria, it remains a major global health problem. Worryingly, the global malaria situation seems to be at crossroads, since data from the past 5 years has evidenced a stagnation of the gains witnessed in the first 15 years of the millennium, and a recent increase in its incidence. Despite undeniable progress in terms of control, recent strategies have clearly been ineffective and about 40% of the world's population still lives exposed to malaria in the 86 countries where it remains endemic. Two thirds of the disease's mortality burden are borne by children under the age of five, an age range where malaria is estimated to be responsible for 5.2% of total global deaths. Over 90% of these malaria-associated child deaths occur in sub-Saharan Africa.

Severe malaria is a complex multisystemic disease that usually appears with one or more of these presentations: hyperparasitaemia, severe anaemia, acute renal failure, metabolic acidosis, respiratory distress, hypoglycaemia, and/or cerebral malaria. Among all these complications, cerebral malaria and respiratory distress are the most lethal. Severe anaemia, although less aggressive, causes the highest cumulative malaria-associated mortality overall due to its high incidence. Both parasite and host determinants appear to contribute to the triggering and progression of severe and cerebral malaria. To summarize, the host's immune response to the parasitic infection, associated with the sequestration of parasitized red blood cells in the microvasculature of vital organs such as the brain, results in an altered inflammatory response with alterations in cytokine levels and antibody production, endothelial activation, microvascular obstruction, metabolic alterations and a rupture and dysfunction of the blood-brain barrier. Notwithstanding this, we are far from fully understanding the complete pathophysiology of the process, and how the interaction between

the parasite and the host is translated into a clinical phenotype that can range in a wide clinical spectrum from asymptomatic infection to death. It is vitally important to delve into all aspects of this process to improve the management of children with severe malaria. Paradoxically, the current initiatives to eliminate the disease may actually increase the percentage of children and adults exposed to more severe disease. A decrease in the prevalence of the infection could negatively impact the rate and speed of acquisition of immunity, that critically relies on the continuous exposure to repeated infections. It is important to take this changing epidemiological context into account as the expression of the disease may start to change, with older children at increased risk of severe and fatal disease and a higher proportion of the severe cases presenting with cerebral malaria.

The vast majority of the severe sequelae and deaths from malaria are caused by *Plasmodium (P.) falciparum*. In the absence of rapid and effective treatment, infection with this parasite can easily progress to severe and lethal forms. The current treatment of choice for severe malaria in both children and adults is parenteral artesunate, which in several studies has been shown to be more effective than quinine. However, and in spite of this good effectiveness, the prevalence of lethal outcomes remains excessively high (8.5% in children and 15% in adults for severe malaria; 18% and 30% respectively for cerebral malaria). Since the host immune response plays a central role in the triggering, severity and prognosis of malaria, different immunomodulatory strategies to supplement first line antimalarial treatments have been tested in an attempt to improve clinical prognosis. To date several putative adjunctive strategies have also been disappointingly included in clinical trials of the treatment of severe malaria, but without success.

The pathophysiology of severe malaria is complex and seeking to intervene on a single pathophysiological route may not be sufficient to reduce mortality. Searching for multiple

routes, using multiple interventions or alternatively, using a single intervention that acts at different pathophysiological levels, may be a more promising alternative in the quest for an effective adjuvant therapy. Given the current scientific evidence, it is essential to deeply explore the role that the different actors play in the clinical translation of the infection, from the parasite to the host and how the result may influence the clinical management of severe cases.

Materials and Methods

This thesis is based on research conducted at the Barcelona Institute for Global Health (ISGlobal)/Hospital Clínic-Universitat de Barcelona in Spain, at the Centro de Investigação em Saúde de Manhiça (CISM) in Mozambique, and in collaboration with the Sandra-Rotman Centre for Global Health in Toronto, Canada.

This thesis is structured in six articles: four published in international peer-reviewed journals, one accepted but not yet published, one additional article currently in preparation for submission. These 6 articles include the following:

1. A review of adjunctive therapies that have been tested for severe malaria, including a discussion of promising preclinical studies as well as candidate therapeutics for future clinical investigation
2. A retrospective analysis of data using the morbidity surveillance system in place at Manhiça district hospital comparing the prevalence and incidence of post-malarial anaemia among Mozambican children with severe malaria treated with either quinine or artesunate.
3. A cross-sectional study investigating the epidemiology of *P. falciparum* resistance markers in different endemic areas of Asia and Africa including Manhiça, a semi-rural area in southern Mozambique. Resistance-associated mutations being

investigated include the genetic markers *kelch13*, *multidrug resistance 1 (Pfmdr1)* and *plasmepsin 2 (Pfpm2)*.

4. A case-control study investigating the differential expression of biomarkers in children with severe malaria compared to children with uncomplicated malaria and the association of these biomarkers with specific manifestations of severity.
5. A clinical trial investigating the safety and tolerability of rosiglitazone as an adjuvant treatment to routine antimalarial therapy in children with uncomplicated malaria, compared to the use of placebo.
6. A commentary on the main challenges in conducting clinical trials of adjuvant therapies in severe malaria which includes proposals for solutions to address these difficulties.

Five of the six articles have been led (First author) by the author of this thesis and cover a variety of methodologies, including original research, reviews and comments.

Key results

The studies included in this thesis provide important results for the understanding of the pathophysiology and clinical epidemiology of malaria. In addition, this work may provide relevant insights for future studies of adjuvant therapies for the treatment of severe malaria in children.

The first article reviews the current literature on adjuvant treatments for severe and cerebral malaria. This review not only summarizes data from clinical trials conducted in humans, but also highlights some studies conducted in animal models that may offer novel strategies and candidates for future evaluation in patients. Unfortunately, there have been numerous attempts to test the safety and added benefit of multiple adjunctive therapeutics that have not resulted in clear evidence of their usefulness to reduce mortality associated with severe

malaria. This may reflect our incomplete understanding of the pathophysiology of malaria, but also the difficulties in conducting clinical trials in patients with severe malaria. To allow the detection of significant differences in severe and fatal outcomes, clinical trials should be designed with well-specified population groups, inclusion criteria and interventions, precise sample sizes, and clearly defined analytical objectives.

The second article presents the results of a retrospective comparative analysis aiming to explore differences in the prevalence of post-malarial anaemia among children with severe malaria treated with parenteral artesunate or intravenous quinine. This analysis was possible using data from the morbidity surveillance system in place at Manhiça district hospital and selected children under 15 years of age admitted with a diagnosis of malaria between 2003 and 2017, alive at the time of hospital discharge and with at least one haematocrit (PCV) measurement within 28 days after discharge and identified by passive case detection. The results obtained show that there are no significant differences between the two treatment groups in terms of the percentage of post-malarial treatment anaemia. However, given the high prevalence of anaemia in Manhiça, this study highlights the need to investigate and monitor this condition in all children affected by severe malaria regardless of the treatment received.

The third article represents an important update on the prevalence of artemisinin and piperaquine resistance molecular markers, specifically *P. falciparum kelch13* (a marker of artemisinin resistance), *Pfmdr1* and *Pfpm2* (which are related to resistance to different partner drugs such as lumefantrine or piperaquine, included in artemisinin-based combinations, respectively). The results of this study show how parasites expressing currently validated *kelch* resistance mutations 13 are present in Asia, but are not yet detectable in countries in Africa, including Mozambique. On the other hand, isolates with multiple copies of the *Pfmdr1* and *Pfpm2* genes were shown to be more frequent than previously reported in Africa, especially in

countries such as Burkina Faso and Uganda, although in Mozambique the proportion was much lower. This study underlines the need for regular research and surveillance of the resistance profiles of *P. falciparum* in Africa and the continued search for new antimalarial compounds with proven efficacy.

The fourth article presents the results of a case-control study conducted to identify host molecules differentially expressed in children with severe malaria compared to those with uncomplicated malaria. Participants were children under 10 years of age who came to the Manhiça District Hospital in Mozambique between September 2014 and May 2016. Cases and controls were nested by sex, age (+/- 6 months) and parasitaemia, with 56 case-control pairs finally included in the analysis. The results of this study demonstrate that the levels of some biomarkers that lead to host inflammation and endothelial activation are differentially expressed in severe malaria cases, the measurement of which could therefore be used as potentially public-health relevant prognostic marker. Furthermore, it also shows that the expression of certain biomarkers is associated with specific manifestations of severity. These results can help gain a better understanding of the pathophysiology of malaria, opening new avenues for research into new diagnostic and therapeutic tools. Importantly, this study specially underlines the central role that angiopoietin-2 may play in the future in that respect.

The fifth article is a clinical trial conducted in Manhiça at the request of the National Bioethics Committee of Mozambique as a prelude to the further evaluation of rosiglitazone, an oral antidiabetic, in a clinical trial of the treatment of severe paediatric malaria. A prospective, randomized (2:1), double-blind, placebo-controlled, Phase IIa trial comparing rosiglitazone as an adjuvant treatment added to standard treatment of children with uncomplicated malaria was conducted. Thirty children were recruited, 20 of whom received rosiglitazone and 10 placebo, all of whom were closely followed with clinical, haematological, biochemical and electrocardiographic assessments. The results of this study support further evaluation of

rosiglitazone as an adjunct therapy for severe malaria and may contribute to accelerating the improvement of treatment and reduction of the negative effects of severe malaria in children.

The last article is an opinion piece that reflects on the challenges, cost, and feasibility of conducting randomized controlled trials in severe malaria with adequate power to identify the effects on mortality of the interventions studied. Difficulties in identifying and recruiting patients with severe malaria, reductions in mortality rates, and the high human and logistical costs required to conduct these trials may result in inappropriate rejection of new therapies due to lack of demonstrated benefit. With this in mind, a number of measures are proposed to facilitate their implementation, including: decreasing the sample sizes needed by using host biomarkers for risk stratification in children in randomized clinical trials; using alternative, but validated, surrogate markers of mortality; and finding safe Food and Drug Administration (FDA)-approved drugs that modulate the underlying causal pathways in severe malaria. This commentary may provide insights on how to rethink new strategies in the search for adjuvant therapies to improve the prognosis of patients with severe malaria.

Conclusions and recommendations

Mortality and morbidity rates associated with *P. falciparum* infection remain exceptionally high despite the availability of effective antimalarial treatments. In fact, after adequate treatment with artesunate, between 8.5 and 15% of patients diagnosed with severe malaria still die and up to 50% of survivors of cerebral malaria may develop long-term neurological sequelae. The Global Technical Malaria Strategy (GTS) 2016-2030 report proposed a reduction of at least 90% in malaria incidence and mortality by 2030. However, without further interventions to accelerate the current rate of reduction, this objective will not be achieved. Thus, there is an urgent need to develop adjuvant therapies to be used in conjunction with antimalarials to improve the prognosis of the disease. Since the first studies conducted in the 1980s with corticosteroids, different alternatives have been investigated, ranging from the use of

immunomodulators (immunoglobulins, anti-TNF therapies…), anticoagulant drugs or iron reducers; to strategies to reduce the neurological effects on the central nervous system (prophylactic phenobarbital or mannitol), improve anaemia or maintain the hydroelectrolytic balance. Unfortunately, to date, none of these interventions has been able to demonstrate a clear benefit in reducing the mortality and sequelae associated with severe malaria. This is probably the result of the complex and still unknown interactions between socio-demographic factors, the parasite and the host. From this interrelationship emerges the clinical expression of malaria infection, on a spectrum ranging from asymptomatic infection to death. It is therefore essential to carry out further research to understand the phenomenon in its entirety.

It is also necessary to continue working on a more complete understanding of the therapeutic effects of the currently available antimalarial drugs. The approval of intravenous artesunate as a first line of treatment for severe malaria marked a paradigm shift in the management of this disease. Although it proved to be more effective and safer in the short term than quinine, its safety profile, beyond the period of hospitalization, was not fully defined. Once artesunate began to be used in non-immune patients in non-endemic countries, anaemia was reported in some of these patients. With a peak between two and four weeks after treatment, this anaemia with haemolytic characteristics may, in some serious cases, require a blood transfusion. However, these data may not be extrapolated to children in sub-Saharan Africa that bear the brunt of the disease and where anaemia is also highly prevalent. The results of our study, in line with others conducted in African countries, show that the introduction of artesunate as the treatment of choice has not led to an increase in anaemia compared to the period when quinine was the first-line drug. However, it is not clear which is the effect in the need of transfusions after the introduction of artesunate. What has however become evident is the need of monitoring -in the short term- haematological parameters in all patients regardless of the antimalarial treatment received.

The effectiveness of such treatments depends largely on the sensitivity of the parasite to them, and *P. falciparum* has been shown to develop resistance against the majority of existing antimalarial drugs. There are serious concerns about the widespread dissemination (and particularly to Sub-Saharan Africa) of the already detected partial resistance to artemisinins detected in the Greater Mekong Sub-region, given that this family of drugs includes the most powerful and effective compounds currently available. However, mutations such as those affecting the *Kelch 13* gene, which confer "partial" resistance to the parasite, well-documented in South East Asia have not been yet detected in Sub-Saharan Africa, and the results of this thesis support this observation. On the other hand, we found that the percentage of mutations in *Pfmdr1* and *Pfpm2*, was higher than previously reported. This underlines the urgent need to establish resistance monitoring networks and to promote the research on new antimalarial drugs to widen our currently limited malaria pharmacopeia.

Research requires, among other things, a deeper understanding of the pathophysiology of malaria in all its manifestations. This should help to clarify the mechanisms that determine, for example, why one particular patient rapidly responds to treatment and others develop life-threatening disease. Evidence supporting the fundamental role of host immunological and endothelial mediators in determining the severity of a malaria episode is rapidly emerging. The results of the case-control study described in this thesis may help to identify which of these biomarkers play a more critical role, such as inflammatory markers as Il-8 or sFlt-1; or markers of endothelial activation such as angiopoietins 1 and 2. Our study shows, among other findings, that angiopoietin-2 plasmatic levels are higher in children with severe malaria compared to those with uncomplicated malaria and, as a result, angiopoietin-2 could become both a diagnostic and therapeutic target for this disease. Indeed, rosiglitazone has demonstrated anti-inflammatory, antioxidant and neuroprotective properties in experimental models, and it is able to reduce the levels of angiopoietin-2. It has also been shown to be safe in studies in adult patients with uncomplicated malaria. The clinical trial included in this thesis

suggests that rosiglitazone is also safe and well tolerated in Mozambican children with uncomplicated malaria. This evidence was necessary for rosiglitazone to be further investigated in clinical trials in children for the treatment of severe malaria.

To be successful, future clinical trials of adjunctive therapies must address some important limitations that have pervaded previous studies. These limitations can be mitigated by risk stratification of participants and the application of specific severity criteria. Immune activation and host endothelial markers, such as angiopoietin-2, could be used to identify those high-risk participants who would be most likely to benefit from adjuvant therapies. In the commentary that closes this thesis, the use of angiopoietin-2 as a surrogate marker of mortality is discussed. In addition, it also recommends that preference be given to drugs that are known to be safe in the target population (including children), approved by regulatory agencies for other indications, and that can act on pathophysiological pathways related to severity, such as is the case of rosiglitazone.

The set of studies and data from this thesis may help to provide a better understanding of the critical role of host response and parasite interaction in the pathogenesis of severe malaria and may facilitate the search for new diagnostic methods and future effective adjuvant therapies.

RESUMEN (Español)

La malaria es la enfermedad parasitaria más importante a nivel mundial y, sólo en el año 2018, causó alrededor de 228 millones de infecciones, entre dos y cuatro millones de casos de malaria grave y alrededor de 405000 muertes. Estos datos demuestran que, a pesar de los renovados esfuerzos por erradicarla, la malaria continúa siendo un enorme problema de salud pública a nivel global que, incluso en los últimos tiempos y de manera alarmante, ha revertido la tendencia hacia el descenso y aumentado su incidencia. A pesar de los innegables avances en términos de control, las estrategias implantadas en los últimos años han sido claramente inefectivas y alrededor del 40% de la población mundial todavía vive expuesta a la malaria en alguno de los 86 países donde continúa siendo endémica. Los niños menores de cinco años, en concreto, soportan el 67% de la carga de mortalidad de la enfermedad, en una franja etaria en la que se calcula que la malaria es la responsable del 5.2% del total de fallecidos. Alrededor del 90% de esas muertes se producen en África subsahariana.

La malaria grave es una compleja enfermedad multisistémica que, normalmente, se manifiesta con una o más de estas presentaciones: hiperparasitemia, anemia grave, insuficiencia renal aguda, acidosis metabólica, distrés respiratorio, hipoglicemia y/o malaria cerebral. Entre todas estas complicaciones, la malaria cerebral y el distrés respiratorio son las que presentan una mayor letalidad. La anemia grave, aun siendo menos agresiva, es la que presenta en conjunto una mortalidad más elevada debido a su alta prevalencia. Tanto las características del parásito como del huésped y la interacción entre ambos contribuyen al inicio y la evolución de la malaria grave y cerebral. De modo muy esquemático, se podría decir que la respuesta inmune del huésped a la infección parasitaria, asociada al secuestro de eritrocitos parasitados en la microvasculatura de órganos vitales como el cerebro, desemboca en una cascada inflamatoria alterada asociada a: activación endotelial, alteración en los niveles de citoquinas y en la producción de anticuerpos, obstrucción microvascular, alteraciones metabólicas y ruptura y disfunción de la barrera hematoencefálica. En cualquier caso, estamos lejos de

comprender en su totalidad la fisiopatología del proceso y cómo esa interacción entre el parásito y el huésped se traduce en un espectro que abarca desde la infección asintomática hasta la muerte. Es pues de vital importancia ahondar en todos los vértices de ese proceso para mejorar el manejo y pronóstico de niños con malaria grave. Paradójicamente, el actual afán por eliminar la enfermedad podría hacer que aumentara el porcentaje de niños y adultos expuestos a cuadros de mayor gravedad y mortalidad. Esto se justificaría porque la disminución de la prevalencia de la infección podría afectar de manera negativa a la inmunidad adquirida, desarrollada gracias a la exposición continuada a través de infecciones de repetición. Es importante tener este hecho en cuenta en un contexto epidemiológico cambiante en el que la expresión de la enfermedad podría variar ocasionando, por ejemplo, que los cuadros de malaria grave varíen sus manifestaciones clínicas y se hagan más comunes en edades más avanzadas.

La mayoría de las secuelas graves y de la mortalidad asociada a la malaria está provocada por el *Plasmodium Falciparum*. En ausencia de un tratamiento rápido y efectivo, la infección por este parásito puede progresar con facilidad hacia formas graves y letales. El tratamiento de elección actual para la malaria grave, tanto en niños como en adultos, es el artesunato parenteral que, en diferentes estudios, ha demostrado una mayor eficacia que la quinina intravenosa. Sin embargo, y a pesar de su eficacia, la prevalencia de cuadros mortales continúa siendo excesivamente elevada (8.5 % niños y 15% en adultos para malaria grave; 18% y 30% respectivamente para malaria cerebral). Teniendo en cuenta estos datos y considerando que la respuesta inmune del huésped juega un papel central en el desencadenamiento, severidad y pronóstico de la malaria, se han probado diferentes estrategias inmunomoduladoras para intentar mejorar su pronóstico. Sin embargo, hasta el día de hoy se han realizado múltiples ensayos clínicos con diferentes fármacos que, desgraciadamente, no han demostrado su eficacia como terapia adyuvante. La fisiopatología de la malaria grave es compleja y pudiera ser que la intervención sobre una sola ruta fisiopatológica no fuera suficiente para reducir la

mortalidad. Buscar múltiples vías, usando intervenciones múltiples o, alternativamente, utilizando una sola que actúe a diferentes niveles fisiopatológicos, podría ser la respuesta adecuada para encontrar una terapia adyuvante efectiva. De uno u otro modo, dada la actual evidencia científica disponible es primordial ahondar en el papel que los diferentes actores juegan en la traducción clínica de la infección, desde el propio parásito hasta el mismo huésped, pasando por terapias en uso hasta el perfil farmacológico de nuevas intervenciones.

Materiales y Métodos

Esta tesis se basa en investigaciones realizadas en el Instituto de Salud Global de Barcelona (ISGlobal)/Hospital Clínic-Universitat de Barcelona en España, el Centro de Investigación en Salud de Manhiça (CISM) en Mozambique y en colaboración desarrollada con el Sandra-Rotman Centre for Global Health de Toronto, Canada.

Esta tesis está estructurada en seis artículos: cuatro publicados en revistas internacionales peer-reviewed, uno aceptado, pero todavía no publicado, y uno en fase de preparación. Son los siguientes:

1. Una revisión sobre las terapias adyuvantes que se hayan estudiado para el tratamiento de la malaria grave, incluyendo estudios pre-clínicos con resultados prometedores, así como terapias candidatas para ensayos clínicos futuros.
2. Un análisis retrospectivo de datos utilizando el sistema de vigilancia de la morbilidad en curso en el hospital distrital de Manhiça, un área semirrural del sur de Mozambique, para comparar la prevalencia e incidencia de anemia entre niños mozambiqueños con malaria grave tratados con quinina o artesunato.
3. Un estudio transversal que analiza la epidemiología de marcadores de resistencias de *P. falciparum* en diferentes áreas endémicas de Asia y África (incluyendo Manhiça). Las mutaciones asociadas a resistencias que son

investigadas incluyen los marcadores genéticos del parásito *P. falciparum kelch13, P. falciparum multidrug resistance 1* (*Pfmdr1*) y *P. falciparum plasmepsin 2* (*Pfpm2*).

4. Un estudio de casos y controles que estudia los diferentes niveles de biomarcadores en niños con malaria grave en comparación con niños con malaria no complicada y, además, la asociación de dichos biomarcadores a manifestaciones específicas de gravedad.
5. Un ensayo clínico aleatorizado que investiga la seguridad y la tolerancia de la rosiglitazona como tratamiento adyuvante al tratamiento antimalárico de rutina en niños con malaria no complicada, en comparación con el uso de placebo en niños tratados con dicho tratamiento estándar.
6. Un comentario sobre los principales retos al realizar ensayos clínicos de terapias adyuvantes en malaria grave y la propuesta de posibles soluciones para afrontar dichas dificultades.

Cinco de los seis artículos han sido liderados por el autor de esta tesis como primer nombre y abarcan metodologías variables, que incluyen investigación original, revisiones o comentarios.

Resultados clave

Los estudios incluidos en esta tesis proporcionan resultados importantes para la compresión de la fisiopatología y epidemiología clínica de la malaria y, sobre todo, abren puertas para mejorar el estudio de futuras terapias adyuvantes para el tratamiento de la malaria grave en niños, para los cuales todavía no existen intervenciones efectivas que reduzcan la carga de morbi-mortalidad asociada con la enfermedad.

El primer artículo revisó la evidencia actual de tratamientos adyuvantes para la malaria grave y cerebral. Con dicho fin, esta revisión no solo recapituló los datos de ensayos clínicos

realizados en seres humanos, sino que también, puso de relieve algunos estudios realizados en modelos animales que pueden ofrecer estrategias novedosas y candidatas para su futura evaluación en pacientes. Desafortunadamente, ha habido numerosos intentos que no se han llegado a traducir en el descubrimiento de terapias con un claro beneficio en la mejora de complicaciones y mortalidad asociadas a la malaria grave. Esta situación puede ser el reflejo de nuestra incompleta comprensión de la fisiopatología de la malaria, pero también, de las dificultades para realizar ensayos clínicos en pacientes con malaria grave. Para permitir la detección de diferencias significativas en términos de esas complicaciones y esa mortalidad, los ensayos clínicos deberían diseñarse con grupos de población, criterios de inclusión e intervenciones bien definidos, tamaños de muestra precisos y objetivos analíticos claramente establecidos.

En el segundo artículo se presentan los resultados de un análisis retrospectivo realizado para comparar las diferencias en cuanto a prevalencia de anemia entre niños con malaria grave tratados con artesunato parenteral o quinina intravenosa. Para ello, se utilizaron los datos del sistema de vigilancia de morbilidad del hospital distrital de Manhiça y se incluyeron a niños menores de15 años ingresados con diagnóstico de malaria entre los años 2003 y 2017, vivos al momento del alta hospitalaria y con al menos una medición de hematocrito en un plazo de 28 días después del alta identificada por detección pasiva. Los resultados obtenidos evidencian que no existen diferencias significativas entre ambos grupos de tratamiento en cuanto al porcentaje de anemia post-malaria. La diferencia entre ambos grupos de transfusiones sanguíneas queda menos clara. En cualquier caso, dada la alta prevalencia de anemia en Manhiça, este estudio resalta la necesidad de investigar y hacer seguimiento de esta patología en todos los niños afectados por malaria grave independientemente del tratamiento que hayan recibido.

El tercer artículo representa una actualización importante en cuanto a la prevalencia de marcadores de resistencia a artemisininas y piperaquina, concretamente de los genes *P. falciparum kelch13, Pfmdr1* y *Pfpm2*. Los resultados de este estudio muestran cómo las mutaciones de resistencia del *kelch13* actualmente validadas están presentes en Asia, pero no lo están todavía en países endémicos de África, incluyendo Mozambique. Por otra parte, se demostró que los aislamientos con múltiples copias de los genes *Pfmdr1* y *Pfpm2* eran más frecuentes de lo que se había informado anteriormente en África, especialmente en países como Burkina Faso y Uganda, aunque en Mozambique la proporción es mucho más baja. Este estudio subraya la necesidad de realizar investigaciones periódicas de los perfiles de resistencia de *P. falciparum* en territorio africano y de continuar la búsqueda de nuevos compuestos antimaláricos con eficacia demostrada.

El cuarto artículo presenta los resultados de un estudio de casos y controles realizado con el objetivo de identificar moléculas del huésped expresadas de forma diferencial en niños con malaria grave y malaria no complicada. Se reclutaron niños menores de 10 años que acudieron al Hospital distrital de Manhiça entre septiembre del año 2014 y mayo del año 2016. Los casos y los controles se anidaron por sexo, edad (+/- 6 meses) y parasitemia (en cruces) con 56 pares incluidos, finalmente, en el análisis. Los resultados de este estudio demuestran que los niveles de ciertos biomarcadores que traducen inflamación del huésped y activación endotelial se expresan de manera diferencial en los casos de malaria grave. Además, muestra que la expresión de ciertos biomarcadores está asociada a manifestaciones específicas de gravedad. Este análisis subraya, especialmente, el papel central que la angiopoyetina-2 (un biomarcador de activación endotelial) puede desempeñar en esa búsqueda.

El quinto artículo es un ensayo clínico llevado a cabo en Manhiça a petición del Comité Nacional de Bioética de Mozambique y como preludio de la evaluación ulterior de la rosiglitazona en un ensayo clínico de malaria grave pediátrica. Se realizó un ensayo de fase IIa,

prospectivo, aleatorizado, doble ciego y controlado por placebo, evaluando la seguridad de la rosiglitazona como tratamiento adyuvante añadido al tratamiento estándar de niños con malaria no complicada. Se reclutaron 30 niños de los que 20 recibieron rosiglitazona y 10 placebo, a todos los cuales se realizó un seguimiento exhaustivo con evaluaciones de tipo clínico, hematológico, bioquímico y electrocardiográfico. El tratamiento con rosiglitazona no indujo a la hipoglucemia ni alteró significativamente los parámetros clínicos, bioquímicos, hematológicos o electrocardiográficos de ningún paciente. Los resultados de este estudio apoyan la continuación del estudio de la rosiglitazona como terapia adyuvante para la malaria grave y pueden contribuir reducir los efectos negativos de este síndrome en niños.

En el último artículo se reflexiona sobre los retos, el coste y la viabilidad de llevar a cabo ensayos controlados aleatorizados en malaria grave con la potencia adecuada para identificar efectos significativos en la mortalidad de las terapias adyuvantes estudiadas. Las dificultades para identificar y reclutar pacientes con malaria grave, la reducción de las tasas de mortalidad y el alto coste humano y logístico necesario para llevar a cabo estos ensayos pueden dar lugar a un inadecuado rechazo de nuevas terapias debido a la falta de beneficios demostrados. Teniendo esto en cuenta, se proponen una serie de medidas para facilitar su realización como son: la disminución de los tamaños de muestra de los ensayos clínicos mediante el uso de biomarcadores del huésped para la estratificación y cribaje de niños de riesgo; el uso de objetivos analíticos alternativos, pero validados de mortalidad; y la búsqueda de medicamentos seguros aprobados por la Agencia de Medicamentos y Alimentación (*Food and Drug Administration*, FDA) que modulen las vías causales subyacentes a la malaria grave. Este comentario puede ayudar a repensar nuevas estrategias en la búsqueda de terapias adyuvantes que mejoren el pronóstico de los pacientes con malaria grave.

Conclusiones y recomendaciones

Las tasas de mortalidad y morbilidad asociadas a la infección por *P. falciparum* siguen siendo, a pesar de la disponibilidad de tratamiento antimaláricos efectivos, excepcionalmente elevadas. De hecho, tras recibir tratamiento con artesunato, entre el 8.5% y el 18% de los pacientes diagnosticados con malaria grave mueren y hasta el 50% de los sobrevivientes de malaria cerebral pueden desarrollar secuelas neurológicas a largo plazo. El informe de la Estrategia Técnica Mundial contra la malaria 2016-2030 propone una reducción de al menos un 90% en su incidencia y en su mortalidad para el año 2030. Sin embargo, sin nuevas intervenciones que aceleren el ritmo de reducción actual no se logrará este objetivo. Así pues, existe una necesidad urgente de desarrollar a corto plazo terapias adyuvantes que sean capaces de mejorar el pronóstico de la enfermedad.

Desde los primeros estudios realizados en los años ochenta con corticoesteroides se han buscado diferentes alternativas terapéuticas que van desde el uso de inmunomoduladores (inmunoglobulinas, terapias anti-TNF…), fármacos anticoagulantes o reductores del hierro; hasta estrategias para reducir los efectos neurológicos en el sistema nervioso central (fenobarbital o manitol), mejorar la anemia asociada o mantener el equilibrio hidroelectrolítico. Por circunstancias particulares de cada caso, hasta la fecha, ninguna de esas intervenciones ha conseguido demostrar un beneficio claro a la hora de reducir la mortalidad y las secuelas asociadas a la malaria grave. Como se ha dicho previamente, este cuadro es el resultado de la compleja interacción entre factores socio demográficos, el parásito y el huésped. De esa interrelación emerge la expresión de la infección por *P. Falciparum* en un espectro clínico que va desde la infección asintomática hasta la muerte. De este modo, para comprender ese fenómeno en su totalidad, se hace primordial la investigación en todos los factores de dicha ecuación.

En esa línea de razonamiento, también resulta indispensable ahondar en los efectos terapéuticos de las actuales antimaláricos disponibles. La aprobación de la utilización del artesunato intravenoso como primera línea para el tratamiento de la malaria grave marcó un antes y un después en el manejo de esta enfermedad. Pero, si bien demostró ser más efectivo y más seguro a corto plazo que la quinina, su perfil de seguridad más allá del periodo de hospitalización, no quedó definido por completo. Tras el comienzo del uso del artesunato en pacientes no inmunes en países no endémicos, empezaron a reportarse casos de anemia en algunos de dichos pacientes. Con un pico entre las dos y las cuatro semanas tras el tratamiento, esta anemia de características hemolíticas llega a necesitar, en algunos casos graves, de transfusión sanguínea. Sin embargo, dichos datos podrían no ser extrapolables a poblaciones infantiles del África subsahariana, que soportan el mayor peso de la enfermedad y donde, además, existe una elevada prevalencia de anemia. Los resultados de nuestro estudio, en consonancia con otros realizados en países africanos, muestran que, tras la introducción del artesunato como tratamiento de elección, no se han constatado diferencias en la prevalencia e incidencia de anemia en comparación con el periodo en el que la quinina era el fármaco de primera línea. Queda claro, en cualquier caso, la necesidad de monitorizar los parámetros hematológicos en todos los pacientes independientemente del tratamiento antimalárico recibido.

La efectividad de dichos tratamientos depende, en buena parte, de la sensibilidad del parásito hacia ellos. En concreto, el *P. falciparum* ha demostrado ser resistente a la mayoría de la farmacopea antimalárica existente. Desde hace unos años, se corre el riesgo de que esa resistencia acabe afectando al uso de las artemisininas, los compuestos más potentes y efectivos en la actualidad. De hecho, en ciertas regiones de Asia ya se ha evidenciado la presencia de mutaciones, tales como las que afectan al gen Kelch 13, que confieren una resistencia «parcial» al párasito. En África todavía no existe evidencia de dichas mutaciones y los resultados de esta tesis confirman este hecho. Sin embargo, se ha constatado que el porcentaje de mutaciones de

los genes *Pfmdr1* y *Pfpm2,* asociados a resistencias a fármacos como la lumefantrina y la piperaquina, es mayor que el reportado con anterioridad. Esto subraya la necesidad imperiosa de establecer redes de monitorización de resistencias y de continuar investigando en nuevos fármacos antimaláricos con efectividad contrastada.

Para ello, se debe, entre otras cosas, ahondar en la comprensión de la fisiopatología de la malaria en todas sus manifestaciones. En los últimos años, se ha ido generando más y más evidencia sobre el rol fundamental de los mediadores inmunológicos y endoteliales en la determinación de la gravedad de un episodio de malaria. Los resultados del estudio de casos y controles de esta tesis pueden ayudar a identificar cuáles de esos biomarcadores desempeñan un papel más crucial, como podría se el caso de ciertos factores de inflamación (Il-8, Flt-1) y activación endotelial (angiopoyetina-2). Nuestro estudio demuestra, entre otros hallazgos, que la angiopoyetina-2 está más elevada en aquellos niños que presentan un cuadro de malaria grave en comparación con aquellos con malaria no complicada y, como consecuencia, podría convertirse en una diana tanto diagnóstica como terapéutica para esta enfermedad.

La rosiglitazona ha demostrado sus propiedades antiinflamatorias, antioxidantes y neuroprotectoras en modelos experimentales. Y entre otras acciones, es capaz de reducir los niveles circulantes de la angiopoyetina-2. También ha demostrado su seguridad en estudios en pacientes adultos con malaria no complicada. El ensayo clínico de esta tesis añade a este corpus de conocimiento la confirmación de que este fármaco es seguro y tiene una buena tolerancia en pacientes menores de doce años. En concreto, en niños mozambiqueños con malaria no complicada. Esta evidencia es de vital importancia para que se continúe investigando la eficacia de la rosiglitazona en ensayos clínicos con niños con malaria grave.

Para que en el futuro esos ensayos lleguen a mejor puerto, se debe hacer frente a las limitaciones a las que se enfrentan en la actualidad. Esas limitaciones pueden mitigarse

mediante la estratificación de riesgo de los participantes y la aplicación de criterios de gravedad específicos. Para identificar a esos participantes de alto riesgo que tendrían más probabilidades de beneficiarse de las terapias complementarias, se podrían utilizar marcadores de activación inmunitaria y endotelial del huésped, tales como la angiopoyetina-2. En el comentario que cierra esta tesis se propone utilizar este marcador para dicho fin y tenerlo en cuento como objetivo primario de análisis, alternativo y sustitutivo de la mortalidad. Además, se recomienda disponer, siempre que sea posible, de los medicamentos que actúan directamente en vías fisiopatológicas y que ya están aprobados por la FDA u otros organismos reguladores para otras indicaciones, y de los que se sabe que son seguros en las poblaciones destinatarias, incluidos los niños.

El conjunto de estudios y de datos de esta tesis puede ayudar a tener una mayor comprensión de la función crítica de la respuesta del huésped y su interacción con el parásito en la patogénesis de la malaria grave y puede facilitar la búsqueda de nuevos métodos diagnósticos y de futuras terapias adyuvantes con eficacia contrastada que ayuden a disminuir la morbi-mortalidad asociada a la malaria grave.

1. INTRODUCTION

Malaria is a protozoan disease transmitted by *Anopheles* female mosquitoes and resulting from the infection of a vulnerable human host by a *Plasmodium (P.)* parasite. Five species cause all malaria infections in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. Knowlesi*, of which *P. falciparum* and *P. vivax* stand out as the most important species (For a more comprehensive and global view of malaria disease, please see **Annex 1**).

1.1. Historical perspective

Malaria is an old disease, the history of which may be tracked back to the prehistory of humankind, perhaps first originated in Africa and, afterwards, expanded to the rest of the planet. There is early documentation of possible malaria cases from about 2700 BC in China. Historically, it was believed that malaria fever was caused by fumes ("miasmas") emanating from swamps. As a matter of fact, the word malaria comes from a derivation of the Italian *mal'aria* ("bad air") and the disease is also known as paludism, a French name derivate from the Latin word *palus* (swamp).

Beyond the recognition that malaria was a cause of intermittent fevers, our understanding of the basis of the disease started at the end of the 19th century when the life cycle of the malaria parasite started to be drawn (1). Charles Louis Alphonse Laveran was the first person to describe the blood stages of the parasite in patients infected with malaria in 1880 in Algeria. Soon after, the sexual stages of a malaria-like parasite, *Haemoproteus Columbae*, were observed in birds by MacCullum in 1897. In the same year, Ronald Ross showed that the infection, in this case the avian malaria, was transmitted by the bite of infected mosquitoes. Simultaneously, Grassi, Bignami and Bastinelli confirmed that human malaria was also transmitted in this way. The next relevant discoveries to understand the whole cycle took some further decades, when exoerythrocytic development of parasites in the liver were described by Krotoski in 1962.

The beginning of the race opened up by Laveran in the understanding of the human parasite culminated more than a century later with the characterization of the complete genome of both *P. falciparum* (2) and its main vector *Anopheles Gambiae* (3). The progress of new technologies in molecular biology, genomics or proteomics may help to develop new tools to control and address an ancient scourge which some time ago was spread among almost the entire world (see also section 1.8).

1.2. Epidemiology

1.2.1 Geographic Distribution

Nearly half of the world's population (~3200 million people) is at risk of malaria which is currently endemic in 86 countries (4). The areas with ongoing malaria transmission are mainly in sub-Saharan Africa (SSA) but also in South-East Asia (SEA), Eastern Mediterranean, Western Pacific, and the Americas. However, fifteen countries in SSA and India carry almost 80% of the global malaria prevalence and five specific countries currently account for nearly half of all malaria cases worldwide: Nigeria (25%), Democratic Republic of the Congo (DRC) (11%), Mozambique (5%), India (4%) and Uganda (4%) (4). In the last two decades there has been a significant reduction in the incidence rate of malaria with some important advances and changes in the geographic distribution of malaria in the world (Figure 1). Indeed, the first fifteen years of the millennium witnessed a 37% and 60% reduction in the malaria incidence and mortality rates, respectively (4). However, and worryingly, the number of cases per 1000 population at risk has stood similar during the past three years. As World Health Organization (WHO) has rightfully highlighted, the situation of malaria is at crossroads (5).

According to WHO, *P. falciparum* accounted for almost all malaria cases in Africa, but was also present in regions of the Western Pacific (71.9%), the Eastern Mediterranean (69%) and SEA (62.8%). On the other hand, *P. vivax* is the main parasite in the region of the Americas, also abundantly present in the Indian subcontinent and SEA, and the Pacific. With the

exception of some specific regions of the Horn of Africa, *P. vivax* is very uncommon across the rest of the African continent. It is important to take in mind that mixed infections may coexist in places where more than one species circulates. There is also a major concern related to the continued emergence of parasite resistance to antimalarial medicines especially in some SEA countries such as Myanmar, Cambodia and Thailand where *P. falciparum* has become a serious public health concern because of this reason.

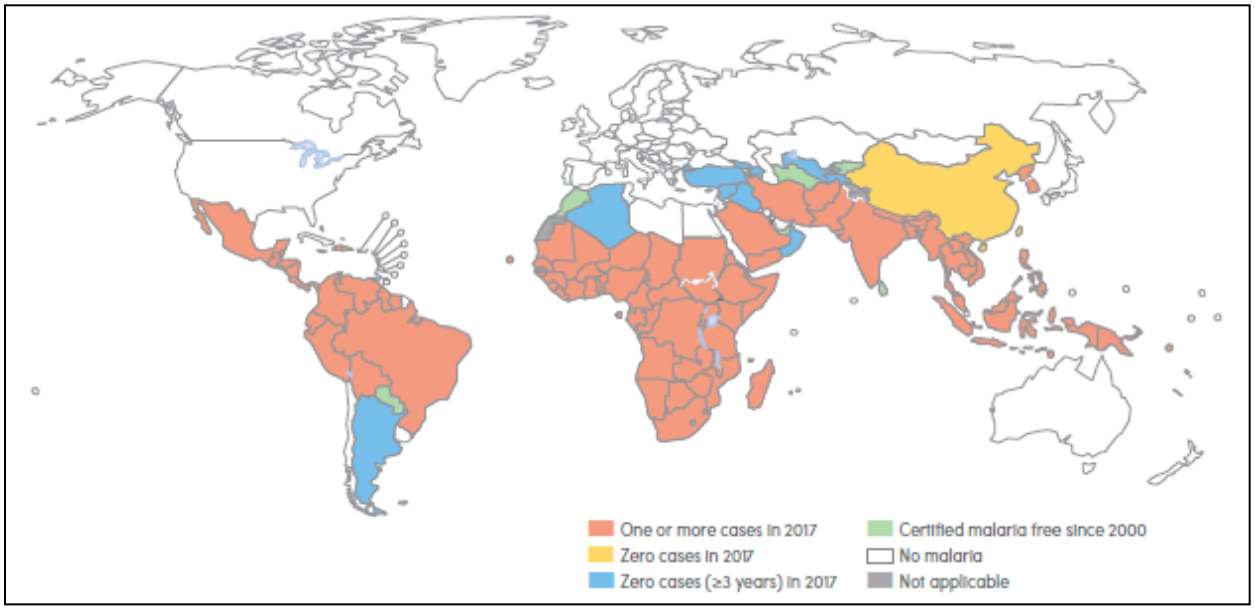


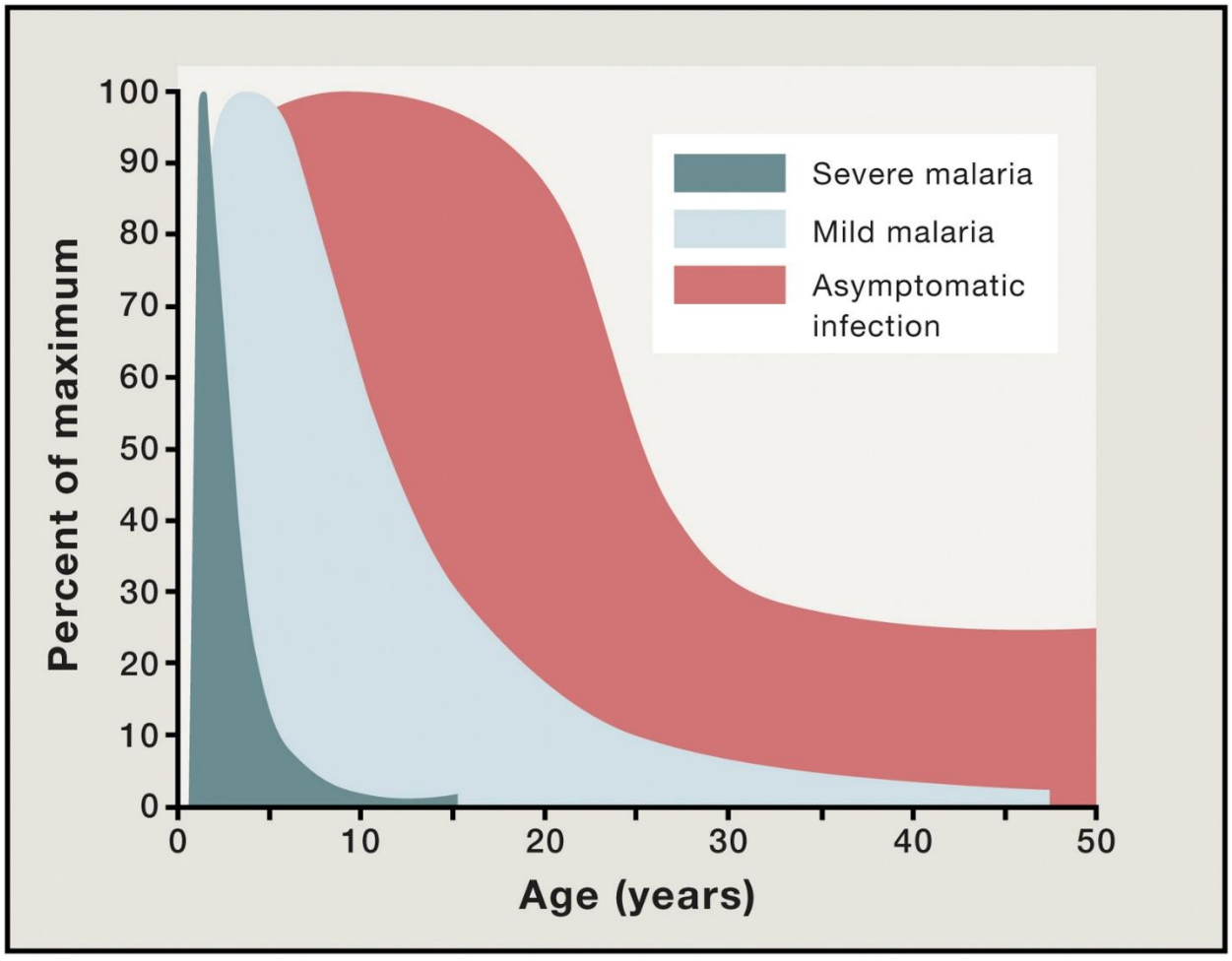
Figure 1: Countries with indigenous cases in 2000 and their status by 2017 (Source: WHO database)

1.2.2 The global burden of malaria, immunity and impact of severe disease

Malaria is the most important parasitic disease in the world causing around 228 million infections and an estimated 405,000 deaths, annually (4). The greatest burden of severe and fatal disease is borne by children under 5 years (particularly in SSA) who accounted for 67% of all malaria deaths worldwide in 2018 (4). This burden makes the optimal management of malaria a global health priority and is a continuous reminder of the need to develop new-or aggressively implement existing- effective antimalarial interventions (4, 6, 7).

Humans are unable to develop full immunity to malaria infection. However, acquisition of clinical immunity, which confers protection from life-threatening malaria episodes, may be achievable through repeated exposure to infective mosquito bites. In areas of high transmission, where children are repeatedly exposed to infective mosquito bites from birth, most children will acquire clinical immunity to severe malaria (SM) if they survive their first years of life (Figure2) (8).

Figure 2: Immunity to clinical spectrum of malaria infection



In areas of low transmission, however, SM can occur at any age, and is more common among adults, because clinical immunity to malaria takes longer to build, is quick to wane, or simply never occurs. It has been argued that a decrease in the intensity of malaria transmission may put children and adults at risk of severe and fatal disease, precisely as a result of interfering with the natural acquisition of such immune responses (9). Mechanisms involved with the development of those responses are complex and still poorly understood (10). Indeed, we lack a complete understanding on how immunity is acquired and why some individuals develop severe disease and others do not (11). There is scarce evidence of well-defined biomarkers of protection and/or vulnerability to severe disease and further research is needed to fill this crucial knowledge gap.

The global annual incidence of SM, which is associated with higher morbidity and mortality, has been estimated from around two to four million cases per year (12). In low-resource settings access to health services is often severely limited, and represents a major constraint to survival for those who develop severe malaria. The case fatality rate (CFR) for SM is heavily dependent on the possibility of reaching the health system, and can range between 20% with in-hospital care, to >90% when the patient remains at home (13). Prompt recognition and risk stratification of those children with a more adverse prognosis is crucial to reduce the number of malaria-related fatalities and to avoid misallocation of limited health resources. Understanding the gaps in malaria pathophysiology, diagnosis and treatment would help to achieve those goals by improving the management of most vulnerable children.

1.3 The biology of malaria

1.3.1 Malaria parasite

Malaria parasites are protozoan who belong to the phylus *Apicomplexa* and the genus *Plasmodium*. There are over 120 species of this eukariotyc organism but only five of them are considered to cause disease in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P.*

Knowlesi. The five species differ in geographic distribution, clinical features, patterns of drug resistance and epidemiology. *P. falciparum* is transmitted in tropical and subtropical regions, mainly in SSA, where it is the predominant species. *P. falciparum* accounts for the highest proportion of severe disease and almost all deaths attributed to this infection. Unlike the other species, which specifically invade specific stages of maturation of the erythrocyte, *P. falciparum* is able to invade red blood cells (RBCs) of any age, thus translating into a greater virulence and faster potential to multiply and reach higher peripheral parasitaemias. Furthermore, *P. falciparum* is the only species which is able to invade with multiple parasites a single RBC simultaneously.

P. vivax is the most widely distributed malaria species and accounts for most of the cases in the Americas and a significant proportion of those in the Indian Subcontinent, SEA and the Pacific. Although has been traditionally considered to cause uncomplicated malaria, there is now evidence of its potential to cause severe disease and complications (14). *P. malariae and P. ovale* only cause uncomplicated cases of malaria. *P. Knowlesi*, the only zoonotic parasite transmitted from primates to humans, is prevalent in some areas of Malaysia, where it is more likely to cause severe malaria than *P. falciparum*.

1.3.2 Malaria vector

Malaria is transmitted by female anopheline mosquitoes, where the parasite undergoes its sexual reproduction, a significant component of its life cycle. There are more than 400 different species but only around 30-40 are thought to transmit the infection, predominantly the *Anopheles Gambiae* species. Four major features of these mosquitoes have shaped the global distribution of malaria infection: longevity of the mosquito, feeding behaviour, susceptibility of the vector to infection with the parasite and mosquito density. This density is strongly correlated with patterns of rainfall, being greater with higher precipitations. It is also higher in coastal areas and lowlands where there are more freshwater breeding sites. The presence of the *Anopheles* mosquito is rare above 2000 meters of altitude and it is favoured by

temperatures ranging around 20-30 ºC. Global climate change may modify the paradigm of vector distribution and its control in the next few years.

1.3.3 Human Host

Mosquitoes transmit malaria during a blood feed by inoculating the parasite into the human host who is its main reservoir. After the mosquito bite, the clinical outcome will depend on multiple parasite and host factors but also on geographic and social ones (15). Among all of those factors are the drug resistance and multiplication rate of the parasite; presence (or absence) of naturally acquired immunity, and age of the host (see section 1.2.2); access to treatment and transmission intensity.

Some genetic factors may also influence the manifestation of the disease until such a point that it has been argued that "no infectious disease has shaped the human genome more than has malaria" (16). The geographic distribution of sickle cell disease, glucose-6-phosphate dehydrogenase deficiency, haemoglobins C and E, and ovalocytosis shows substantial overlap to that of malaria, which may translate an evolutionary survival mechanism. As an illustrative case, sickle cell trait (presence of the haemoglobine variant HbAS), present in up to 25% of the population of certain African countries, provides partial protection against SM, although the incidence of infections may be similar from that in non-affected population. Similarly, the Duffy null phenotype blood group (absence of the erythrocytic membrane Duffy antigens A and B) is predominant in Africa, while Duffy positive is more frequent in SEA. When Duffy antigens are missing the *P. vivax* parasite cannot invade RBCs and people are resistant to this infection. This situation has modified Duffy blood types seen in populations where malaria is common. For example, in areas of West Africa with a high frequency of Duffy null phenotype, there is a low incidence of *P. vivax* malaria.

1.3.4 Lifecycle of *Plasmodium falciparum* parasite

The lifecycle of *P. falciparum* is summarized in Figure 2. Female mosquito of the *Anopheles* species (previously infected with the sexual stages of malaria parasite) transmit malaria after biting humans for feeding. During the blood feed the mosquito inoculates microscopic motile *sporozoites,* the infective stages of the parasite, which circulate in the bloodstream to invade hepatocytes and then replicate. This is called the exoerythrocytic stage of the lifecycle, which is the silent part of the cycle because, at this point, there are not yet any symptoms. For *P. Falciparum,* the development in the liver takes around 8-14 days and during that time a single *sporozoite* can rise to 10000 to 30000 merozoites. *Merozoites* are released into the bloodstream after the bursting of the hepatic schizonts to start the so called erythrocytic stage of the lifecycle. For other species like *P. Vivax* or *P. Ovale* this stage can be followed, months and even years later, with the reappearance of *merozoites* and clinical symptoms as some parasites can remain in the liver as dormant forms -called *hypnozoites*- ready metabolically inactive but ready to be reactivated (16).

Each *merozoite* entering the bloodstream will try to invade a RBC and multiply. By the end of the intraerythroctytic lifecyle the *P. falciparum* parasite can multiply from a single one to 16-32 parasites who will reach again the bloodstream after the bursting of the erythrocytic schizont. This asexual cycle inside the RCB is usually synchronous, and lasts around 48 hours for *P. falciparum, P. vivax* and *P. Ovale* and around 72 hours for *P. malariae,* also coinciding with the appearance of clinical symptoms, thus determining the periodicity of fevers. The *merozoites* released will reinvade new RBCs and perpetuate the blood stage of the cycle. Most of the *merozoites* undertake this form of replication but a small percentage will follow a parallel pathway to develop into the sexual stages which are essential from a parasite survival standpoint. The female and male gametocytes will be taken up by a new mosquito and will complete the sexual reproduction in the vector's midgut, where fertilization occurs and a zygote is formed. This zygote will develop into a motile form called ookinete which travels across the gut wall and attains the bloodstream where it forms an oocyst. Inside the oocyst

new sporozoites will develop and will travel to the mosquito salivary glands where they will wait to be injected to humans after a blood meal and start a new lifecycle. The vector-based component of the parasite's life cycle will approximately last for two weeks.

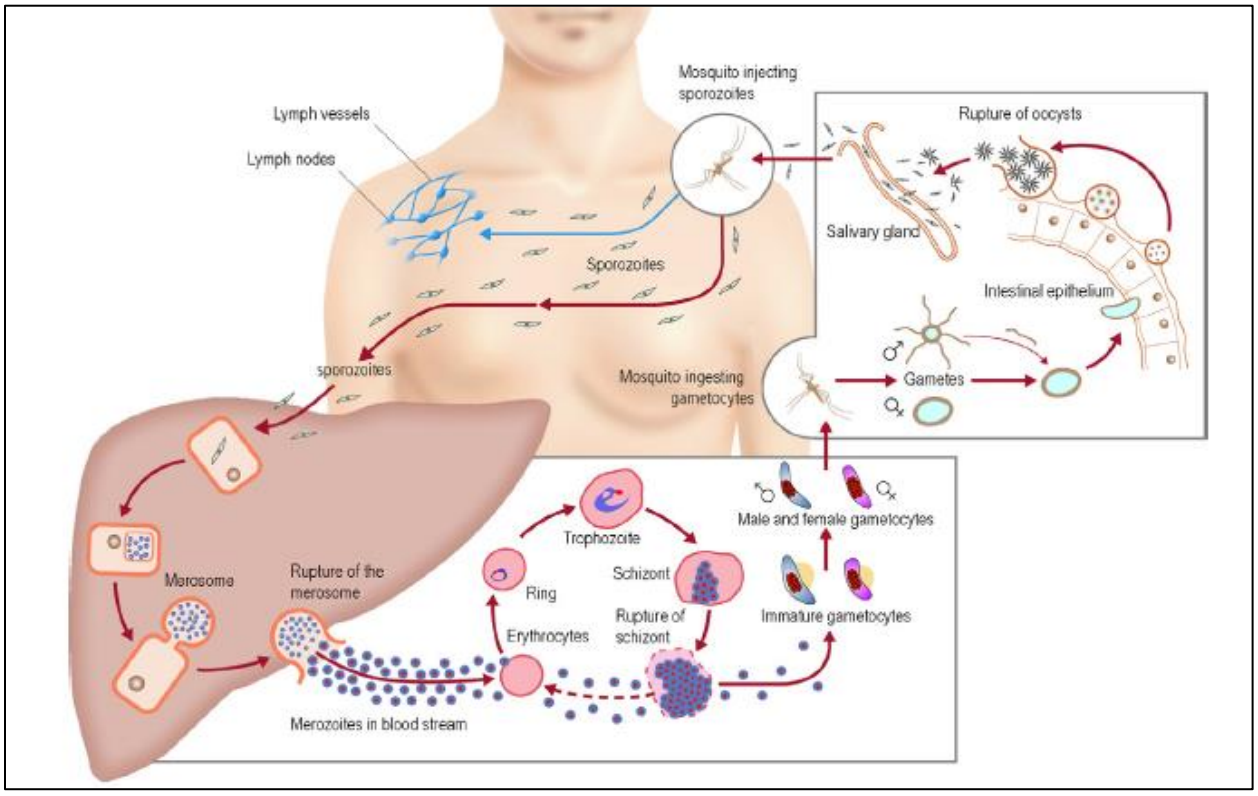


Figure 3: Lifecycle of *P. falciparum* in human body and anopheline mosquito

1.4 The pathobiology of severe and cerebral malaria

Both parasite and host determinants contribute to the onset and outcome of SM and CM. However, the mechanisms underlying this process are far from being completely understood and there is still no clear evidence of which combination of factors may lead to progression to uncomplicated malaria, severe disease or death (11).

Pronounced proinflammatory responses are characteristic of SM, and excessive activation of the immune system is central to the pathophysiology of CM. Host innate immune responses to infection, combined with the sequestration of parasitized erythrocytes (PEs) in the

microvasculature of vital organs such as the brain, result in dysregulated inflammation, endothelial activation, microvascular occlusions, metabolic derangement, and ultimately dysfunction and breakdown of the blood-brain-barrier (BBB) (17). Likewise, sequestered PEs, perfusion abnormalities, microhaemorrhages, oedema, tissue ischemia and focal disruptions of the BBB are common fundoscopic and autopsy findings in CM patients and correlate well with disease severity (18-20). Oxidative stress and axonal injury in the vicinity of brain haemorrhages and in areas of vascular occlusion have also been observed in CM post-mortem studies, and may contribute to neurological dysfunction pre-mortem and in CM survivors (21-23). Pre-mortem magnetic resonance imaging studies of CM patients have also evidenced an important role of raised intracranial pressure as a potential mechanism leading to death (24) .

P. falciparum sequestration is suggested to be mediated through the adherence of mature forms of infected RBCs (iRBCs) to host receptors expressed on the endothelium lining of host capillaries, on uninfected erythrocytes to form rosettes (25) and on platelets to form platelet-mediated clumps (26). *P. falciparum* isolates derived from infected individuals exhibits a wide range of binding affinities to numerous host receptors such as intercellular adhesion molecule ICAM-1, CD36 and C1QBP/gC1qR, being the latter associated with SM (27). Endothelial protein C receptor (EPCR) upregulated expression has also been involved as a critical cytoadhesion phenotype (28). Cytoadhesion is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (29), a family of large and highly polymorphic proteins, encoded by about 60 *var* genes per haploid genome classified into groups A, B or C and expressed on the surface of infected erythrocytes which have a key role on parasite virulence (30, 31). The genetic structuring of the *var* repertoire (2, 30, 32) is considered to be of functional importance and has been linked to malaria severity as well as cytoadhesive and serological properties of iRBCs. This evidence and the relatively rapid rate of acquisition of immunity to SM compared to mild malaria (33) suggest that SM is caused by parasites expressing a relatively conserved subset of PfEMP1 variants associated with high

pathogenicity, low host immunity as well as syndrome-specific disease signatures (34-36). Some studies have shown a possible link between clinical prognosis, endothelial dysfunction and sequestration by demonstrating the association between elevation of endothelial biomarkers such as Ang-2 or s-ICAM1 with death, CM and malarial retinopathy [10]. However, the relationship between parasite PfEMP1 expression, endothelial activation, and SM seems to be formed by multiple and heterogeneous pathogenic mechanisms which are far from being completely understood (36, 37).

Endothelial dysfunction, together with inflammation and RBCs sequestration, is a key component of the pathological triumvirate which leads to SM (11). Different markers of endothelial activation have been related to disease severity such as the Angiopoietin (Ang)-Tie axis (see figure 4) (38). Tie-2 is the receptor of both Ang-1 and Ang-2. The union of Ang-1 to Tie-2 promotes endothelial stability and vascular quiescence and, moreover, have anti-inflammatory and anti-apoptotic effects (38). However, Ang-2 antagonizes these actions and, when released from the endothelial cells, triggers a pro-inflammatory and pro-coagulant state. Different studies have shown that dysregulation of the Ang-Tie2 axis with low levels of Ang-1 and high levels of Ang-2 and sTie-2 can differentiate between uncomplicated and SM and are associated with poor disease outcomes (37, 39-45).

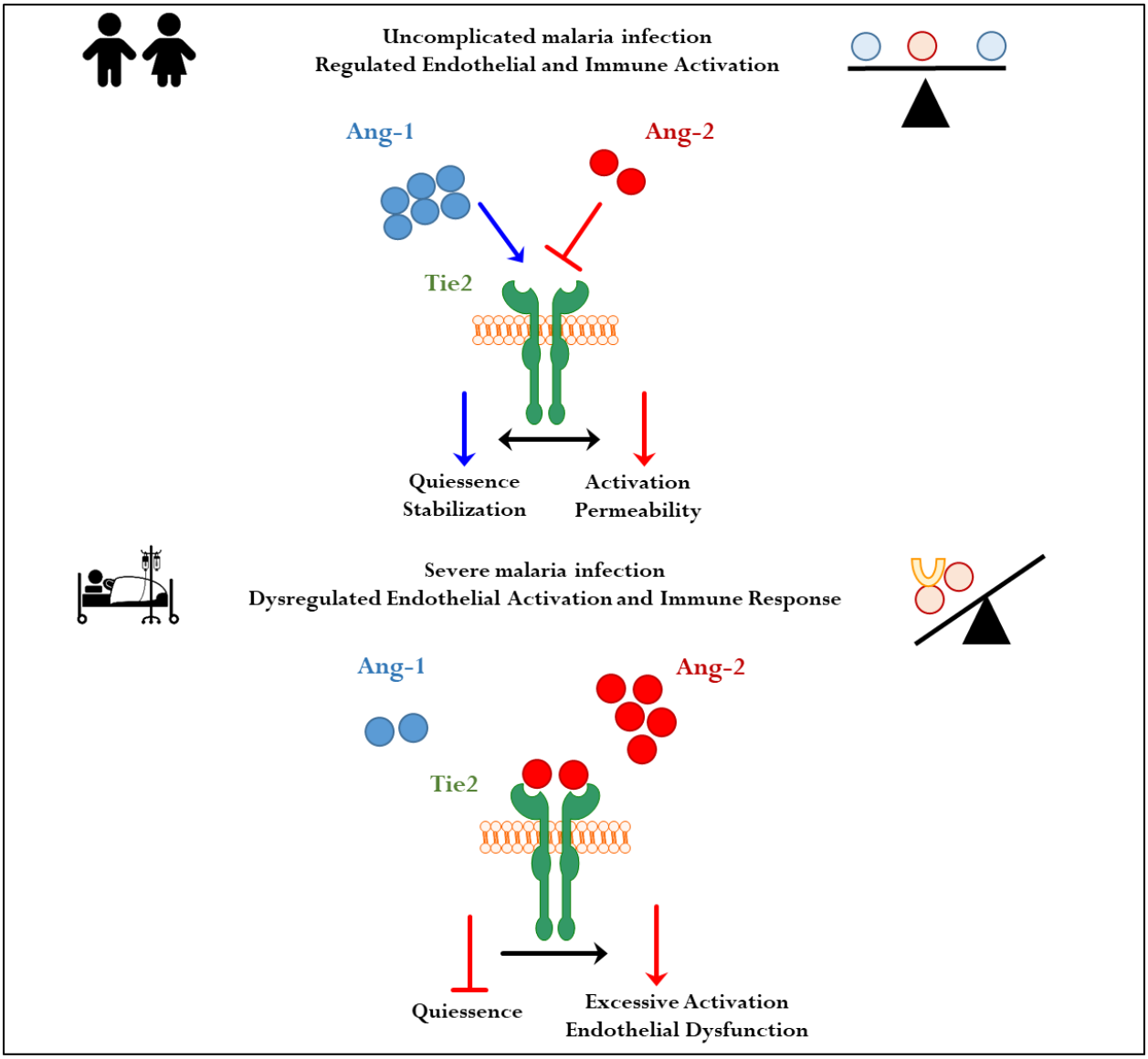


Figure 4. Role of the Ang/Tie axis plays in regulation of endothelial activation and immune response in severe malaria infection. During normal physiology or self-resolving infections, the endothelium shifts between states of quiescence (i.e stabilization of the endothelium) and activation (i.e permeable endothelium) to adapt and accommodate pathogen sequestration and elimination as well as vascular remodeling. Angiopoietin-1 activates Tie2, which promotes endothelium stabilization, while angiopoietin-2 inhibits these events, thereby promoting activation.

SM, including SMA and CM, has been also co-related with a deregulated pro-inflammatory state (41, 42, 46-48). Interleukin (IL-6) and Interleukin (IL-8) are both elevated in children with SM (47-49). Higher levels of soluble triggering receptor expressed on myeloid cells 1

(sTREM-1) have been observed in children with SM when compare with uncomplicated malaria (50) and those elevated levels correlated well with poor prognosis (42, 51). 10 kDa interferon γ-induced protein (IP-10) is associated with CM and can discriminate well those children with prolonged clinical recovery times and higher mortality (42, 51). The soluble FMS-like tyrosine kinase-1 (sFt-1) contributes to malaria disease by an unknown mechanism but high levels of this biomarker have been associated to SM (42, 51). The soluble tumor necrosis factor receptor 1 (sTNFR-1) is an apoptotic factor whose elevated levels in CSF have been associated with CM in children (52). Brain-derived neurotrophic factor (BDNF) is the most important and more abundant neurotrophic factor in the central nervous system (CNS) and low circulating levels have been associated with disease severity and poor clinical outcomes in children with SM (53). Renal impairment is an independent predictor of poor outcome in children with SM (54) and, in children, a more frequent event that previously thought (55, 56). Levels of Cystatin C (Cys C), a biomarker of kidney functional status, has been recently associated to SM and increased mortality (56). Acidosis is a defining criteria of SM and hyperlactataemia is a well-known and widely studied parameter related to disease severity and poor prognosis in SM (57-68).

High parasite biomass is thought to trigger the pathological interaction between endothelial dysfunction, inflammation and RBCs sequestration leading to SM (11). HRP2 translates well the total body parasite biomass in acute falciparum malaria (69, 70). Higher plasmatic concentrations of Histidine rich protein-2 (HRP-2) have been described in patients with SM in comparison with UM and have been associated with specific severity syndromes, disease progression, and mortality (71-78). A meta-analysis has confirmed the powerful relationship between parasite burden and severity and, moreover, has revealed that, in combination with *var* disease signatures, it would appear to be an excellent predictor of SM in both children and adults (36).

1.5 Diagnosis of malaria

Malaria clinical manifestations are rather non-specific, and it is difficult to distinguish from other illnesses on the sole basis of a clinical approach. Current guidelines recommend parasitological confirmation of all suspected malaria cases before starting early, specific and appropriate treatment. Thick and blood films are still the gold standard for malaria diagnosis. Thick films proved sensitivity and thin films allow differentiation of species and quantification of malaria parasites. However, rapid diagnostic tests (RDTs) are now the most widely available option as they provide simple, sensitive and specific diagnosis based on the detection of *P. falciparum* proteins (typically histidine-rich protein 2 (HRP-2)), pan-malaria or species-specific lactate dehydrogenase (LDH), or aldolase antigens in blood samples, which can be obtained easily through finger-prick (16). They represent an affordable, cost-effective and easy-to-use technology with minimal training required which make RDTs a valuable option to improve the management of malaria cases, especially in areas with limited laboratory resources. They are also a valuable option in epidemic investigations and surveys. Nucleic acid amplification-based tests can detect low density malaria infections but their use is restricted to epidemiological research and surveys' mapping and they do not have a practical role in the clinical management of malaria (for a more comprehensive summary of the current state of malaria diagnostic tools, please see **Annex 2**).

1.6. Clinical features of malaria

There are five species which cause malaria infections in humans, being *P. falciparum* and *P. vivax* (and occasionally *P. Knowlesi*) the ones associated to more severe symptoms. *P. falciparum* is the main responsible for the vast majority of severe cases and deaths. However, there is now increasing evidence that *P. Vivax* can also cause severe manifestations such as severe anaemia and pulmonary oedema, coma or hypoglycaemia (16, 79, 80), or even death (81). Thus, *P.vivax* malaria can no longer be considered a purely "benign" malaria, although there is

a need of more epidemiological studies to better define the real impact of its potential deleterious consequences (16).

The incubation period varies depending on the species, ranging between 12 and 30 days. *P. falciparum* has typically the shortest one. The classical malaria episode usually starts with the known as cold stage, characterized by a strong feeling of coldness with shivers, lasting around 15-60 minutes. This phase is followed by the hot stage, presenting a rapid increase in body temperature that can easily reach 40º C and may last for 2-6 hours. It can also be associated with flushed, dry skin, and often headache, nausea, and vomiting. Next phase is called the sweating stage where the fever drops rapidly, and the patient presents with drowsiness, weakness and profuse sweating during 2-4 hours. Traditionally, malaria has been defined in relation to these paroxysms which can last for 9 to 10 hours. They usually start during the day and leave the patient in a relatively acceptable state between crises. The periodicity of the paroxysms coincides with the parasite's intraerythrocytic cycles and the synchronization of schizont rupture.

In *P. knowlesi* infection, with a cycle of 24 hours, fever can manifest every day, termed as quotidian fever. *P. vivax* and *P. ovale* with 48 hours cycles have the paroxysms every third day (tertian malaria). *P. malariae* is the cause of quartan malaria with fever reappearing intermittently every fourth day (every 72h). *P. falciparum* infection can be expressed as tertian or subtertian or quotidian fever, depending on the synchronization and size of the parasite bulk. This nomenclature is nowadays outdated and not used in clinical practice. The malaria paroxysms may recur for a few cycles, with no further complications (uncomplicated malaria) or alternatively, and less frequently, progress to a more severe form of the disease.

1.6.1 Uncomplicated malaria

An uncomplicated malaria case may be defined as a patient with a clinical diagnosis of malaria with a *Plasmodium* asexual parasitaemia > 0 parasites/μL and not fulfilling the criteria for SM. Some studies have looked at the malaria attributable fraction, and they propose alternative

parasitaemia thresholds (for example 2500 parasites/microliters in children beyond one year of age). The huge percentage of infections by malaria parasites are considered to be benign or mild and only a small proportion of *P. falciparum* infections will lead to severe manifestations. The first symptoms of the disease are non-specific including general malaise, fatigue, arthromyalgias, headache, abdominal discomfort, nausea, vomiting or orthostatic hypotension which are followed by irregular fever. In endemic areas malaria is the most common cause of fever. In children, respiratory symptoms are also frequent and the infection can be misdiagnosed. In areas of stable transmission, young children with recurrent infections can present with an enlarged spleen and chronic anaemia. Most patients with uncomplicated malaria only have few abnormal physical findings. A wide range of haematological alterations have been reported in relation to malaria infections which may help to distinguish between uncomplicated and SM. Apart from well-characterized anaemia and thrombocytopenia, different leukocyte alteration patterns have been reported including normal white blood cell count (WBCC), leukopenia and leucocytosis. In the presence of important abnormalities in these parameters, which are normally determined by automated haematology analysers, it may be recommended to additionally obtain a peripheral blood smear, and whenever possible, conduct a bone marrow examination. The microscopic observation of the peripheral blood smear may not only quantify the real magnitude of the WBBC elevation but can also detect qualitative abnormalities in all haematological series and suspect an associated co-infection (for an example, see an interesting case report, in **Annex 3**). Electrolyte imbalances like hyponatremia, hypo or hyperpotassaemia and more often mild or moderately transaminasemias may also be present.

Relapse is defined by the WHO as the '"recurrence of asexual parasitaemia in *P. vivax* or *P. ovale* infections arising from hepatic hypnozoites" and occurs when treatment does not eliminate them allowing them to persist in the liver. These relapses are usually frequent during the first year, when the hepatic *hypnozoites* reactivate and liberate *merozoites* into the bloodstream. *P. malariae* infection is clinically similar to the others but less aggressive and

tends to express in a more chronic way, and can lead to renal problems such as nephrotic syndrome. Although this species has not a hypnozoitic stage infection can reactivate years after the original infection.

1.6.2 Severe malaria

It is estimated that ≤ 1% of children with malaria will progress to SM. For research and epidemiological purposes, SM can be defined as the confirmation of a malarial infection in the presence of one or more of a series of syndromes or conditions, including impaired consciousness, acidosis, hyperlactataemia, hypoglycaemia, severe anaemia, acute kidney injury, jaundice, pulmonary oedema, significant bleeding, hyperparasitaemia, or shock (Table 1) (12).

Table 1: Clinical defining features of severe malaria (12)

Impaired consciousness	A Glasgow Coma Score <11 in adults or a Blantyre coma score <3 in children
Acidosis	A base deficit of >8 meq/l or, if unavailable, a plasma bicarbonate of <15 mM or venous plasma lactate>5 mM. Severe acidosis manifests clinically as respiratory distress – rapid, deep and laboured breathing
Hypoglycaemia:	Hypoglycaemia: Blood or plasma glucose <2.2 mM (<40 mg/dl)
Severe malarial anaemia:	Severe malarial anaemia: A haemoglobin concentration <5 g/ dL or a haematocrit of <15% in children <12 years of age (<7 g/dL and <20%, respectively, in adults) together with a parasite count >10 000/µl
Renal impairment (acute kidney injury):	Plasma or serum creatinine >265 µM (3 mg/dL) or blood urea >20 mM
Jaundice:	Plasma or serum bilirubin >50 lM (3 mg/dL) together with a parasite count >100 000/µl
Pulmonary oedema	Radiologically confirmed, or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose gums or venepuncture sites; haematemesis or melaena
Shock	Compensated shock is defined as capillary refill ≥3 s or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Hyperparasitaemia:	P. falciparum parasitaemia >10%

SM is a complex multi-system disease that may be differently defined according to the age group it affects, as clinical manifestations may vary between adults and children. As example, jaundice and pulmonary oedema is more frequent in adults and seizures and hypoglycaemia in children (12). However, it should be noted that the differences between those age groups might not only be due to disparities in pathology but also to the under-recognition of complications in young children with SM, as is the case for acute kidney injury (56). Higher parasitaemias are associated with poorer prognosis, although, low parasitaemias may also manifest as SM. As the peripheral parasitaemia may not reliably translate the total parasite biomass, new tools to measure the real burden are required.

It is also important to highlight that there is a biological link between malaria and susceptibility to invasive bacterial infection. It has been estimated that, in Africa, around 6.4% of cases of SM can have a concomitant invasive bacterial co-infection, being the most prevalent pathogens *Non-typhoidal Salmonella species, S. pneumoniae, E. coli, S. aureus, Group A streptococci* and in infants*, Group B streptococci* (82) (to consult an illustrative case, please see **Annex 3**). Patients with SM should be started on arrival on antibiotherapy although their effect on mortality and/or clinical outcomes have not been tested in any randomized controlled trial (RCT).

Malaria complications develop very fast in children and may lead to death only a few hours after the first symptoms. In fact, more than 75% of fatal events in Africa occur during the first 48 hours after onset of disease and in the first 24 hours of hospital admission (83). Different clinical scoring systems have been developed to risk stratify and predict mortality in patients affected by infectious diseases in low-resource settings (84). Among then, the Lambaréné Organ Dysfunction Score (LODS), which combines coma, prostration, and deep breathing, has demonstrated to be highly sensitive and specific to discriminate between survivors and non-survivors of SM (84, 85). Furthermore, models adding specific biomarkers such as sFlt-1 and Ang-2 to LODS have demonstrated an improved predictive power (51). This highlights the potential of new approaches, combining clinical manifestations and biomarker evaluation, to generate simple and useful diagnostic and prognostic tools.

Most children with SM can be identified by a combination of just three overlapping syndromes with differ in biological, clinical and epidemiological characteristics: Cerebral malaria (CM), severe anaemia and acidosis/hyperlactatemia (clinically manifested as respiratory distress).

1.6.2.1 Cerebral malaria

CM, perhaps the most feared complication of malaria, is characterized by severe impairment of consciousness (deep coma) in the absence of other alternative explanations or diagnoses in a patient with confirmed malaria parasitaemia. Impaired consciousness is defined by a Glasgow coma score of less than 11 of 15 in adults and children > 5 years (deep coma being ≤8), or a Blantyre coma score of less than 5 (deep coma being ≤2) in children who are too young to speak (12). Impaired consciousness, together with severe respiratory distress, has one of the highest mortality rates of the severe complications with CFRs, both for adults and children, approaching 20% (86, 87). Beyond impaired consciousness, CM can also present with repeated seizures or other neurological abnormalities. CM is an exclusion diagnosis, thus requiring exclusion of other common causes of coma, such as meningitis, hypoglycaemia, or a transient post-ictal state. Indeed, CM can only be confirmed in the presence of coma which persists longer than one hour after a seizure, irrespective of anticonvulsant medications (12). An accurate diagnosis of CM is challenging, important in terms of patient management and epidemiological surveillance, but also, to enrol patients in pathogenesis and/or therapeutic studies specifically targeting CM cases. Visualizing any of the changes typical of what is known as malarial retinopathy (patchy retinal whitening, focal changes of vessel colour, white-centred retinal haemorrhages or papilledema) can contribute to the *in vivo* confirmation of CM (88). Unfortunately, although the diagnosis of those retinal changes is relatively straight forward, the lack of specialized ophthalmology resources in low-income countries hampers such diagnosis. Therefore, it appears that there is a need for a low cost, easy to use, retinal-screening device that could be straightforwardly used by non-specialist clinicians and routinely implemented in the assessment of comatose patients, when CM is a possible diagnosis. Clinical

manifestation of CM, as other SM syndromes, may substantially differ between adults and children (Table 2) depending on the degree of neurological involvement which may vary from specific brainstem anomalies to a diffuse cortical encephalopathy.

On arrival or during their admissions, patients may develop abnormal respiratory pattern, tone abnormalities, decerebrate or decorticate posturing, opisthotonos (89), or cranial nerve abnormalities (90). Impaired consciousness may be transient or long-lasting, and patients with a good evolution regain full consciousness during the first 2-3 days. Generalized seizures are common in children being referred or directly observed in up to 60-80% of African children. Convulsions may also be generalised or focal, single or recurrent, and at any level of body temperature. There is a higher risk of neurological sequelae or death in those children affected by prolonged or refractory convulsions, or in those patients who experiment seizures after the start of antimalarials (68, 91). When seizures appear, it is necessary to perform a lumbar puncture for ruling out bacterial meningitis whenever the clinical situation of the patient allows. In malaria cases, cerebrospinal fluid (CSF) is normally acellular and sterile, with a slight increase in proteins and elevated opening pressure (92).

CM is also associated with long-term cognitive and neurological deficits in up to one-third of survivors, including hemiparesis, cerebellar ataxia, cortical blindness, hypotonia, spasticity, aphasia, seizure disorders, behavioural disorders and attention-deficit hyperactivity disorder (ADHD) (93-101). The devastating short-term effects that an acute malarial infection can have on any given individual have been historically well characterized, and there are also abundant data on the subacute and chronic sequelae derived from severe malarial episodes, which are understandable in the context of the sudden and profound insult that such an aggressive infection may have in the central nervous system and other key organs. However, much scarcer information exists regarding other more subtle or prolonged deleterious effects of malarial infection and disease on well-being, beyond its acute phase, particularly in regard to common mental disorders and neuro-psychiatric health which may have an important

individual and public health impact (for a more comprehensive view on this, please see **Annex 4**)

Table 2 : Differential characteristics from cerebral malaria in children and adults (adapted from (86))

Clinical characteristics	Children	Adults
Neurological signs and symptoms		
Convulsions	Referred history present in up to 80% of cases, and directly observed during admission in up to 60%. Recurrent convulsions include focal motor (>50%), generalized tonic-clonic (34%), partial with secondary generalization (14%), subtle, or purely electroencephalographic (15%). *Status epilepticus* is frequent (68, 91)	Present in up to 20% of patients, mainly generalized tonic-clonic. *Status epilepticus* is rare (102, 103)
Neurological abnormalities	Prostration. Brainstem changes in >30%, often associated with intracranial hypertension(104). Malaria retinopathy present in >60%(105). Cerebral oedema visible in CT scan in up to 40% (106)	Typically, symmetric upper motor neuron signs. Brainstem abnormalities or malaria retinopathy rare(102, 107)
Coma	Appears rapidly, often following a convulsion(68)	Develops more gradually, often after an insidious 2-3 day-long phase of drowsiness, confusion and/or agitation. May be triggered by a tonic-clonic convulsion(102)
Evolution		
Conscience recovery	Fast, between 24-48 hours(90, 108)	More slowly, >48 hours(109)
Mortality	20-75% of all deaths within first 24 hours of admission(90, 110). Many cases never reach the hospital	20-50% of all deaths within first 24 hours
Neurological sequelae	Frequent (10-50%)(87). Most common include: ataxia (2.5%), hemiparesis (4.4%), tetraparesis (3.5%), deafness (1.9%), cortical blindness (2.3%) and aphasia (2.1%). Epilepsy (111, 112). Neurocognitive abnormalities (113, 114)	Infrequent (<5%). Isolated cranial nerve abnormalities, multiple mononeuritis, polyneuropathies, extrapyramidal tremors and other cerebellar signs(102)

1.6.2.2 Severe anaemia

Severe malarial anaemia is the number one cause of mortality due to malaria, globally (12). While its associated CFR is low if blood derivatives are available for transfusion, its very high incidence translates into the highest number of malaria-associated deaths. Although SM is mainly caused by *P. falciparum*, severe anaemia may present in all types of malaria infections (115). Severe anaemia is considered when haemoglobin is <5 g/dl or a haematocrit is <15% in children and a haemoglobin <7 g/dl or a haematocrit of <20% in adults (12). Anaemia develops very quickly in SM reaching a nadir around one week after admission and with a slow recovery in the next weeks. Such anaemia is the result of a double etiological mechanism involving destruction of infected and uninfected erythrocytes and bone marrow dyserythropoiesis, ultimately leading to tissue ischaemia and hypoxia. Prevalence of anaemia in malarial endemic areas is very high, particularly in children, and has a multifactorial aetiology (116). Malaria associated anaemia decreases with age and increases with exposure (11) and is associated with clinical findings as hyperdynamic circulation, respiratory distress and pallor. Mortality raises sharply when concentrations of haemoglobin falls below 3 g/dl, although it may also be high with higher haemoglobin concentrations approaching the normal range, particularly in the presence of other concomitant complications (115). In high malaria transmission areas, anaemia is a major cause of admission and can present in up to 17.3% of children admitted with SM with a CFR of 5.7 % (117).

The management of SM is based on blood-transfusions and iron supplementation although it is necessary to establish a clear evidence in the best use of both therapeutical approaches (12, 118, 119). The effect of antimalarial treatment in malarial anaemia and haemoglobin recovery dynamics needs also to be fully understood (115) (figure 3). The burden of anaemia in endemic settings might be reduced through proactive prevention activities, including vector control, deployment of insecticide-treated bed nets, prompt and accurate diagnosis of illness and appropriate use of effective antimalarials in strategies such as intermittent preventive treatment (IPT) or seasonal malaria chemoprevention (115).

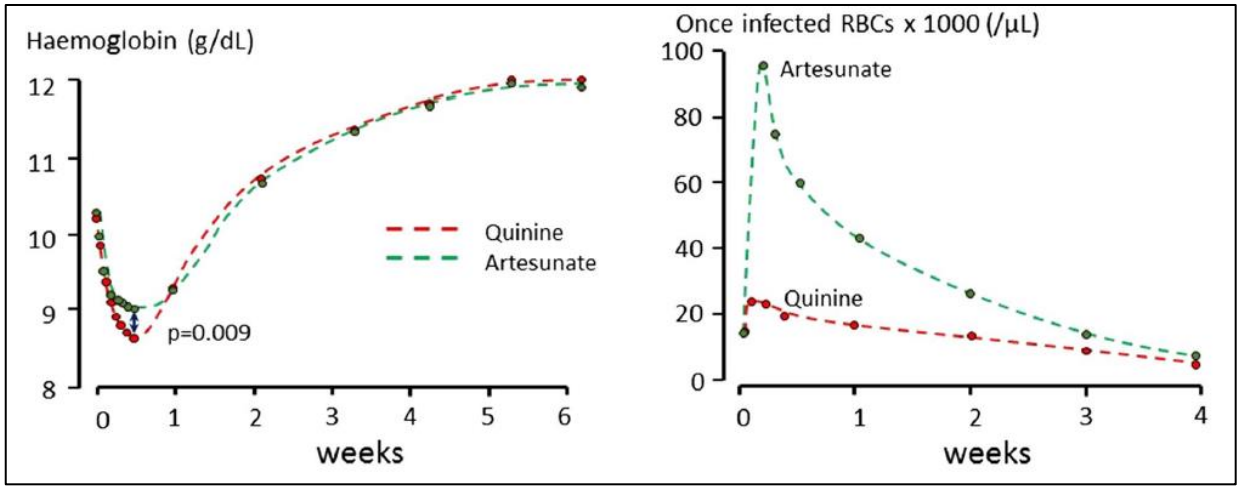


Figure 5: Reduction in haemoglobin concentrations and corresponding increases in pitted erythrocytes in relation to anti-malarial drug treatment (artesunate or quinine) in African children (From Fanello et al, 2017)

1.6.2.3 Respiratory distress

Respiratory distress is a common manifestation of SM which develops in up to 25% of adults and 40% of children with severe falciparum malaria (120). It usually presents with deep (acidotic) and laboured breathing, tachypnoea, low chest indrawing and sustained nasal flaring. Acidotic breathing is a sign of poor prognosis and is caused by the accumulation of plasmatic lactate (66), and the decrease of bicarbonate levels (121) due to the sequestration of parasites and obstruction of microcirculation. The mortality of this syndrome is high and can reach 15%, being the best independent prognostic marker for a fatal outcome when it presents with other syndromes as CM or severe anaemia (67, 122). Contrary to these two syndromes, there is no relationship between age and exposure and respiratory distress (11). Patients with respiratory distress pose a diagnostic challenge in low-resource settings where there is a generalized scarcity of technical and specialist resources. Most of the patients with SM and respiratory distress present normal oxyhaemoglobin concentrations and non-pathological chest X-ray, translating the metabolic (rather than pulmonary) origin of the syndrome. However, some of the episodes may coexist with superimposed infections, with both infections contributing synergistically to respiratory distress. The real prevalence of this

overlap is not well known, so it is important to develop new strategies which may help to improve its management, as it could be the case of lung ultrasound, a cheap, easy-to-use and accurate diagnostic tool which may help to distinguish between malaria and other life-threating diseases as bacterial pneumonia or sepsis (123, 124). Critical care support is increasingly available in these settings but far from being widely implemented. Mechanical ventilation could help to dramatically improve the prognosis of patients with respiratory distress (120).

1.7. Case management

1.7.1 History of malaria treatment

Malaria infection has played a unique role in the history of humans. It is thought that *Plasmodium* parasites have infected humans for more than 50000 years with devastating effects, making malaria one of the ancient scourges of humankind. Only in the 20th century malaria has been blamed for causing between 150 million and 300 million deaths (2 to 5 percent of all casualties) (125).

Reports from China from ~2000 years ago describe the use of herbal remedies based on the plant *Artemisia Annua* to treat intermittent fevers. Beyond this, the first effective treatment for malaria was obtained four centuries ago from the bark of the cinchona tree, growing in Peru, on the slopes of the Andes Mountains. Cinchona bark contains quinine and was subsequently introduced into Europe as the treatment of "the ague" and intermittent fevers. However, the quinine alkaloid was only isolated nearly two centuries after the widespread use of the cinchona bark. For many centuries, quinine was the sole available treatment for malaria.

New antimalarial drugs were developed during the 20th century under the umbrella of the military history. During the First World War, many advances were made to find additional treatments to the scarce antimalarial therapeutic arsenal such as, for example, quinacrine. Following the synthesis of this and other several compounds, a big breakthrough came in 1934, when chloroquine, a new class of antimalarial of the 4-aminoquinoline family, was

synthetized for the first time in Germany. At the beginning of the Second World War was recognized as a powerful antimalarial and by the end of the war, was considered the first line therapy for malaria treatment. Considering its safety, high efficacy and low price, chloroquine became during decades the cornerstone of malaria treatment. The rapid appearance of chloroquine resistant parasites drove the development of other antimalarial compounds like proguanil (1946), amodiaquine and primaquine (1950's), the antifolate sulfadoxine-pyrimethamine (SP) (1967), halofantrine or mefloquine (developed by the United States army during the Vietnam War). In the late seventies, piperaquine replaced chloroquine as the first-line treatment in China but its extensive use as monotherapy hampered its, while lumefantrine was introduced during the eighties. During the first decade of the 21st century only three drugs have been approved for their use as antimalarials (126) out of the 850 new therapeutic products registered in that period, which reflect the little interest of pharmaceutical companies in this disease. However, in the light of the appearance of artemisinin resistance there has been a surge in the search of new antimalarials and, at least, 13 drugs are currently in clinical development (127) (to see an example of research in new compounds in which the candidate has participated, please see **Annex 5**). Furthermore, this situation has driven a change in the current perspective on combination therapies and new alternatives with three or more existing drugs are now under evaluation.

Artemisia annua has been a component of Chinese herbal medicine for more than two thousand years and the infusions prepared from wormwood have been widely used for the treatment of fevers during that time. As part of the secret 523 Project lead by Professor Youyou Tu, artemisinin was isolated in China from the *Artemisia annua* (128) plant and now, this new class of antimalarial drug is the first choice to treat uncomplicated and SM. The discovery of this compound has changed the paradigm of malaria treatment shifting from quinolone-based to artemisinin-based therapies and opening a new direction in the development of antimalarial drugs.

1.7.2 Management of uncomplicated malaria

Malaria management is based on prompt diagnosis and treatment and its main objective is to avoid the fateful consequences this infection can provoke (see **Annex 1**). Currently, this management is mainly based in two pillars: a) the use of the most widely accessible diagnostic tools as microscopy and RDTs (see **Annex 2**); b) the use of rapid and effective antimalarial drugs such as the artemisinin derivatives (to have a global view of the current available antimalarial pharmacopeia, please see **Annex 6**).

One of the major problems related to the treatment of uncomplicated malaria is the limited availability of paediatric friendly formulations which may hinder the prognosis of the disease in the most vulnerable population. Consequently, it is necessary to develop new effective drugs and make them available in a child-friendly formulation (to see an example of research in these compounds, please see **Annex 7**). Another important challenge to bear in mind is related with the risk of drug resistance against malaria therapy.

1.7.3 Antimalarial Drug resistance

Emergence of antimalarial drug resistance threatens effective antimalarial drug treatment, malaria control, and elimination and it has been reported for all antimalarials. Antimalarial drug resistance in *P. falciparum* has traditionally emerged in low-transmission settings, particularly in SEA and South America, and then, has expanded to high-transmission settings in Africa (129, 130). This is the route that chloroquine and SP resistance followed (Figure 4) and it is expected that artemisinin resistance will follow a similar route (131, 132). Of the five human malaria species, *P. falciparum* and *P. vivax* have developed resistance to antimalarial drugs. There is no conclusive evidence about chloroquine resistance in *P. malariae* and its existence is still under debate (133).

Artemisinin-based combination therapies (ACTs) seem to be less effective in SEA, particularly in the Greater Mekong Sub-region (GMS), where partial artemisinin resistance (PAR) and piperaquine resistance have been reported (131, 134-145). PAR is characterized by a delayed

clearance of parasitemia during the first three days of treatment with artemisinin monotherapy or ACT (131).

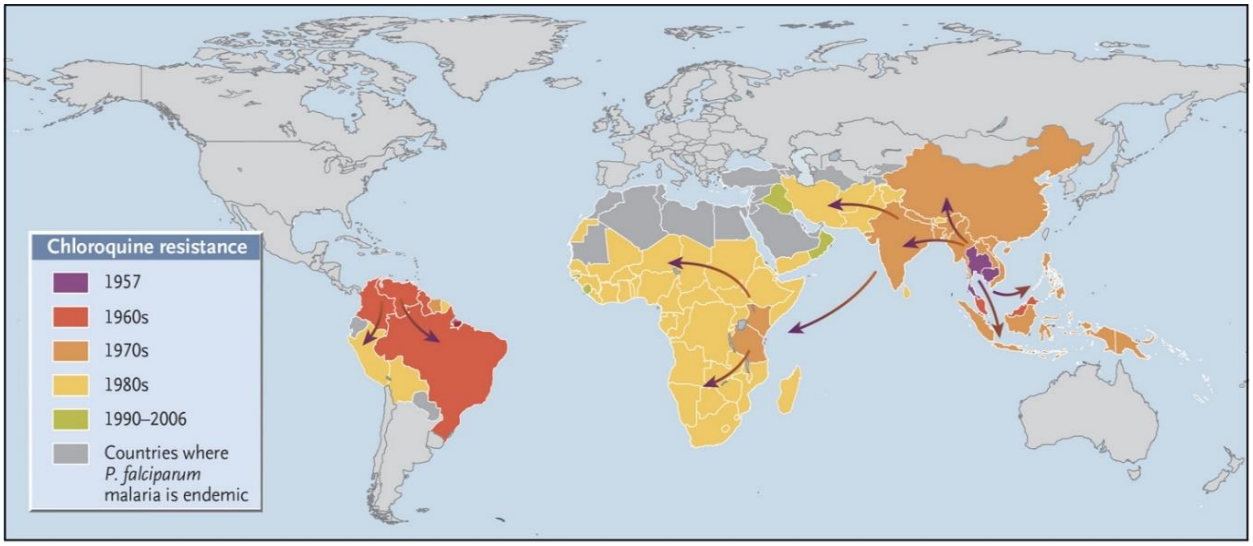


Figure 6: History of Chloroquine-Resistant *P. falciparum* Malaria (Source: WWARN)

The containment and the elimination of these multidrug resistant parasites in SEA have become a priority for the WHO to avoid their spread to Africa as was the case with previous generations of antimalarial drugs (e.g. chloroquine, SP) (146). Resistance to other antimalarial drugs such as SP, mefloquine and primaquine (more debatable) have also been described (131). While sporadic case reports of PAR have been described in SSA, there is no evidence that such a phenotype has reached this region (147).

De novo appearance of resistance needs the spontaneous emergence of mutations or gene duplications which may arise in the sexual stages in the mosquito, in the preerythrocytic liver stages or in the erythrocytic stages. Drugs with extended half-life intervals and prolonged time of subtherapeutic blood levels (such as piperaquine or SP) or those requiring a limited number of genetic errors (such as atovaquone, mefloquine or SP) are more prone to trigger resistance (131). Others factors such as incorrect public policies (for instance the extensive use in China of piperaquine, as a monotherapy, in mass treatment and mass prophylactic campaigns; or the addition of pyrimethamine and chloroquine for salt consumption), unregulated drug market,

counterfeited or substandard drugs or different pharmacokinetic properties in certain population′s subgroups may also facilitate the emergence of drug resistance. Host immunity and mosquito, parasite and host genetics are also factors to consider in the development of antimalarials drug resistance (131).

To strength the surveillance and research on new antimalarials it is important to catalogue their use across the world, monitor their probable loss of efficacy, and measure the frequency of molecular markers associated with resistance. In particular, mutations in the propeller domain of a *Kelch* gene located on the chromosome 13 (*Kelch13*), and amplification of a cluster of genes encoding both *Plasmepsin 2* (*Pfpm2*) and *Plasmepsin 3* proteins, have been recently shown to be associated with artemisinin and piperaquine resistance, respectively (148-150).

1.7.4 Primary treatment of severe and cerebral malaria

P. falciparum is responsible for the majority of malaria associated morbidity and mortality. In the absence of prompt and effective treatment, *P. falciparum* infection may progress to severe and potentially fatal forms. Parenteral artesunate is now widely accepted as the standard of care for the treatment of SM, both in adults and children, following the landmark SEAQUAMAT and AQUAMAT trials that demonstrated its superiority over quinine (151, 152). Recently, intramuscular artesunate administration has proven to be non-inferior to intravenous artesunate in reducing parasitemia ≥99% at 24 hours in children with SM (153). Therefore, treatment with potent artemisinin-derivatives alone is insufficient to prevent death or neurological disability in all patients with SM. Parenteral treatment must be switched to oral as soon as the patient is able to swallow the medication (see table in **Annex 1**). Additionally, there is a rectal form of artesunate which is indicated as a pre-referral option for children under six years of age living in remote areas waiting for immediate transfer to a higher-level (154).

Malaria complications may develop very fast and may lead to death only a few hours after the first symptoms appear. Patients with SM may be adequately monitored with frequent

measures of vital signs and haematological and biochemical parameters such as glycaemia, haemoglobin or renal and hepatic function. Whenever feasible, monitoring parasite density is desirable until confirming parasite clearance (12) (to see more about treatment of SM, see **Annex 1**)

1.7.5 Adverse side effects and anaemia in severe malaria treatment

In terms of efficacy, there is no doubt that artesunate is superior to quinine, and moreover, it seems that it has a better short-term safety profile than quinine (155). However, there are less data about longer-term complications or side effects of this drug. In real-life conditions, those complications after discharge may be undetected under routine health assistance, considering the fragile health infrastructure in most malaria endemic areas (156).

It was not until the introduction of artesunate as first-line therapy for SM that some reports, mainly among returning travellers from non-endemic countries, raised awareness about post-artesunate delayed haemolysis (PDAH) as a potential side effect that seemed to have been previously overlooked. This haemolytic event typically appeared 2-4 weeks after treatment in around 15-30% patients, and in most severely ill patients usually required blood transfusions (157-165). The pathophysiology of this process has not been fully understood but splenic clearance of erythrocytes by pitting or drug-induced autoimmune mechanisms may be involved in this phenomenon (166, 167). However, data regarding this complication cannot be directly generalized to endemic countries where vulnerable age groups, patient characteristics, clinical manifestations, or quality of care among other things may importantly differ (168). Contrary to the evidence from non-endemic countries, some studies performed in SSA have not reported PADH as a relevant complication, but data are still insufficient to understand its main determinants (169).

1.7.6 The role of adjunctive therapy in severe malaria treatment

As we have explained before, the host immune response plays a central role in the onset, severity and outcome of malaria infections and this has promoted the search for adjunctive therapies based on the available pathophysiological knowledge to improve clinical prognosis. Adjunctive therapies are used in combination with primary antimalarial treatment, with the aim of improving efficacy, or reducing disease-associated complications. To date, several putative adjunctive therapies have been tested in SM, albeit without success. An effective adjunctive therapy must be safe, have a clear benefit over antimalarial use alone, be effective as a late-stage intervention, be minimally invasive, inexpensive and ideally feasible to implement in low-resource endemic settings, where the bulk of SM occurs. The objective of adjunctive therapy should be the improvement of clinical outcomes, and/or reduction of mortality, in addition -if possible- to the prevention of long-term neurocognitive deficits. Adjunctive therapies based on modulating host response to infection, could reduce malaria-associated morbidity, mortality and could enhance and extend the clinical utility of current antimalarials.

1.7.7 PPAR-γ agonists and rosiglitazone

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a member of the family of nuclear hormone receptors that function as ligand-activated transcription factors via their heterodimerization with another nuclear receptor, retinoic X receptor (170-172). PPARγ agonists are promising candidates for adjunctive malaria treatment as they have been reported to have anti-inflammatory, anti-oxidant, and neuroprotective properties (172-177) (see figure 7). The PPARγ agonist rosiglitazone is in the thiazolidinedion class of drugs and is approved for the treatment of type II diabetes (178). Rosiglitazone acts by increasing insulin sensitivity rather than increasing insulin levels, does not induce hypoglycaemia, and has an established safety profile in adults (178-182).

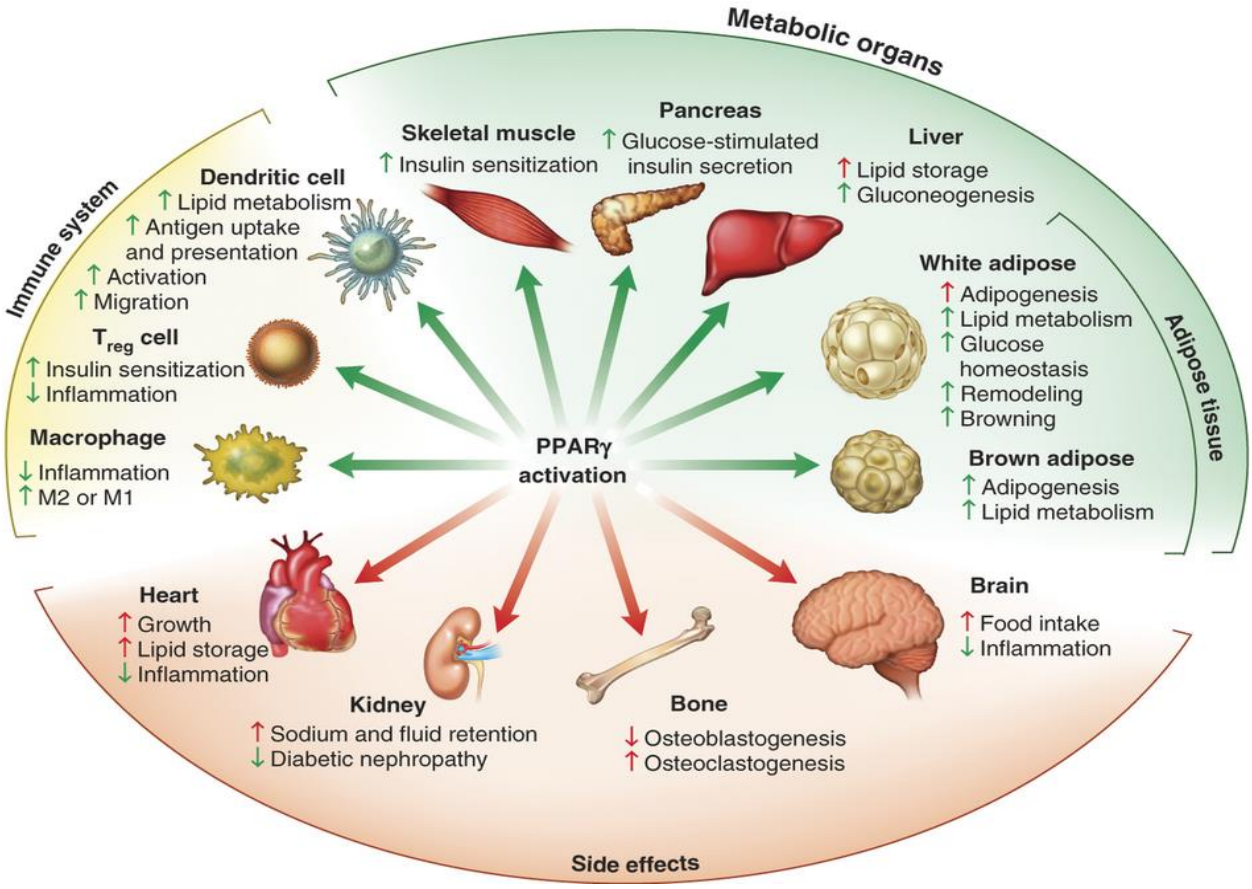


Figure 7: Known effects of PPARγ activation. Activation of PPARγ results in beneficial effects (green arrows) as well as adverse side effects (red arrows) (183).

Rosiglitazone has been shown to enhance macrophage phagocytosis of *P. falciparum* parasitized erythrocytes, and to reduce parasite-induced pro-inflammatory cytokine secretion from monocytes and macrophages *in vitro* (184). In a pre-clinical *in vivo* model of experimental cerebral malaria (ECM), rosiglitazone improved survival over artesunate alone, enhanced parasite clearance, reduced systemic inflammation and endothelial activation, prevented vascular leak, enhanced neuroprotective pathways, and protected mice from malaria-induced cognition and motor impairments (175, 185). Following these results, a RCT in adults demonstrated that rosiglitazone was safe and well tolerated and led to significantly improved parasite clearance times, lower levels of pro-inflammatory mediators and evidence of enhanced endothelial quiescence (186). Considering that rosiglitazone can target multiple

pathways implicated in the pathobiology of SM, and that there is significant evidence on its safety due to its widespread use for diabetes in adults, this drug appears as a promising candidate for an effective adjuvant therapy.

1.8. Malaria control and prevention

The World Health Assembly held in Mexico in 1955 witnessed the launch of the "Global Malaria Eradication Program" (GMEP) by the World Health Organization (WHO). With this campaign, which ended in 1969, malaria was successfully eliminated in areas like the south of the United States of America, the south of Europe, the North of Africa, the Middle East, some regions of East Asia and the north of Australia. These achievements were made possible through the combination of measures such as environmental actions, development of effective antimalarial drugs and use of powerful insecticides like dichloro-diphenyl-trichloroethane (DDT). Although this success confirmed that well-funded antimalarial interventions may have a big impact, the global resurgence of malaria after GMEP reflected the fragility of that success, which has been mainly related to the weakening of malaria control programs (187). In addition, other causes of that failure can be attributed to the intrinsic potential for malaria transmission and to vector or drug resistance.

It was not until the first decade of the 21st century when renewed vision, activities and efforts against malaria eradication appeared, as were the cases of the Global Malaria Forum led by Bill & Melinda Gates; and the Global Malaria Action Plan coordinated by The RBM Partnership to End Malaria. Nowadays, an important number of countries have moved towards elimination campaigns with significant results. However, the ambitious goals established for the next decades need sustained investment to maintain progress and support research activities for new diagnostic and treatment tools.

Although the world's malaria situation is currently at crossroads, the global health community can be congratulated for spectacular reductions in the global burden of malaria, particularly in the first 15 years of the millennium. In the last decades, a number of different successful

interventions implemented may have played an important role in improving the overall malaria situation: a) Measures to eliminate the mosquito vector as larvicides, insecticides (including Indoor residual spraying (IRS) with long-lasting insecticides), or environmental procedures; b) Measures to limit contact between humans and mosquitoes, with long-lasting insecticide treated bednets (LLINs); c) Improved and more efficacious treatments, and chemoprophylaxis strategies as intermittent preventive treatment, and seasonal malaria chemoprevention; d) The first effective malaria vaccine (currently being deployed in a pilot way in 3 African countries). For a deeper insight in these strategies, please consult **Annex 1** and **Annex 8**.

2. HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

The hypotheses of this thesis include:

1) The patterns of post-malarial anaemia and the need of blood transfusions in children with severe malaria treated with quinine or artesunate may be different.

2) The proportion of multidrug resistant parasites (i.e. *P. falciparum kelch13* mutants and gene copy number of both *Pfmdr1* and *Pfpm2*) surrogate markers of the risk of resistance to the main front-line antimalarials currently in use may vary according to geographical area.

3) Levels of biomarkers reflecting host inflammation and endothelial activation will be higher among severe malaria cases (as compared to uncomplicated malaria cases), and could allow for a good differentiation of sick patients upon arrival.

4) Rosiglitazone would be a safe and well-tolerated drug that would allow reduction in the expression of biomarkers of severity and have a positive effect on the prognosis and neurocognitive sequelae of severe malaria patients.

2.2 Objectives

General objective

The overarching goal of this PhD thesis is to explore and better characterize the mechanisms involved in the clinical expression of malaria and some of the determinants of its adverse outcome in a semi-rural hospital in Southern Mozambique. Additionally, it aims to identify diagnostic and therapeutic targets which may allow to improve the prognosis of severe malaria disease

Specific objectives

1) To provide a comprehensive literature review on the existing evidence related to adjunctive therapies for severe and cerebral malaria (**article 1**)

2) To investigate the prevalence of multidrug resistant parasites (*P. falciparum kelch13* mutants and carriers of copies of both *Pfmdr1* and *Pfpm2* genes) in different sites from Southeast Asia (Vietnam) and Africa (Benin, Burkina Faso, DRC, Gabon, Mozambique, Uganda) (**article 2**)

3) To evaluate the patterns of post-malarial anaemia in children with severe malaria in the first four weeks after hospital discharge, in order to better characterize the mid-term safety profile of intravenous quinine and artesunate (**article 3**):
   - To estimate the differences in terms of decrease of haematocrit in children treated with intravenous artesunate in comparison to intravenous quinine
   - To estimate the need of blood transfusions in children treated with intravenous artesunate in comparison to intravenous quinine.

4) To identify parasite and host molecules in plasma differentially expressed in children with severe and uncomplicated malaria (**article 4**):
   - To identify those molecules that would allow an adequate risk-stratification of malaria severity.
   - To describe the relationship between parasite and host biomarkers.

5) To determine the safety, tolerability, pharmacokinetics and pharmacodynamics of rosiglitazone (0.045mg/kg/dose) twice daily in children with uncomplicated malaria in addition to standard of care antimalarial treatment versus standard of care treatment plus placebo for four days (**article 5**)

6) To comment on solutions that could facilitate the choice and evaluation of adjuvant therapies for severe malaria in randomised controlled trials (**article 6**)

3. MATERIALS AND METHODS

3.1 Thesis Research Context

This thesis is based on the research work undertaken under the umbrella of the Barcelona Institute for Global Health (ISGlobal)/ Hospital Clinic-Universitat de Barcelona, in Spain. ISGlobal is a research centre, fruit of an innovative alliance between the "Caixa Foundation", academic institutions and government bodies, aiming to contribute to the efforts undertaken by the international community to address the most pressing challenges in global health.

All original research articles included in this thesis (four articles) are based on studies conducted at the *Centro de Investigacão em Saude de Manhiça* (CISM) in Mozambique, with whom ISGlobal has a longstanding partnership. CISM was created in 1996 as part of a collaborative programme between the Mozambican and Spanish governments through the "*Agencia Española de Cooperación Internacional para el desarrollo*" (AECID) and the Eduardo Mondlane University of Medicine (Maputo) and the Hospital Clínic of Barcelona, to promote and conduct biomedical research on those diseases with high morbidity and mortality among the local population.

Article 2 included in this thesis is part of a multicenter clinical trial named "Phase IIb Study to Investigate the Efficacy of Artefenomel (OZ439) & piperaquine (PQP) Co-administered to Adults & Children with Uncomplicated *P. falciparum* Malaria" funded and coordinated by Medicines for Malaria Venture (MMV).

3.2 Study area and research facilities

3.2.1 Manhiça and CISM

The District of Manhiça is a rural area located 90 km away from the capital Maputo in Mozambique (figure 8). CISM was created in 1996 with the objective of conducting biomedical research in those diseases that affect the most poor and vulnerable. Manhiça is the paradigm of a poor, resource-constrained rural SSA setting, with a population predominantly young (18% of which is less than 5 years of age) (188).

CISM has been running a Demographic Surveillance System (DSS) since the year 1996, covering at the moment the totality of the district's population which includes a full census regularly (biannually) updated of the population covered, and a detailed registry of all major demographic events (births, deaths, pregnancies, in and out-migrations) occurring within the study area. The Manhiça study area at the time of this thesis covered 500 km^2 (one fifth of the whole Manhiça district, see figure 8) ~94000 inhabitants and around 20000 households. Currently, the study area has been expanded to the entire district, covering 2300 km^2 and 202,000 inhabitants. All households within the area are geo-positioned using global positioning system (GPS) and all individuals in the DSS receive a Permanent Identification number (Perm-ID) allowing monitoring of longitudinal demographic information, which is collected electronically through three basic procedures: (i) annual household visits (ii) maternity and morgue visits to record births and deaths, and (iii) contact with key community informants. The data collected in the Manhiça DSS comprises households and individual features, socio-economic status, vital data (including cause and date of death), migration, health history, and vaccination status among others.

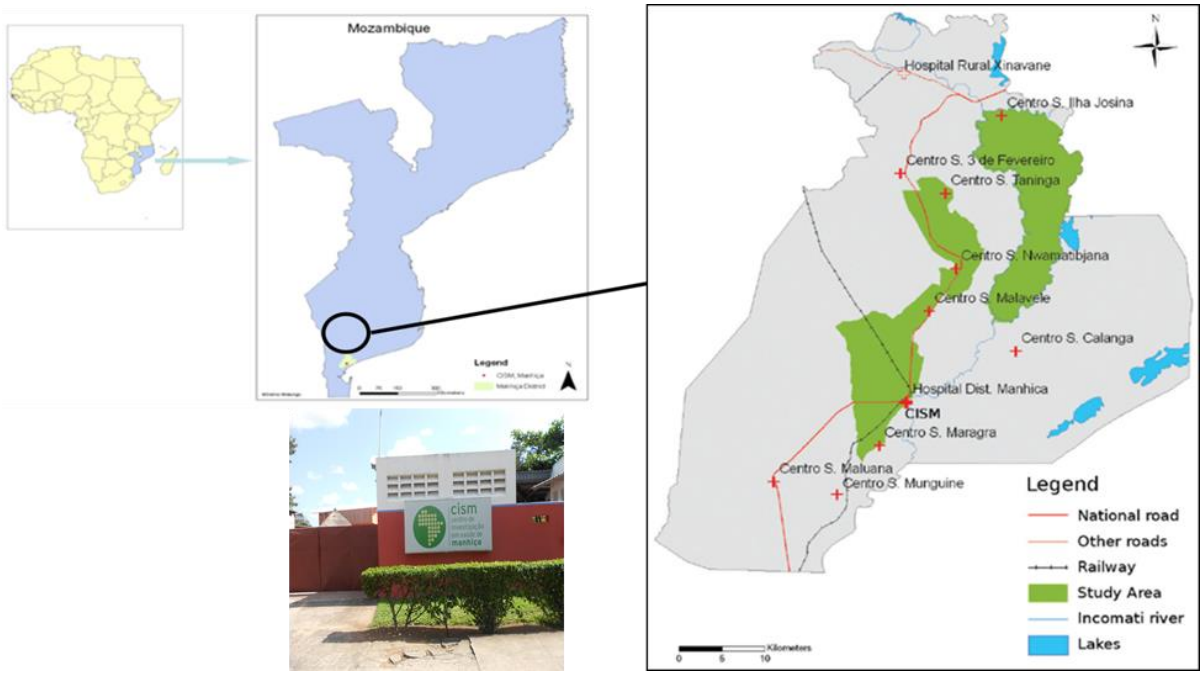


Figure 8: Mozambique, Maputo province, Manhiça district and CISM study area.

Additionally, CISM put in place in 1998 a morbidity surveillance system at Manhiça District Hospital (MDH) and 8 other peripheral health posts (189), to document paediatric morbidity and mortality. Morbidity surveillance includes the systematic collection (using standardized forms) of demographic, clinical history, clinical exam, outcome and treatments for all children <15 years of age visiting the outpatient department or being admitted to the paediatric wards in the hospital. Data on over 75,000 paediatric admissions and more than 1.2 million outpatient visits have been collected over the past 23 years. Malaria screening (for all children with fever or a history of fever in the preceding 24 hours) and microbiological surveillance are also routinely conducted, and blood cultures are systematically collected for all admissions <2 years of age, and for older children with suspected severe disease (figure 9). The DSS and MSS are able to link demographic data and clinical data to conduct biomedical research in priority health fields.

The Centre includes a fully equipped laboratory (including parasitology, haematology, biochemistry, microbiology including blood cultures, (including biosafety level III premises), molecular biology (including PCR and RT-PCR) and immunology (figure 9). The site has a dedicated freezer room, with six -80ºC freezers. Contamination rates in the past years have ranged between 5-13% of all processed blood cultures (190). A detailed description of CISM and the study area can be found elsewhere (191).

Figure 9. (A) parasitology department at CISM; (B) laboratory of bacteriology in CISM.

Over the past 23 years, CISM has conducted a series of studies with important impact on public health policies in the country, including studies on malaria preventive tools (RTS,S malaria candidate vaccine (192); Intermittent preventive treatment in infants (IPTi)/ Intermittent preventive treatment in pregnancy (IPTp) (193, 194), the treatment of malaria (195, 196), co-adjuvant treatment for malaria (197) and the detailed description of the burden and epidemiology of childhood diarrhoea, and viral and bacterial infections in children with acute respiratory symptoms (198-202), at the basis for Mozambique's application for *Haemophilus Influenzae b (Hib)*, *Pneumococcal* and rotavirus vaccines to GAVI.

3.2.2 Manhiça District Hospital

The MDH, upgraded in 2011 from the Manhiça Health Centre, is the referral health facility for the entire Manhiça District. This public hospital has 110 beds, including a 26-bed paediatric ward, an 8-bed basic intensive care facility, and a day hospital (6-bed) where children can be temporarily admitted and observed prior to a final admission decision. It also includes a malnutrition specialized hospitalization unit (6-bed). The hospital has a maternity ward, a surgery room (where caesarean sections can be performed, together with basic emergency surgery), a fully digital (film-free) X-ray machine, and a clinical trials unit. It has been estimated that around 85% of the deliveries in the area (+/-5000 per year) are institutional deliveries, and a facility ("waiting home") is available at MDH for pregnant women with risk factors for a complicated delivery to settle by the hospital in attendance of labour, facilitating a supervised delivery. The MDH is supported in terms of staffing and resources by CISM. The MDH admits around 3500 children annually and receives over 50,000 outpatient visits (Figure 10). The main causes of admission at paediatric ward of MDH are malaria, pneumonia, diarrhoea, malnutrition, and neonatal pathologies (203). The prevalence of Human immunodeficiency virus (HIV) among hospitalized children is around 25.7% (204).

Figure 10. (A) antenatal care and outpatient wards in Manhiça District Hospital (MDH), outpatient department; (B) MDH paediatric ward

3.2.3 Research Clinical Trials Unit

The Research Clinical Trials Unit (RCTU) is located within the hospital grounds and has been running since 2005. In this RCTU, 3 large studies ("Artekin" (196); "Coartem dispersible" (195) and "Drug" (205)) were conducted, which led to the clinical development of two antimalarial drugs (DHA-PQP (Eurartesim), and paediatric dispersible Coartem). Currently, the unit has four different rooms, including a fully equipped hospitalization ward, with space for 6 beds (figure 10). The unit has also two rooms for developing clinical and basic laboratory procedures, equipped for the assistance and follow-up of the patients recruited in the different trials. A fourth room is used as kitchen and waiting room for patients and relatives. The RCTU is fully staffed and supported by highly qualified CISM personnel.

In close collaboration with CISM's Regulatory Unit, the RCTU develops its activities of recruitment, follow-up and assistance of patients enrolled in different clinical trials and supports study-specific hospital care with a high dependency unit for intensive care and monitoring of patients. During the realization of this thesis, the candidate has been involved in the different malaria clinical trials ongoing in the unit:

- **KIDS**: A Phase II, open-label, multicentre, pharmacokinetic, pharmacodynamics and safety study of a new paediatric Eurartesim dispersible formulation and crushed film coated Eurartesim tablet, in infant patients with *Plasmodium falciparum* malaria (206) (See **Annex 7**)

-**MagOZ**: Phase IIb study to investigate the efficacy of OZ439 & PQP co-administered to adults & children with uncomplicated *P. falciparum* malaria (207) (See **Annex 5**

- **ROSI**: Rosiglitazone adjunctive therapy for severe malaria in children (197) (See **Article 5** of this thesis)

Figure 11: Research Clinical Trials Unit (RCTU)

3.2.4 Morbidity in the study area

By linking the information obtained through its morbidity surveillance system to the demographic data available for the DSS area, CISM has provided detailed descriptions of the health status of the community. HIV prevalence in adults in the area is among the highest in the world (208, 209). In recent years, a cohort of around 4,000 HIV-positive children has been routinely followed at the HIV outpatient's clinic at MDH. CISM has also conducted aetiological surveillance for common infections affecting children and infants in the area like diarrhea, respiratory tract infections or bacteraemia (198-202). Bacteraemia rates peaked at $1730/10^5$ child-years at risk in infants less than one year old, $782/10^5$ in those 1–4 years old, and $49/10^5$ in children aged 5 years and older. Additionally, the main causes of invasive bacterial disease in non-neonate infants included *Streptococcus pneumoniae* (23%), *Non-typhoidal salmonella* (23%), *Escherichia coli* (13%), *Haemophilus influenzae type b* (13%) and *Staphylococcus aureus* (8%) (190)

3.2.5 Malaria in the study area

Mozambique is a country of high malaria transmission, and the greatest burden of severe and fatal disease is borne my children under the age of 5. In 2011, the prevalence of malaria in children under 5 in rural areas was 46.3%. Malaria transmission is perennial in this district of Mozambique with some seasonality. *P. falciparum* accounts for over 90% of all malaria cases (210). In 2003–2005, malaria accounted for 30.5% of all paediatric outpatient visits (210) and 49% of all paediatric admissions (211). Almost 19% of all in-hospital paediatric deaths were due to malaria (211). According to WHO criteria, 13.2% of admissions had SM, being prostration (55%), respiratory distress (41.1%) and severe anaemia (17.3%) the 3 most prevalent clinical presentations (211). Recent changes in the epidemiology of malaria in SSA have encompassed a steady decline in malaria incidence, coupled with a decrease in severe disease, which reached its nadir in 2010, with only 3 deaths related to malaria in the whole of the year (Guinovart et al, in preparation). Since that year, SM cases is on the rise, with older

children presenting and with a higher proportion of cases presenting cerebral malaria (Guinovart C, personal communication, see figure below).

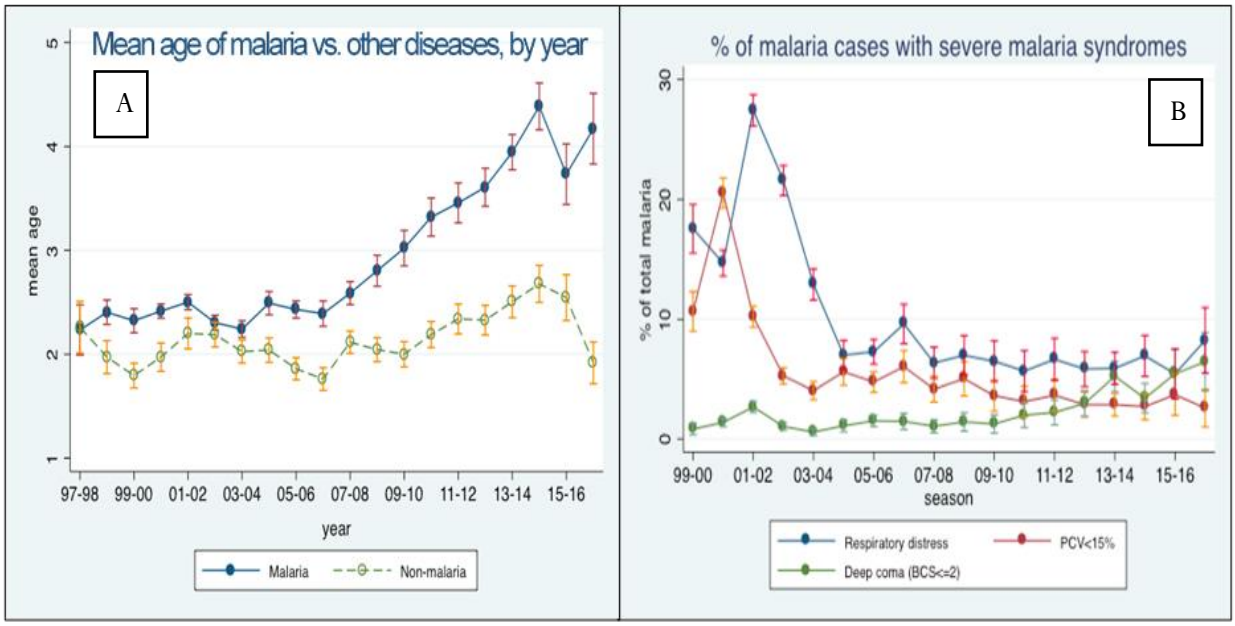


Figure 12: (A) Mean age of malaria vs other diseases, by year; (B) % of malaria cases with severe syndromes

3.2.6 Mortality surveillance in the area

Since the year 2016, the possibility of conducting minimally invasive autopsies (MIA; otherwise also known as Minimally Invasive Tissue Sampling, or MITS) is a reality at the Manhiça District Hospital. Indeed, the site is part of the CHAMPS (Child Health and Mortality prevention Surveillance) Network, a large endeavour aiming to better characterize the causes of child mortality in different epidemiological settings in Africa and Asia (212). In this respect, all child deaths (<5 years of age) occurring at the district are amenable to be approached for a specific consent to conduct a minimally invasive autopsy to investigate cause of death. The candidate has been directly involved in the implementation activities of this project in Manhiça.

3.3 Overview of the articles included in the thesis and role of the candidate in each piece of the work

The thesis is presented as a collection of six articles, five of them already published in peer-reviewed international journals, and one manuscript under preparation for publication. The author of this thesis has led five of the six articles included in this thesis and is first author in those five manuscripts. The thesis also includes an additional article in which the candidate is a co-author. This thesis is based on work undertaken through a partnership between the Barcelona Institute for Global Health (ISGlobal) and Centro de Investigação em Saúde de Manhiça (CISM), in Mozambique. The partnership benefited from collaborations with the Mozambican Ministry of health, the director of Mozambique's "Programa Nacional de control da Malária", the district and local health service authorities, and the director and personnel from Manhiça's district hospital. Some of the work presented in this thesis also involves other research institutions, as some of these studies were conducted as part of multi-centre studies. Two of the studies received support from the University Health Network (UHN) (article 5) and MMV (article 3), respectively. The work in the clinical trial presented in this thesis was performed in close collaboration with the Sandra Rotman Centre for Global Health (Toronto, Canada). During the realisation of this thesis, the candidate had a fellowship from the program Río Hortega of the Instituto de Salud Carlos III (ISCIII) (CD16/00024).

The 6 articles included in the thesis are:

Article 1: **Varo R**, Crowley VM, Sitoe A, Madrid L, Serghides L, Kain KC, Bassat Q: *Adjunctive therapy for severe malaria: a review and critical appraisal*. Malar J 2018, 17:47.

Article 2: Varo R, Quintó L, Sitoe A, Madrid L, Acácio S, Vitorino P, Valente AM, Mayor A, Camprubí D, Muñoz J, Bambo G, Macete E, Menéndez C, Alonso PL, Aide P, Bassat Q. *Post-malarial anemia in Mozambican children treated with quinine or artesunate: A retrospective observational stud.* Int J Infect Dis. 2020 Jun 2; 96:655-662. doi: 10.1016/j.ijid.2020.05.089.

This is a retrospective analysis of data collected in the context of routine clinical practice through the Manhiça MSS from children younger than 15 years who were admitted to MDH during a 14-year long period (2003-2017). The MSS in place at MDH and its updates over time have been approved by the Mozambican Ethics Committee (ref 017/CNBS/03 and CIBS_CISM/05/13). The analytical plan of this specific analysis was assessed and approved by Manhiça's Internal Scientific committee (ref CCI/120/JUN2016).

Article 3: Leroy D, Macintyre F, Adoke Y, Ouoba S, Barry A, Mombo-Ngoma G, Ndong Ngomo JM, **Varo R**, Dossou Y, Tshefu AK, Duong TT, Phuc BQ, Laurijssens B, Klopper R, Khim N, Legrand E, Ménard D. *African isolates show a high proportion of multiple copies of the Plasmodium falciparum plasmepsin-2 gene, a piperaquine resistance marker.* Malar J 2019, 18:126.

This article reports the results of an ancillary study of a Phase IIb clinical multicentre study which was conducted to evaluate the efficacy of a single oral dose of OZ439–PPQ in Asian

and African patients presenting with uncomplicated falciparum malaria. The motivation of this study was to investigate the profile of *P. falciparum* resistance to different antimalarials drugs. The implementation of the study, screening and recruitment of the study participants were coordinated at Manhiça site field by the author of this thesis, together with the supervision of data collection and sample processing at the laboratory facilities. The study was funded by MMV. The study was approved by the relevant Institutional ethics committees, national Institutional Review Boards and, where relevant, local regulatory authorities at each of the participating sites. Participants provided written informed consent prior to inclusion. In Mozambique the different amendments of the clinical study protocol MMV_OZ439_13_003 were approved by: Comité Nacional Bioética em Saúde (CNBS), Maputo, Mozambique ref 438/CNBS/14; Departamento Farmacéutico, Ministerio da Saúde, Mozambique ref 1132/380/DF 2015. The author of this thesis led the implementation of this trial in the Manhiça site.

Article 4: *Host Biomarkers are associated with severe malaria in Mozambican children: a case–control study:* original research (under preparation)

A case-control study in children under 10 years of age presenting at MDH with severe and uncomplicated malaria enrolled between September 2014 and May 2015 aiming to investigate host-response molecules in plasma differentially expressed in children with severe and uncomplicated malaria. The implementation of the study, screening and recruitment of the study participants were supported at the field by the author of this thesis, together with the supervision of data collection and sample processing at the laboratory facilities. Data cleaning and data analysis was led by the author of this thesis. This study was reviewed and approved by the Mozambican National Bioethics Committee (CNBS) (Ref. 71/CNBS/2014) and the Clinical Research Ethics Committee of the Hospital Clínic, Barcelona, Spain (Ref.

HCB/2013/8749).Written informed consent was obtained from patients prior to participation.

Article 5: Varo R, Crowley VM, Sitoe A, Madrid L, Serghides L, Bila R, Mucavele H, Mayor A, Bassat Q, Kain KC: *Safety and tolerability of adjunctive rosiglitazone treatment for children with uncomplicated malaria*. Malar J 2017, 16:215

This clinical trial was a request from the Mozambican National Bioethics Committee, and as a prelude to rosiglitazone's further evaluation in a RCT in paediatric severe malaria (ClinicalTrials.gov: NCT02694874). It was a prospective, randomized, double-blind, placebo-controlled, Phase IIa trial of rosiglitazone adjunctive treatment in addition to Mozambican standard of care in children with uncomplicated malaria. The implementation of the study, screening and recruitment of the study participants were coordinated at the field by the author of this thesis, together with the supervision of data collection and sample processing at the laboratory facilities. Data cleaning and data analysis were also led by the author of this thesis. This study was funded in part by the Canadian Institutes of Health Research (CIHR) Foundation Grant FDN-148439 (KCK) and a Canada Research Chair in Molecular Parasitology (KCK). This study was reviewed and approved by the Mozambican National Bioethics Committee (CNBS) (Ref. 230/CNBS/15), the pharmaceutical department of the Mozambican Ministry of Health (Ref. 374/380/DF2016), the Clinical Research Ethics Committee of the Hospital Clínic, Barcelona, Spain (Ref. HCB/2015/0981), and the University Health Network Research Ethics Committee, Toronto, Canada (UHN REB Number 15-9013-AE). All participants and their parents/legal guardians were given detailed oral and written information about the trial, and children were recruited only after a written informed consent was signed by their parents/legal guardians.

Article 6: Varo R, Erice C, Johnson S, Bassat Q and Kain KC. *Clinical trials to assess adjuvant therapeutics for severe malaria (*accepted in Malaria Journal).

During the performance of these studies, the author of this thesis obtained a fellowship from the program Rio Hortega of the ISCIII (grant Nº: CM16/00024). The printing of this thesis was supported by the PhD student program at ISGlobal. CISM receives core funding from the Spanish Agency for International Cooperation and Development (AECID). ISGlobal is supportted by the Spanish Ministry of Science and Innovation through the ´Centro de Excelencia Severo Ochoa 2019-2023 Program (CEX2018-000806-S), and by the Generalitat de Catalunya through the CERCA Program.

4. RESULTS

Varo *et al. Malar J (2018) 17:47*
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Malaria Journal

REVIEW

Open Access

Adjunctive therapy for severe malaria: a review and critical appraisal

Rosauro Varo[1,2*†], Valerie M. Crowley[3†], Antonio Sitoe[1], Lola Madrid[1,2], Lena Serghides[4,5,6], Kevin C. Kain[3,7,8‡] and Quique Bassat[1,2,9,10*‡]

Abstract

Background: Despite recent efforts and successes in reducing the malaria burden globally, this infection still accounts for an estimated 212 million clinical cases, 2 million severe malaria cases, and approximately 429,000 deaths annually. Even with the routine use of effective anti-malarial drugs, the case fatality rate for severe malaria remains unacceptably high, with cerebral malaria being one of the most life-threatening complications. Up to one-third of cerebral malaria survivors are left with long-term cognitive and neurological deficits. From a population point of view, the decrease of malaria transmission may jeopardize the development of naturally acquired immunity against the infection, leading to fewer total cases, but potentially an increase in severe cases. The pathophysiology of severe and cerebral malaria is not completely understood, but both parasite and host determinants contribute to its onset and outcomes. Adjunctive therapy, based on modulating the host response to infection, could help to improve the outcomes achieved with specific anti-malarial therapy.

Results and conclusions: In the last decades, several interventions targeting different pathways have been tested. However, none of these strategies have demonstrated clear beneficial effects, and some have shown deleterious outcomes. This review aims to summarize evidence from clinical trials testing different adjunctive therapy for severe and cerebral malaria in humans. It also highlights some preclinical studies which have evaluated novel strategies and other candidate therapeutics that may be evaluated in future clinical trials.

Keywords: Adjunctive, Treatment, *Plasmodium falciparum*, Malaria, Severe, Cerebral, Experimental, Human, Murine

Background

The global burden and impact of severe malaria

Malaria is the most important parasitic disease in the world, causing an estimated 212 million infections and 429,000 deaths annually [1]. The greatest burden of severe and fatal disease is borne by children, particularly in sub-Saharan Africa [1]. Humans are unable to develop full immunity to malaria infection. However, acquisition of clinical immunity, which confers protection from life-threatening malaria episodes, is possible but requires repeated exposure to infective mosquito bites. In areas of high transmission, where children are repeatedly exposed to infective mosquito bites from birth, most children will acquire clinical immunity to severe malaria (SM) if they survive their first years of life [2]. In areas of low transmission, however, SM can occur at any age, and is more common among adults, because clinical immunity to malaria takes longer to build, is quick to wane, or simply never occurs. It has been argued that a decrease in the intensity of malaria transmission may put children and adults at risk of severe and fatal disease, precisely as a result of interfering with the natural acquisition of such immune responses [3].

In low-resource settings access to health services is often severely limited, and represents a major constraint to survival for those who develop SM. The case fatality rate (CFR) for SM is heavily dependent on the possibility

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of reaching the health system, and can range between 20% with in-hospital care, to > 90% when the patient remains at home [4]. It has been estimated that the global annual incidence of SM can be as high as 2 million cases per year [5].

The pathobiology of severe and cerebral malaria

Both parasite and host determinants contribute to the onset and outcome of severe and cerebral malaria (CM). Host innate immune responses to infection, combined with the sequestration of parasitized erythrocytes (PEs) in the microvasculature of vital organs, such as the brain, result in dysregulated inflammation, endothelial activation, microvascular occlusions, metabolic derangement, and ultimately dysfunction and breakdown of the blood–brain-barrier (BBB) [6]. Sequestered PEs, perfusion abnormalities, haemorrhages, oedema, tissue ischemia, and focal disruptions of the BBB are common fundoscopic and autopsy findings in CM patients and correlate well with disease severity [7–9]. Oxidative stress and axonal injury in the vicinity of brain haemorrhages and in areas of vascular occlusion have also been observed in CM post-mortem studies, and may contribute to neurological dysfunction pre-mortem and in CM survivors [10–12].

There is continued debate within the malaria community as to the utility of animal models and their applicability to human pathophysiology. Notable differences between human CM and *Plasmodium berghei* CM that are generally agreed upon, include the lack of pronounced sequestration of infected red blood cells (iRBCs) and the accumulation of immune cells including leukocytes, monocytes, macrophages, and T cells, in the brains of mice with experimental cerebral malaria (ECM) [13, 14]. In murine models of ECM, intravital microscopy studies have revealed that neurological signs in ECM are associated with vascular leakage and dysfunction of the neuro-immunological BBB, rather than the physiological BBB [15].

Recently, endothelial protein C receptor (EPCR), a host receptor involved in anticoagulation and endothelial cytoprotection, has been identified as a receptor for *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) suggesting a link between severe disease and coagulopathy [16]. In addition, dysregulation of the haem-haemopexin axis has been associated with poor clinical outcome and disease severity [17, 18]. Both of these new insights into SM pathogenesis open the door for new therapeutic options.

Primary treatment of severe and cerebral malaria

Severe malaria is a complex multi-system disease that may be differently defined according to the age group it affects, as clinical manifestations may vary between adults and children. However, it should be noted that the differences between those age groups might not be due to disparities in pathology but due to the under-recognition of complications in young children with SM, as is the case for acute kidney injury [19]. For epidemiological purposes, SM can be defined as the confirmation of a malarial infection in the presence of one or more of a series of syndromes or conditions, including impaired consciousness, acidosis, hyperlactataemia, hypoglycaemia, severe anaemia, acute kidney injury, jaundice, pulmonary oedema, significant bleeding, hyperparasitaemia, or shock [5]. CM, perhaps the most feared complication of malaria, is characterized by severe impairment of consciousness (deep coma) in the absence of other alternative explanations or diagnoses. Impaired consciousness, together with severe respiratory distress, has one of the highest mortality rates of the severe complications [5]. Beyond impaired consciousness, CM can also present with repeated seizures or other neurological abnormalities. CM is associated with long-term cognitive and neurological deficits in up to one-third of survivors, including hemiparesis, cerebellar ataxia, cortical blindness, hypotonia, spasticity, aphasia, seizure disorders, behavioural disorders, and attention-deficit hyperactivity disorder (ADHD) [20–25].

Plasmodium falciparum is responsible for the majority of malaria-associated morbidity and mortality. In the absence of prompt and effective treatment, *P. falciparum* infection may progress to severe and potentially fatal forms. Parenteral artesunate is now widely accepted as the standard of care for the treatment of SM, both in adults and children, following the landmark SEAQUAMAT and AQUAMAT trials that demonstrated its superiority over quinine [26, 27]. Recently, intramuscular artesunate administration has proven to be non-inferior to intravenous artesunate in reducing parasitaemia $\geq$ 99% at 24 h in children with SM [28]. However, even with the improved efficacy of artesunate, CFR for SM (8.5% in children and 15% in adults) and in particular CM (18 and 30%, respectively) remain high [26, 27]. Therefore, treatment with potent artemisinin-derivatives alone is insufficient to prevent death or neurological disability in all patients with SM. Adjunctive therapy, based on modulating host response to infection, could reduce malaria-associated morbidity, mortality and could enhance and extend the clinical utility of current anti-malarials. General declines of malaria and SM burden, decreases in the CFR for malaria and difficulties in detecting reductions in mortality rates may hinder the evaluation of those interventions due to the need of recruiting large numbers of patients [29]. In this respect, it is necessary to creatively innovate in the design of clinical trials with more

precise sample sizes, more accurate clinical predictors and surrogate endpoints for mortality like plasma lactate concentration [29–31]. It is recommended that patients with SM with signs of serious bacterial infection receive intravenous antibiotics [5]. However, their effect on mortality and/or clinical outcome have not been tested in any randomized controlled trial (RCT).

The role of adjunctive therapy in severe malaria treatment

The host immune response plays a central role in the onset, severity and outcome of malaria infections and this has promoted the search for immunomodulatory adjunctive therapy to improve clinical outcome. Adjunctive therapy is used in combination with primary antimalarial treatment, with the aim of improving efficacy, or reducing disease-associated complications. To date, several types of putative adjunctive therapy have been tested in SM without success. Malaria immunopathogenesis is complex and targeting a single pathway may be insufficient to reduce mortality or improve neurological outcomes. Targeting multiple pathways, either by the use of multiple interventions (which is more complicated to deliver and increases the risk of adverse events, drug interactions and costs), or alternatively, by using a single intervention that targets multiple pathways implicated in the pathobiology of SM, could potentially lead to improved outcomes. Effective adjunctive therapy must be safe, have a clear benefit over anti-malarial use alone, be effective as a late-stage intervention, be minimally invasive, inexpensive, and ideally feasible to implement in low-resource endemic settings, where the bulk of SM occurs. The objective of adjunctive therapy should be the improvement of clinical outcome, and/or reduction of mortality, in addition if possible of the prevention of long-term neurocognitive deficits. This review aims to summarize recent evidence highlighting various approaches currently being pursued as adjunctive therapy for SM and CM. The review will focus on therapy tested in humans in RCTs, and it will also mention some preclinical studies that have evaluated some novel strategies and candidate therapeutics that may be evaluated in future clinical trials.

Search methodology

RCTs were identified through electronic searches of PubMed without any language or date restrictions and limited to humans. PubMed was searched (accessed 15 June 2017) through the use of a broad sensitive filter using following combinations: "malaria AND adjunctive therapy" (124 results), and "severe malaria AND adjunctive therapy" (81 results) and "cerebral malaria AND adjunctive therapy" (61 results). The references of the retrieved papers were used to search for additional studies. Adjunctive therapy assessed in RCTs is summarized in Table 1. These RCTs cover a period of 33 years (from 1982 to 2015). Thirty-two RCTs were included in the Table. RCTs that did not report data on clinical outcomes or those performed in patients without severe or cerebral malaria were excluded. In the text, some studies performed in uncomplicated malaria are discussed. Clinicaltrials.gov was also searched for ongoing RCTs or completed RCTs with no published data. To identify relevant preclinical models PubMed was searched (accessed 15 June 2017) using the following search terms: "experimental cerebral malaria" (453 results) and "experimental cerebral malaria AND adjunctive therapy" (21 results). Studies were included if they were published after 2010, peripheral parasitaemia at time of adjunctive therapy administration was more than 5%, and the intervention had a benefit after the onset of symptoms.

Adjunctive therapy for the treatment of severe and cerebral malaria in humans

Immunomodulation

Based on the critical role of the host response in determining the onset, severity and outcome of *P. falciparum* infection, different adjunctive therapy has been evaluated to modify this pathophysiological pathway.

Corticosteroids With the aim of reducing swelling and inflammation in the brain, corticosteroids were one of the first treatments proposed as an adjunctive therapy for SM based on successful case reports. However, dexamethasone failed to demonstrate a decrease in mortality in two clinical trials testing different doses in adults with SM, although the small sample sizes and lack of power do not allow ruling out a clear effect on mortality [32–34]. Furthermore, one of the studies showed an increased risk of adverse events (prolonged coma, pneumonia and gastrointestinal bleeding) within the dexamethasone group compared to those receiving placebo [32]. No additional RCT have tested corticosteroids in SM, and the use of dexamethasone is currently not recommended in its management.

Intravenous immunoglobulin Similarly to what occurred with corticosteroids, treatment with intravenous immunoglobulin was associated with increased deleterious outcomes compared to the placebo group, including higher mortality and more neurological *sequelae* in children [35]. The clinical failure of this therapy may reflect the lack of success to reverse cytoadherence and sequestration [35].

Curdlan sulfate Curdlan sulfate (CS), a sulfated 1 → 3-β-D glucan, previously shown to be a potent human immunodeficiency virus (HIV) entry inhibitor, and known to

Table 1 List of randomized controlled trials of adjunctive therapy in severe malaria

Author, year, country, references	Antimalarial	Adjuvant therapy	Dosage and route	Study design	Type of malaria	Ages	Sample size	Outcome
Immunomodulation								
Warrell et al. 1982, Thailand, [31]	IV quinine	Dexamethasone	IV; children 0.6 mg/kg at the start followed by 7 doses of 0.2 mg/kg at 6-h intervals; adults 0.5 mg/kg at the start followed by 7 doses of 10 mg each (total duration of treatment 48 h)	RCT, DB, PC	CM	6–70 years	100	Failed to decrease mortality. Increased risk of adverse events (prolonged coma, pneumonia and gastrointestinal bleeding)
Hoffman et al. 1988, Indonesia, [32]	IV quinine	Dexamethasone	IV; initial dose, 3 mg/kg; total, 11.4 mg/kg per 48 h	RCT, DB, PC	CM	1.5–42 years	38	No differences in mortality, parasite and fever clearance times or incidence of complications
Taylor et al. 1992, Malawi, [34]	IV quinine	Immunoglobulin (IFAT antimalarial Ab)	IV; 400 mg/kg over 3 h	RCT, DB, PC	Coma	1–12 years	31	Increased mortality but not statistically significant. No differences in parasite and fever clearance times or incidence of complications
Havlik et al. 2005, Thailand, [36]	IV artesunate	Curdlan sulphate	IV; 4 mg/kg over 30 min/8 h (adjusted dose according to APTT)	RCT, DB, PC	SM but not CM (Phase IIB); SM and CM (Phase IIC)	12–60 years	Phase IIB: 44; Phase IIC: 26	No differences in mortality or parasite clearance times. Trend to improve duration of coma and fever clearance time
van Hensbroek et al. 1996, The Gambia, [37]	IM quinine and IM artemether ± oral pyrimethaminesulfadoxine	anti-TNF mAb	IV; 5 mg/kg over 15 min	RCT, DB, PC	CM	1–9 years	624	No differences in mortality, coma recovery or complications. Lower fever clearance time. Trend towards faster parasite clearance time. Higher rate of neurological sequelae
Di Perri et al. 1995, Burundi, [38]	IV quinine	Pentoxifyline	IV; 10 mg/kg/24, 72 h	RCT	CM	< 14 years	56	Lower mortality not statistically significant. Significant reduction in coma recovery

Table 1 continued

Author, year, country, references	Antimalarial	Adjuvant therapy	Dosage and route	Study design	Type of malaria	Ages	Sample size	Outcome
Das et al. 2003, India, [39]	IV quinine	Pentoxifylline	IV; 10 mg/kg/24, 72 h	RCT	CM	> 18 years	52	Improved mortality, not statistically significant. Significant reduction in coma recovery time
Hemmer et al. 1997, Germany, [40]	1. IV quinine + doxycycline; 2. oral mefloquine or halofantrine	Pentoxifylline	IV; 5 mg/kg/24 h for 5 days	RCT, DB, PC	UM and CM	22–69 years	51	No differences in mortality, clinical outcomes or laboratory parameters. More side effects
Looareesewam et al. 1998, Thailand, [41]	IV artesunate	Pentoxifylline	IV; low (0.83 mg/kg/h) or high (1.67 mg/kg/h) over 72 h	RCT, DB, PC	SM	16–60 years	45	No significant differences in fever and parasite clearance time or in clinical outcomes
Lell et al. 2005, Kenya, [42]	IV quinine	Pentoxifylline	IV; 10 mg/kg/24 h for 72 h	RCT, DB, PC	CM	9 month–8 years	15	Higher mortality. No difference in coma recovery, incidence of complications or neurological sequelae. Trend to faster fever and parasite clearance times
Decreasing procoagulant effects								
Hemmer et al. 1991, Germany, [52]	1. IV quinine + oral doxycycline or oral mefloquine; 2. IV quinine + oral doxycycline	Heparin or acetylsalicylic acid (ASA)	IV: Heparin 70 U/kg/day SC. for 5 days; ASA 500 mg on days 0, 2, 4	RCT	SM	> 14 years	97	No difference in fever, parasite clearance, or time to discharge
Decreasing cytoadherence and sequestration								
Maude et al. 2014, Bangladesh, [57]	IV artesunate	Levamisole	Oral, 150 mg, single dose	RCT, OL	SM	21–45 years	56	No differences in mortality, parasite clearance time, 'sequestration ratio' or normalization of plasma lactate
Improving liver function								
Treeprasertsuk et al. 2009, Thailand, [80]	IV artesunate	Ursodeoxycholic acid	IV; 750 mg/day, 2 weeks	RCT, DB, PC	SM with jaundice	> 15 years	80	Safe, but no differences between liver test, fever and parasite clearance times

Table 1 continued

Author, year, country, references	Antimalarial	Adjuvant therapy	Dosage and route	Study design	Type of malaria	Ages	Sample size	Outcome
Restricting iron availability								
Gordeauk et al. 1992, Zambia, [81]	IV quinine +oral pyrimethaminesulfadoxine	Deferoxamine	IV; 100 mg/kg/day over 72 h	RCT, DB, PC	CM	20–54 months	83	Lower mortality, not statistically significant. Faster coma recovery time and parasite clearance time
Thuma et al. 1998, Zambia, [82]	IV quinine	Deferoxamine	IV; 100 mg/kg/day over 72 h	RCT, PC	CM	< 6 years	352	Non-significant trend to faster recovery from coma. No statistical differences in mortality
Mohanty et al. 2002, India, [83]	IV quinine and oral doxycycline	Deferiprone	Oral; 75 mg/kg/day in 12 hourly divided doses over 10 days	RCT, DB, PC	SM	13–84 years	45	Faster fever, parasite clearance and coma recovery time. No differences in mortality
Prevention of seizures								
White et al. 1988, Thailand, [85]	IV quinine	Phenobarbital	IM; 3.5 mg/kg, single dose	RCT, DB, PC	CM	6–78 years	48	Fewer convulsions
Crawley et al. 2000, Kenya, [86]	IV quinine	Phenobarbital	IM; 20 mg/kg, single dose	RCT, DB, PC	CM	19–65 months	340	Fewer convulsions. Higher mortality
Decreasing intracranial pressure								
Namutangula et al. 2007, Uganda, [91]	IV quinine	Mannitol	IV; 1 g/kg	RCT, DBO, PC	CM	6–60 months	156	Did not significantly reduce time taken to regain consciousness, sit unsupported, or mortality
Mohanty et al. 2011, India, [92]		Mannitol	IV; 1.5 g/kg over 15 min, followed by 0.5 g/kg every 8 h until the patient regained consciousness or for a maximum period of 72 h	RCT, OL, PC	CM with brain swelling	25–31 years	61	Trend towards higher mortality in mannitol group. Mannitol prolonged coma recovery
Fluid resuscitation								
Maitland et al. 2005, Kenya, [97]	IV quinine	Human albumin/saline	IV; 20 mL/kg of either 4.5% human albumin solution or 0.9% saline vs control (fluids maintenance group)	RCT, OL	SM with either moderate and severe acidosis	> 1 years	150	Safe and resulted in significantly lower mortality. Acidosis did not improve

Table 1 continued

Author, year, country, references	Antimalarial	Adjuvant therapy	Dosage and route	Study design	Type of malaria	Ages	Sample size	Outcome
Akech et al. 2006, Kenya, [98]	IV quinine	Human albumin/gelofusine	IV; 20–40 mL/kg of either 4.5% human albumin solution or gelofusine	RCT, OL	SM	> 3 years	88	Trend to lower mortality, not statistically significant with albumin. No difference between shock and acidosis recovery. Higher neurological sequelae with albumin group
Fluid resuscitation								
Maitland et al. 2011, Uganda, Kenya, Tanzania, [99]	IV quinine	Human albumin/saline	20 mL/kg of either 4.5% human albumin solution or 0.9% saline vs (fluids maintenance group)	RCT, OL	SM	2 month–12 years	1793 SM cases out of 3123 total sample size	Higher mortality in children treated with bolus
Decreasing oxidative stress								
Watt et al. 2002, Thailand, [105]	IV artesunate	N-Acetylcysteine	IV; 300 mg/kg over 20 h	RCT, DB, PC	SM	> 18 years	30	Faster normalization of lactate levels and Glasgow Coma Score
Treeprasertsuk et al. 2003, Thailand, [106]	IV artesunate	N-Acetylcysteine	IV, oral: 3 different regimes	RCT, PC	SM	14–16 years	108	No differences in mortality, fever and parasite clearance time. No differences in adverse events between groups
Charunwatthana et al. 2009, Bangladesh, Thailand, [107]	IV artesunate	N-Acetylcysteine	IV; 300 mg/kg over 20 h	RCT, DB, PC	SM	30–39 years	108	No differences in clearance of elevated plasma lactate levels, coma recovery times, mortality, fever clearance time, and complications or adverse events
Correcting lactic acidosis								
Khrisna et al. 1994, Thailand, [112]	IV quinine	Dichloroacetate	IV; 46 mg/kg, single dose	not stated	SM	> 14 years	45	Decreased lactate concentrations. No evidence of toxicity. Mortality, incidence of complications and clinical/parasitological measures of recovery did not differ

Table 1 continued

Author, year, country, references	Antimalarial	Adjuvant therapy	Dosage and route	Study design	Type of malaria	Ages	Sample size	Outcome
Correcting lactic acidosis								
Khrisna et al. 1995, Ghana, [113]	IM quinine	Dichloroacetate	IV; 50 mg/kg, single dose	RCT, OL, PC	SM	1.5–12 years	18	Decreased lactate concentrations. No differences in mortality, fever or parasite clearance times
Khrisna et al. 1996, Thailand, [114]	IV quinine	Dichloroacetate	IV; 46 mg/kg single dose	RCT, OL, PC	SM	> 14 years	20	No differences in mortality, greater decrease in lactate concentrations
Agbenyega et al. 2003, Ghana, [115]	IV quinine	Dichloroacetate	IV; 50 mg/kg, single dose	RCT, DB, PC	SM	1–12 years	124	Significantly reduced the concentration of blood lactate
Increasing NO availability								
Hawkes et al. 2015, Uganda, [119]	IV artesunate	Nitric Oxide	inhaled, 80 ppm	RCT, B, PC	SM	1–10 years	180	No differences in levels of Ang-2. No differences in mortality, recovery rates or parasite clearance time
Mwanga-Amumpaire et al. 2015, Uganda, [120]	IV artesunate	Nitric Oxide	inhaled, 80 ppm	RCT, OL, PC	CM	2 month–2 years	92	Did not increase Ang-1, did not reduce mortality rate. Similar clinical outcomes and neurological sequelae between groups

Ab antibody, *Ang* angiopoeitin, *APPT* activated partial thromboplastin time, *CM* cerebral malaria, *IM* intramuscular, *IV* intravenous, *mAb* monoclonal antibody, *MO*: months, *NO* nitric oxide, *OL* open-label, *PC* placebo-controled, *PPM* parts per million, *SC* subcutaneous, *SM* severe malaria, *UM* uncomplicated malaria, *YR* years

inhibit *P. falciparum* in vitro, has been tested in two RCTs due to its capacity to modulate the immune response to *P. falciparum* [36]. As a sulfated polysaccharide (similar to heparin), CS would be expected to have some anticoagulant properties, and confer certain direct and non-specific effect on cytoadhesion and rosetting. Neither of the studies demonstrated differences in mortality, possibly on account of small sample sizes, but CS was safe and appeared to reduce the severity of the disease process [37].

Anti-TNF therapy Therapy targeting tumour necrosis factor (TNF) and its effects have also been explored. Two different strategies have been evaluated in RCTs. One trial used monoclonal antibodies to inhibit TNF function. No difference in mortality was shown and moreover, there was an increased risk of neurological *sequelae* in the experimental group [38]. The retention of TNF by the antibody within the circulation may explain this deleterious effect [38]. Pentoxifylline (PTX), a phosphodiesterase inhibitor, can reduce levels of TNF and has been tested in different studies with controversial results. Two studies showed an improvement in survival and a significant reduction in coma recovery time [39, 40]. However, three others studies comparing adjunctive PTX treatment to placebo showed no clinical benefit [41–43]. One of the studies also showed higher than expected mortality rates [43]. Taking into account these data and the small samples of the studies, there is no clear evidence to propose PTX as an adjunctive therapy.

Charcoal Oral activated charcoal (oAC) can modify the immune response against malaria infection. In a study with ECM, oAC demonstrated a significant reduction in pro-inflammatory cytokines and improvement in survival [44]. Furthermore, oAC was safe and well tolerated in humans in a Phase I trial and did not interfere with the pharmacokinetics of parenteral artesunate [44]. A RCT in children with uncomplicated malaria to assess safety and parasite clearance times of oAC in combination with intravenous artesunate has finished in Mali but results are yet to be published (NCT01955382), and no trials including patients with SM have been conducted. Importantly, the route of this intervention, similarly to what occurs with oral medications, may prove to be a further hindrance, as critically ill children are unable to swallow and the use of nasogastric tubes may prove difficult.

PPAR-gamma agonists Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists are attractive adjunctive candidates as they modulate multiple pathways implicated in the pathobiology of SM by reducing excessive inflammation and neurovascular leak, and by enhancing neuroprotective and anti-oxidant mechanisms [45–48]. Rosiglitazone modulates the innate host immune response to malaria [49]. In a murine model of ECM, this drug showed specific benefits by improving survival and reducing neurological impairments [48]. In a RCT in young adults with uncomplicated malaria, rosiglitazone was safe and well tolerated and those receiving rosiglitazone had lower levels of pro-inflammatory biomarkers and faster parasite clearance times [50]. Those patients receiving rosiglitazone also had increased levels of the considered "protective" brain-derived neurotrophic factor (BDNF) and reduced endothelial activation [48, 50]. A phase IIa trial to prove safety and tolerability of rosiglitazone in children under 12 years of age has recently concluded in Mozambique demonstrating the safety and good tolerability of rosiglitazone in children with uncomplicated malaria [51]. Furthermore, a phase IIb trial is now ongoing at the same site to test the efficacy of rosiglitazone as adjuvant therapy to intravenous artesunate for improving clinical and neurological effects of SM (NCT02694874).

Decreasing procoagulant effects

As SM induces a procoagulant state [52], different drugs with anticoagulant potential (in addition to curdlan sulfate, already mentioned in a previous paragraph) have been studied as adjunctive therapy. A prospective randomized study in adults with uncomplicated and severe falciparum malaria examined acetylsalicylic acid and low-dose heparin [53]. Neither of these treatments showed beneficial effect on clinical, haemostatic or parasitic parameters. Sulfated glycosaminoglycans (GAG), including heparin and sevuparin, can disrupt rosette formation and inhibit cytoadherence to endothelial cells, and have been proposed as potential adjunctive therapy [54, 55]. However, only one study examined their effects in a RCT. Sevuparin sodium, a heparan sulfate mimetic, was tested in adults with uncomplicated malaria to determine its tolerability and pharmacokinetics when administered as an intravenous infusion in combination with atovaquone–proguanil, proving to be well tolerated [56]. Sevuparin reduced merozoite invasion as the mean relative number of ring iRBCs was lower in the experimental group vs the control group and the treatment resulted in the desequestration of RBC infected with mature parasites as more of these were detected in peripheral circulation [56].

Decreasing cytoadherence and sequestration

Levamisole is a specific alkaline-phosphatase inhibitor mainly used to treat intestinal helminths. It was suggested as an adjunctive therapy candidate after showing its capacity to decrease iRBC sequestration in falciparum malaria in vivo [57]. However, a RCT in Bangladesh,

which explored the effect of a single levamisole hydrochloride dose (oral, 150 mg, single dose) in adult patients with SM showed no benefit compared to placebo when administered as adjuvant to intravenous artesunate [58]. As in other studies in which intravenous artesunate is used, its fast effect in killing *P. falciparum* parasites may have blurred the benefits of the adjuvant therapy.

Reduction of parasite biomass

Exchange blood transfusions (EBT) and erythrocytapheresis have been used as an adjunctive treatment in SM based on the hypothesis that infusing fresh whole blood or uninfected erythrocytes resulting in replenishing erythrocytes lost to parasitization, and reducing iron and other toxic bioproducts associated with infection, could lead to improved outcomes in patients with very high parasitaemia. To date, no prospective RCT of EBT or erythrocytapheresis has been conducted, and despite their frequent use these interventions remain controversial. Numerous case reports and retrospective studies have been conducted but there is limited evidence that such approaches improve parasite clearance times or enhance survival in artesunate-treated patients [59–67]. EBT and erythrocytapheresis may be options in high-resource settings with cases of imported malaria, although current expert opinion tends not to recommend them as adjuvant therapy [68–70]. Such approaches, however, are unfeasible in resource-constrained settings and in communities where the prevalence of HIV and other blood-borne transmissible diseases is high.

Improving anaemia and liver function

Severe malarial anaemia (SMA) is an important syndrome of SM and is associated with increased clearance of infected and non-infected erythrocytes and dysregulated haematopoiesis. Blood transfusions are not routinely recommended as a treatment for SMA [71–73]. Erythropoietin has immunomodulation effects and has been shown to reduce clinical signs of ECM in murine models, possibly in relation to its capacity to reduce neural hypoxia and cerebral pathology [74, 75]. In murine ECM models, erythropoietin co-administered with artesunate was associated with an improvement in clinical recovery and global survival rates [76]. In an open-labelled study in children with CM, erythropoietin was safe and well tolerated when administered with quinine [77]. A randomized trial of recombinant human erythropoietin (rHuEPO) in children with CM was prematurely stopped in Mali (EPOMAL Study; ClinicalTrials.gov Identifier: NCT00697164), although preliminary data demonstrated the short-term safety of high doses of erythropoietin (1500 U/kg/day rHuEPO) administered for 3 days (NCT00697164, unpublished data, Picot S, pers. comm.).

Malaria-associated liver injury, including unconjugated hyperbilirubinemia, intrahepatic cholestasis, elevated serum aspartate (AST) and alanine aminotransferase (ALT) levels, and jaundice is not uncommon [78–80]. These symptoms often indicate severe illness and are associated with a higher incidence of complications in a malaria infection [80]. Ursodeoxycholic acid (UDCA) is used in the treatment of cholestatic liver disease and was tested as an adjunctive therapy in adult patients with SM and jaundice with the intention of improving liver function [81]. Although UDCA proved to be safe, it did not significantly improve liver tests. Severity of hyperbilirubinemia, concomitant co-infections and early treatment with intravenous artesunate may explain these results [81].

Restricting iron availability

Iron chelators such as desferrioxamine (DFO) or deferiprone were proposed as adjunctive therapy for malaria. As malaria parasites require iron to multiply, reducing the availability of iron could inhibit parasite replication, with the caveat that these agents could contribute to or exacerbate anaemia. A number of small RCTs, not powered to assess mortality, have evaluated the use of iron chelators in SM, showing a tendency to reduce coma and achieve faster parasite clearance times [82–84]. However, data remain insufficient to support the use of iron chelators in the treatment of SM [85].

Prevention of seizures

In CM, seizures are usually associated with a higher mortality and a higher risk of neurological *sequelae* [5]. Based on this reasoning, anticonvulsants have been used to prevent seizures in CM. A first RCT, conducted in children, demonstrated that a single intramuscular injection of phenobarbitone (3.5 mg/kg) could reduce the incidence of convulsions, although it did not improve mortality [86]. A subsequent RCT in Kenya in 340 children with CM [87], showed that a single prophylactic intramuscular dose of phenobarbital (20 mg/kg) could reduce the frequency of seizures compared to children receiving placebo. However, mortality was doubled in the group receiving phenobarbital. Respiratory depression caused by phenobarbital and is interaction with other intravenous anticonvulsants could explain this negative effect [84]. Consequently, seizure prophylaxis with phenobarbital could not be recommended as adjunctive therapy for CM and others trials with appropriate design, bigger sample and distinct anticonvulsant doses are required [88]. A recent study in Malawi,

assessing the effect of enteral levetiracetam vs phenobarbital to control acute seizures in children with CM has recently finished demonstrating that levetiracetam appeared to have a better safety profile than phenobarbital and a similar effect in the control of neurological complications and mortality (NCT01660672, unpublished data, Birbeck GL, pers. comm.).

Decreasing intracranial pressure

Recent studies using magnetic resonance imaging (MRI) in paediatric patients from Malawi demonstrated that children that died from CM had increased cerebral swelling, as compared to those who survived [89]. In fatal cases cerebral swelling progresses to respiratory arrest prior to death. A neuroimaging study in adult and paediatric patients with CM from India showed that both groups had traits characteristic of posterior reversible encephalopathy syndrome [90]. In a RCT in Kenya, mannitol adjunctive therapy controlled intermediate intracranial hypertension but could not prevent the development of intractable intracranial hypertension and did not affect mortality in children with CM [91]. An Ugandan RCT showed that one dose of mannitol had no adverse effects but also no impact on clinical outcomes or mortality in children with SM [92]. More recently, a computed tomography (CT) study demonstrated that brain swelling is a common finding in adults with CM, although brain swelling did not correlate with coma depth or survival [93]. In the same study, patients were randomized to receive either mannitol or placebo. The group receiving mannitol showed a longer coma duration and higher mortality [93]. A limited understanding of the pathogenic mechanism leading to increase brain swelling, inadequate doses of mannitol and small sample sizes may explain these results. In light of these findings, mannitol cannot be recommended as adjunctive treatment for malaria.

Fluid resuscitation

Appropriate fluid management in cases of SM has been controversial and there is no conclusive evidence to guide fluid management [73, 94]. While some studies have proposed an important role for impaired tissue perfusion in the outcomes of SM [95, 96], others have argued that hypovolemia does not occur in cases of severe and moderate malaria [97]. Some studies have explored the effects of fluid infusion in SM patients, and showed that fluid resuscitation with albumin compared with saline and gelofusine may reduce mortality [98, 99]. Recently, a large RCT (FEAST trial) was conducted in six different centres in Africa to compare volume expansion with boluses of albumin or saline to standard maintenance fluids in severely ill children [100]. The study was stopped because of higher mortality in the intervention groups. Fifty-seven per cent of those children had SM (1793 out of 3123 patients) and results in the malaria-confirmed cases were consistent with the larger group [100]. Excess of mortality seemed to be related to refractory shock rather than fluid overload in the boluses groups [101, 102]. Current recommendations indicate the need to individually assess the volume status of each patient to guide treatment, a general contra-indication for colloids, and in children a recommendation to avoid bolus fluids even in case of moderate hypotension and severe dehydration or metabolic acidosis [5].

Decreasing oxidative stress

Severe malaria is associated with oxidative stress that may be harmful due to the damaging effects of free radicals on cells, increased erythrocyte rigidity and impaired microcirculatory flow [103, 104]. N-acetylcysteine (NAC) is a widely used anti-oxidant that scavenges free radicals, and can reduce expression of endothelial ligands in SM [105]. The use of NAC as adjunctive agent to reduce the negative aspects of oxidative stress associated with SM infection has been investigated. A pilot study in Thailand demonstrated a shorter time in normalization of lactate levels and Glasgow Coma Score with NAC [106]. A RCT in 108 adults with SM showed NAC to be safe and well tolerated, but to have no effect on clinical outcomes or mortality [107]. In a placebo-controlled trial, intravenously administered NAC had no effect on mortality or acidosis, and did not reduce erythrocyte rigidity in adults with SM [108]. Involvement of NAC in the metabolism of isoprostanes may have hampered is anti-oxidative effect [108]. Furthermore, as mentioned previously, the rapid action of intravenous artesunate might have blurred its clinical impact.

Correcting lactic acidosis

Metabolic acidosis is central to the pathophysiology of SM and is an independent predictor of fatality in both adults and children [109–112]. Dichloroacetate (DCA) stimulates pyruvate dehydrogenase activity and promotes the removal of pyruvate, the precursor of lactate. In an attempt to neutralize metabolic acidosis, DCA has been tested in small safety trials in children and adults. DCA was shown to reduce initial blood lactate levels, however, whether DCA will improve the outcome of SM remains to be seen [113–116].

Reduced nitric oxide bioavailability

Nitric oxide (NO) is produced from L-arginine and molecular oxygen by members of the nitric oxide synthase (NOS) family [117]. Limited NO levels can contribute to a number of pathophysiological processes involved in SM, including activation of the endothelium,

stimulation of Weibel-Palade-body exocytosis, and increasing the expression of endothelial adhesion molecules (ICAM-1 and VCAM-1) [118, 119]. The use of inhaled NO (iNO) for the treatment of SM in children has been investigated in two RCTs. Both studies used iNO, administered at 80 parts per million for 48–72 h and both studies used markers of endothelial activation as their primary endpoints, namely the rate of decrease of Angiopoietin-2 (Ang-2), or the rate of increase in Angiopoietin-1(Ang-1) [120, 121]. Both studies found administration of iNO to be safe, but did not observe differences in circulating levels of Ang-1 and Ang-2 between treatment arms. It is possible that the dose and/or route of administration of NO was unable to cause a measurable effect on the endothelium or perhaps it is more suitable in the treatment of patients with increased cerebrovascular resistance [120, 121]. Alternative methods to increase NO levels, such as increasing plasma L-arginine levels via intravenous administration or increasing the bioavailability of cofactors required for NOS activity remain plausible interventions for adjunctive treatments [122–124].

Novel strategies for adjunctive therapy delivery (preclinical murine models)

Animal models remain an useful tool to investigate novel adjunctive therapy [13]. Despite the large volume of research in experimental murine models, this discussion will be limited to preclinical studies where improvements have been observed in relation to novel treatments administered at the onset of clinical symptoms in ECM, and exclude studies of prophylactic treatment. This probably best resembles a clinical scenario where patients with severe disease seek treatment. Studies where adjunctive interventions have shown to protect against ECM-induced neurocognitive impairment will also be discussed (Table 2).

Immunomodulation

New strategies to modify the immune response and target different pathways are ongoing. A recent study in ECM tested a new formulation of glucocorticosteroid, whereby β-methasone hemisuccinate (BMS) was encapsulated in lipososomes. Encapsulated BMS was less toxic to mice than the unencapsulated drug, and when

Table 2 Adjunctive therapy administered after the onset of neurological symptoms of ECM

Author, year, reference	Adjuvant Therapy	Route of administration	Outcome of treatment administered after neurological symptoms
Inmunomodulation			
Waknine-Grinberg et al. 2013, [124]	Glucocorticosteroids in liposomes	i.v. injection	Improved survival, prevented ECM symptoms, improved clinical scores
Dende et al. 2015, [127]	Curcumin	oral gavage	Improved survival, reduced parasitemia
Neuroprotection			
Dai et al. 2012, [129]	Lithium chloride	injection (route not described)	Prevention of cognitive and motor deficits. Reduced long-term motor coordination impairment. No effect on survival or parasitemia
Cabrales et al. 2010, [130]	Nimodipine	i.p. injection	Improved survival, improved motor score, reduced pial vasoconstriction
Martins et al. 2013, [132]	Nimodipine	s.c. osmotic pumps	Improved survival, reduced BBB dysfunction, reduced inflammation
Delivering gaseous signaling			
Orjuela-Sanchez et al. 2013, [133]	Glyceryl trinitrate	Transdermal patch	Improved survival, reversal of pial arteriolar vasoconstriction
Improving endothelial function			
Higgins et al. 2016 [140]	Recombinant human Ang-1	s.c. injection	Improved survival, prevents worsening of clinical outcomes, reduced cerebrovascular leak
Wilson et al. 2013, [141]	Atorvastatin	i.p. injection	Improved survival, reduced systemic and cerebral inflammation, reduces endothelial activation and reduced cerebrovascular leak
Dwivedi H et al. 2016, [145]	Vitamin D	i.m. injection	Improved survival, reduced cerebrovascular leak, reduced inflammation

CQ chloroquine, *ECM* experimental cerebral malaria, *i.m.* intramuscular, *IV* intravenous, *NO* nitric oxide, *s.c.* subcutaneous, *SM* severe malaria, *UM* uncomplicated malaria

administered at a late stage of infection it improved survival and prevented the development and progression of the cerebral syndrome [125]. These preclinical studies may lead to the use of new steroids for the treatment of SM.

Curcumin is an anti-inflammatory molecule that scavenges reactive oxygen and nitrogen species [126]. In vitro studies have shown that curcumin has additive anti-parasitic activity when used in combination with artemisinins [127]. When administered in combination with arteether to mice showing symptoms of CM, curcumin improved survival and prevented death due to anaemia [128].

Neuroprotection

Preclinical models have investigated lithium as a potential neuroprotective intervention. Lithium has been proposed to act as a neuroprotective agent by its ability to inhibit glycogen synthase kinase 3 (GSK3β), activate the PI3 K/Akt and MAPK signalling pathways, and by inducing the expression of brain-derived neurotrophic factors in neurons [129]. Lithium chloride administered to mice with ECM significantly increased the activation of Akt, which was associated with the prevention of adverse neurocognitive outcomes. Adjunctive treatment with lithium chloride was associated with better spatial and visual memory, and motor coordination in mice recovering from ECM [130].

Nimodipine is a calcium channel blocker that has been shown to prevent vasospasms, the abnormal physical narrowing of arteries in the sub-arachnoid space. Neuropathological features of CM include haemorrhages in the brain parenchyma [8]. It has been reported that mice with ECM show vasoconstriction and blood flow changes in the pia matter of the brain. Adjunctive treatment with nimodipine, when administered during late-stage infection, improved survival and improved blood flow to the brain [131]. However, potential hazards, such as hypotension, bradycardia and death can occur in humans treated with high doses of nimodipine [132]. Experiments have shown that in ECM, slow continuous administration of adjunctive nimodipine did not increase hypotension [133]. Additional preclinical work is required to determine if nimodipine is an attractive candidate as adjunctive therapy in SM.

Delivering gaseous signalling molecules

Increasing bioavailable NO in CM remains an attractive treatment strategy. A transdermal nitroglycerin patch was tested as an adjunctive therapy in late-stage ECM, where it increased plasma nitrate and nitrite levels (with no effect on blood pressure), and was associated with improved survival [134]. Haem oxygenase-1 (HO-1) catalyzes the degradation of haem and its activity has been shown to protect mice from ECM [135]. Prophylactic inhalation of carbon monoxide (CO), an end-product of this catalysis, prevents mice from developing ECM and malaria-associated acute lung injury [135, 136]. The toxicity of inhaled CO limits its clinical utility. However, CO-releasing molecules that can deliver controlled amounts of CO to tissues are valid alternatives [137]. The CO-releasing molecule ALF492 significantly improved survival in ECM when administered with artesunate beyond the anti-malarial alone and without affecting oxygen transport by haemoglobin [138].

Improving endothelial function

Targeting endothelial activation and preventing microvascular permeability and vascular leak in CM is another potential target for adjunctive therapy [139]. The angiopoietin (Ang)-Tie2 axis critically regulates endothelial cell function [140]. Perturbation of Ang-1, Ang-2 and soluble Tie2 concentrations are associated with disease severity and death in CM in both murine models and human infections [141]. A mechanistic role for the Ang-Tie2 axis was established in ECM, where it was shown that Ang-1-deficient mice were more susceptible to ECM and adjunctive administration of a recombinant Ang-1 construct preserved BBB integrity and improved survival beyond artesunate monotherapy alone [141]. These studies provide preclinical evidence that interventions that target the Ang-Tie2 axis are potential adjunctive therapy for SM.

Atorvastatin, a drug that reduces cholesterol levels, also inhibits the expression of CXCL10, high levels of which have been associated with CM mortality in adult patients [142]. Mice deficient in CXCL10 are partially protected against ECM [143] and mice receiving atorvastatin treatment in addition to artemether upon neurological signs of ECM had improved survival, and increased transcription of Ang-1 and reduced levels of Ang-2 in brain tissues [144].

Vitamin D may improve survival by targeting multiple pathways in both the innate and acquired immune systems [145]. One study showed that simultaneous administration of intramuscular arteether and vitamin D to mice at the onset of neurological symptoms of ECM improved survival. This survival was accompanied by reduced BBB leak and reduced levels of circulating pro-inflammatory cytokines [146].

Inhibition of the angiotensin pathway is another strategy to maintain endothelial integrity by preserving inter-endothelial cell junctions. Blocking the angiotensin II type 1 receptor with Irbesartan or activation of the type 2 receptor with compound 21 in combination with chloroquine resulted in an increased survival rate, higher than when treated with the anti-malarial alone, even when

mice were treated at the onset of neurological symptoms [147].

Conclusions

Malaria remains a major global health problem, associated with high morbidity and mortality. Strategies designed to improve early detection and recognition of cases likely to progress towards to severe disease, so as to trigger immediate treatment, are absolutely necessary. For those individuals who progress to severe forms of the disease despite prompt treatment, new tools are needed to improve outcomes in addition to existing anti-malarials. Preventing long-term *sequelae*, such as improving neurocognitive outcomes in SM survivors, should be an important consideration when it comes to potential adjunctive therapy; however so far, the majority of attempts to enhance the efficacy of anti-malarial drugs with adjunctive therapy have failed. The development of adjunctive therapy would benefit from a more complete understanding of the physiopathology of SM and CM, and how it differs between adults and children. The identification of host biomarkers associated with disease severity and host response to treatment could provide a useful read out of therapeutic efficacy, and empower RCTs to evaluate adjunctive therapy with smaller and better defined cohorts. Therapy tested in preclinical models of SM are still a valuable resource for potential adjunctive therapy; however preclinical models should employ scenarios as similar as possible to clinical practice, targeting the onset of clinical disease symptoms and prevention of long-term *sequelae*. RCTs in humans should also be guided by a rational and good design based on well-defined sample sizes, clinical predictors and study endpoints that permit detection of significant differences in SM outcomes and direct comparison between studies. It is difficult to extrapolate conclusions and conceive future research considering the heterogeneity of the RCTs in terms of anti-malarial used, type of malaria (SM and/or CM, coma), or study characteristics (limited number of patients per study, different and no comparable age of the populations, different treatment doses, studies not designed to identify differences in clinical outcomes or mortality). Further research, with promising candidates that surpass previous constraints of earlier studies, is urgently needed in order to accelerate the identification of new adjunctive therapy for the treatment of SM.

Abbreviations

ADHD: attention-deficit hyperactivity disorder; ALT: alanine aminotransferase; Ang: angiopoietin; Ang-1: angiopoietin-1; Ang-2: angiopoietin-2; AST: aspartate aminotransferase; BBB: blood–brain-barrier; BDNF: brain-derived neurotrophic factor; BMS: β-methasone hemisuccinate; CFR: case fatality rate; CM: cerebral malaria; CO: carbon monoxide; CS: curdlan sulfate; CT: computed tomography; DCA: dichloroacetate; DFO: desferrioxamine; EBT: exchange blood transfusions; ECM: experimental cerebral malaria; EPCR: endothelial protein C receptor; GAG: sulfated glycosaminoglycans; GSK3β: glycogen synthase kinase 3; HIV: human immunodeficiency virus; HO-1: haem oxygenase-1; iNO: inhaled nitric oxide; iRBC: infected red blood cells; MRI: magnetic resonance imaging; NAC: N-acetylcysteine; NO: nitric oxide; oAc: activated charcoal; PEs: parasitized erythrocytes; PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1; PPAR-γ: peroxisome proliferator-activated receptor-γ; RCT: randomized controlled trial; rHuEPO: recombinant human erythropoietin; SM: severe malaria; SMA: severe malarial anemia; TNF: tumour necrosis factor; PTX: pentoxifylline; oAC: oral activated charcoal; UDCA: ursodeoxycholic acid.

Authors' contributions

VMC, RV, QB, and KC conceived the review. VMC and RV performed the literature search and selected the relevant articles. VMC, RV, QB, and KC drafted the manuscript. AS, LS, LM, QB, KK, VMC, and RV critically revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Post-malarial anemia in Mozambican children treated with quinine or artesunate: A retrospective observational study

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ABSTRACT

Objectives: This retrospective analysis performed in Manhiça, Southern Mozambique, aimed to describe the frequency of post-malarial anemia (measured as a decrease of hematocrit ≥10%) and the need for blood transfusions in children with severe malaria treated with intravenous quinine or parenteral artesunate.
Methods: All children <15 years admitted with a parasitologically-confirmed diagnosis of malaria from 1st January 2003 to 31st December 2017, alive at hospital discharge, and with at least one measurement of hematocrit within 28 days after hospital discharge, detected by passive case detection, were included.
Results: The overall prevalence of post-malarial anemia observed in the study was 23.13%, with an estimated incidence rate of 288.84 episodes/1,000 children-month at risk in the follow-up period (28 days after discharge). There were no differences between treatment groups, although the study showed a higher association between blood transfusions and artesunate treatment.
Conclusions: In this setting, children with severe malaria frequently present a meaningful decrease of hematocrit (>=10%) in the first weeks after their episode, sometimes requiring blood transfusions. Because of the high underlying prevalence of anemia in malaria-endemic settings, all children with severe malaria need to be actively followed up, irrespective of the treatment received.

Introduction

Malaria remains one of the most important global parasitic diseases, causing 219 million cases and around 435 000 deaths in 2017 (WHO, 2019). This overwhelming burden makes the optimal management of malaria a global health priority. In terms of curative efficacy, parenteral artesunate is undoubtedly superior to quinine (Dondorp et al., 2005, 2010), and thus is currently recommended globally as the standard of care for the treatment of severe malaria. Meta-analyses have also confirmed that artesunate has a better short-term safety profile than quinine (Sinclair et al., 2012). However, there is a paucity of data on the

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mid-term adverse events after the treatment period. Furthermore, in real-life conditions, late complications after discharge may be undetected under routine health assistance, considering the fragile health infrastructure in most countries where malaria is highly endemic (Rolling et al., 2014). Consequently, it was not until the introduction of parenteral artesunate as the first-line treatment for imported malaria in non-endemic countries that reports of post–artesunate delayed hemolysis (PADH) emerged (Rolling et al., 2015). These reports have shown clinically relevant PADH occurring in around 15-30% of patients, typically peaking 2-4 weeks after treatment, causing a frequent need for blood transfusions in the most severely ill patients (Gomez-Junyent et al., 2017; Jaureguiberry et al., 2015; Kreeftmeijer-Vegter et al., 2012; Kurth et al., 2017; Lahoud et al., 2015; Rehman et al., 2014; Rolling et al., 2015; Rolling et al., 2013; Roussel et al., 2017; Zoller et al., 2011). The primary mechanism behind these episodes of hemolytic anemia probably relates to the splenic clearance of erythrocytes by pitting (Arguin, 2014; Jaureguiberry et al., 2014). However, evidence generated from non-endemic countries cannot be directly generalized to endemic countries where vulnerable age groups, patient characteristics, clinical manifestations, or quality of care, among other things, may significantly differ (Cramer et al., 2011).

Notably, among specific populations of endemic countries, such as for instance young children, anemic episodes related to malaria treatment could have a profound and synergistic impact, due to the underlying concomitant conditions that are already highly prevalent in these settings, and that are known contributors to the high prevalence of anemia, including iron deficiency, hemoglobinopathies, malnutrition or chronic infections (Kassebaum et al., 2014; Moraleda et al., 2017). Data from different malaria studies conducted so far among African children show a lower incidence of post-malarial anemic events than in patients from non-endemic areas (Burri et al., 2014; Fanello et al., 2017; Hawkes et al., 2019; Rolling et al., 2014; Sagara et al., 2014; Scheu et al., 2019). However, the scarce data available regarding the mid-term safety in endemic areas of a drug so widely utilized is of concern, as such a potential side-effect could have a significant public-health impact in countries where malaria and anemia co-exist, mainly because safe blood products are not readily accessible.

This retrospective analysis aimed to determine, in a rural setting in Mozambique, whether the use of parenteral artesunate in comparison with intravenous quinine, for the treatment of malaria in children, was associated with a higher occurrence of post-malarial anemia (defined as a decrease of hematocrit values >=10%) and a higher need for blood transfusions.

Methods

Study design

This is a retrospective analysis of data collected through the Manhiça District Hospital (MDH) outpatient and inpatient pediatric morbidity surveillance system (MSS). All children under 15 years admitted with a diagnosis of malaria from 1 st January 2003 to 31st December 2017, confirmed to be discharged alive from the hospital, and with at least one measurement of hematocrit within 28 days after hospital discharge detected by passive case detection, were included.

Study setting

Mozambique is one of the ten countries with the highest malaria endemicity in the world, accounting for about 4% of the total global malaria prevalence (WHO, 2019). Mozambique's entire population, estimated to be around 29 million people, is at risk of malaria, and estimates suggest that in 2017, there were approximately 10 million cases and 14,700 estimated deaths (WHO, 2019). In-country parasite prevalence rates are variable but can range from <3% to more than 50%. Artesunate has been recommended as the first-line drug for severe malaria in Mozambique since 2011 (although the drug did not become fully available until 2013), and the shift from quinine to artesunate has gradually occurred across the country (Armindo Daniel Tiago et al., 2011).

This study was conducted in Manhiça, Southern Mozambique. For the past 20 years, the *Centro de Investigação em Saúde de Manhiça* (CISM; Manhiça Health Research Centre) has been running a demographic surveillance system (DSS), and around-the-clock MSS at the neighboring MDH, which sees around 75,000 pediatric outpatients and admits an average of ≈S3,000 children annually (Sacoor et al., 2013). Malaria in Manhiça district is perennial, although with a clear seasonality (November-April), coinciding with the rainy season. The district's malaria incidence has markedly changed in the last two decades, ranging from a high initial transmission period (2003–2007) to a moderate-to-low one after that. The leading causes of admission and under-five mortality in Manhiça are malaria, pneumonia, diarrhea, malnutrition, and neonatal pathologies (Sacarlal et al., 2009). The prevalence of anemia in children admitted to MDH has been proven to be high, with undernutrition, iron deficiency, HIV infection, and malaria being the main contributors identified (Moraleda et al., 2017). Data from the Manhiça district, generated by CISM using the outpatient and inpatient pediatric MSS databases, confirm different micro-epidemiological and clinical malaria patterns within its study area (Bassat et al., 2008; Guinovart et al., 2008). A comprehensive characterization of MDH, CISM, and the study area can be found elsewhere (Sacoor et al., 2013).

Hospital surveillance system

Standardized outpatient and admission questionnaires, which include demographic, clinical, laboratory, and outcome data, are routinely completed for all children <15 years of age attending or being admitted to MDH. On arrival, all children with documented fever (>=37.5 °C, axillary), a history of fever in the preceding 24 hours, or suspected anemia, are given a finger-prick blood sample to measure packed cell volume (PCV); thick and thin blood films are prepared or histidine-rich protein 2 (HRP2)-based rapid diagnostic tests (RDT) are conducted to screen for *P. falciparum* infection. HIV status information is not routinely collected. Once the child is discharged alive or has a fatal outcome, up to four final diagnoses, based on the International Classification of Diseases system version 10 (ICD-10), and treatments received are recorded in the questionnaire after reviewing all available results.

Laboratory methods

PCV was measured using a microcentrifuge and a Hawksley hematocrit reader card (Hawksley and Sons Ltd., Lancing, United Kingdom). Thick and thin blood films for malaria diagnosis were processed as previously detailed (Bassat et al., 2008; Bassat et al., 2009). The Lambaréné method, which counts parasites against an assumed known blood volume, is the method used to calculate parasitemia (Planche et al., 2001), which is considered negative if no parasites are detected after examination of 200 oil-immersion fields in a thick blood film. For routine clinical management, CISM's laboratory uses a semiquantitative "cross' system, ranging from 0 (no malaria infection) to 5 (high parasitemia infection) (WHO, 1999).

Definitions

All case definitions were based on admission data from the standardized questionnaires. A malaria case was defined as a child admitted with fever or a history of fever in the preceding 24 hours, *P. falciparum* asexual parasitemia > 0 parasites/μL (1- 5 crosses) (WHO, 1999), and a clinical diagnosis of malaria provided by the discharging clinician. Severe malaria cases were defined according to World Health Organization (WHO) definitions, as previously described (Bassat et al., 2008). The Blantyre coma score (BCS) was used to characterize consciousness. Deep coma and impaired consciousness were defined for BCS ≤ 2 and BCS < 5, respectively. Repeated convulsions were defined when occurring two or more times in a day. Prostration was defined as the inability to sit unaided or look for a mother's breast/feed in children who were not yet able to sit. Respiratory distress was defined as deep breathing or indrawing. Hypoglycemia was defined as glycemia <2.2 μmol/L (WHO, 2014). Moderate anemia was defined as hematocrit < 42% for children ≤28 days and hematocrit < 33% for children > 28 days. Severe anemia was defined as hematocrit < 25% for children ≤ 28 days and hematocrit < 15% for children > 28 days. Post-malarial anemia was defined as a decrease of at least 10% from the hematocrit value at the initial hospital admission for any hematocrit value determined within 28 days after discharge. Rather than considering the initiation of treatment as the start of follow-up, as proposed by Jauréguiberry et al. (Jaureguiberry et al., 2014), we have chosen discharge as the beginning of follow-up for two main reasons: (1) A lack of hematocrit data from admitted patients beyond recruitment because these are not routinely collected and not included in the standardized admission questionnaires; and (2) The short mean length of admission (quinine group: 3.05 days (95% CI: 2.94 -3.16); and artesunate group: 2.62 days (95% CI: 2.14-3.10) (p-value: 0.0173)) with very few episodes leading to hospitalizations longer than seven days. As we considered this decrease as a non-recurring event, the analysis was restricted to the first episode of a decrease of hematocrit ≥10% in children treated for severe malaria (Arguin, 2014). In the absence of systematic measurements for every child, laboratory markers of hemolysis as lactate dehydrogenase or haptoglobin values (not routinely available in this rural setting), or etiological data of anemia such as iron deficiency, hemoglobinopathies, bacteremia, viral infections (Parvovirus B19, Ebstein Barr Virus, HIV) or intestinal parasitic infections, it was only feasible to describe the general occurrence of anemia (irrespective of type) after treatment in this population. Nutritional status was assessed using anthropometrical Z-scores.

Case management

Children diagnosed with malaria were managed according to Mozambican national guidelines. During the initial study period (January 2003–September 2006), first-line treatment for uncomplicated malaria included amodiaquine plus sulfadoxine-pyrimethamine (SP). In September 2006, this changed to artesunate plus SP and from 2009 onwards to artemether-lumefantrine, Coartem®). From January 2003 to May 2013, the first-line treatment for severe malaria included parenteral quinine (with an initial loading dose of 20 mg/kg plus subsequent 10 mg/kg doses, three times a day) for a minimum of six doses if completed with treatment with SP, or 21 doses when used as monotherapy. Treatment was switched to oral as soon as the child clinically improved and was able to tolerate it. In this period, artesunate was not available in Manhiça. In 2013, quinine was progressively replaced by artesunate (2.4 mg/kg immediately, then at 12, 24 h and then once daily until oral medication could be taken reliably, for a minimum of three doses). In 2015, and for children weighing less than 20 kg, the dose was increased to 3 mg/kg following an update in WHO recommendations (WHO, 2015). Blood transfusions were restricted to children with a PCV < 12%, hemoglobin <4 g/dL (when available), or to children with higher values but with clinical signs of decompensation (respiratory distress or signs of heart failure) or neurological impairment (Bassat et al., 2008). Facilities for intensive care are not available at MDH. All clinical assistance and treatment of admitted children are free of charge. Children requiring specialized care were transferred to Maputo Central Hospital.

Data management and statistical methods

This study includes all children under 15 years admitted with a diagnosis of malaria during 2003-2017, alive at hospital discharge, and with at least one measurement of hematocrit within 28 days from hospital discharge, detected by passive case detection.

Qualitative variables were compared using a χ^2 test or Fisher's exact test. Quantitative variables were compared using the Student t-test. Variables with statistically significant differences between treatment groups were used for model estimates adjusted for imbalances in baseline characteristics in children with post-malarial anemia.

Incidence rates were reported as the number of events per 1000 Children-month at risk (CMAR), with 95% confidence intervals (CIs). Differences in time to first-or-only episodes within 28 days after hospital discharge between treatment groups were analyzed by the Log-rank test and Cox regression. Negative binomial regression models were estimated to compare incidence rates of multiple episodes between treatment groups. The association of treatment with at least one episode within 28 days after hospital discharge was assessed by logistic regression models. Also, the equivalence of the cumulative incidence functions between the two treatment groups, accounting for the risk of dying before reaching specified outcomes, was assessed by the weighted log-rank test as previously proposed (Fine and Gray, 1999); competing risk regression of sub-hazard ratios was done according to Gray (Gray, 1988) with death as the competing risk to evaluate the association between outcomes and treatment adjusted for other covariates.

Anthropometrical Z-scores were computed using the LMS method and the British and WHO Child Growth Standards composite data file for term births implemented by the zanthro command in Stata (StataCorp, 2017). Statistical comparisons were performed at a two-sided significance level of 0.05, and 95%. Confidence Intervals were calculated for all estimations. All analyses were performed using Stata/SE software version 14.1 (StataCorp, 2017)

Ethical approval

This study retrospectively assessed data collected in the context of routine clinical practice. The MSS in place at MDH has been approved by the National Bioethics Committee for Health of Mozambican (CNBS-IRB00002657). The analytical plan of this specific analysis was assessed and approved by CISM's Internal Scientific Committee.

Results

During the 15-year study period (1st January 2003 to 31st December 2017), 23523 children <15 years of age were admitted to MDH. On admission, 9523/23523 (40.48%) children had a malaria diagnosis according to the case definition. Among these, 9461/9523 (99.34%) were alive at discharge; 62/9523 (0.65%) hospital deaths were registered. Fifty-five out of the 62 malaria deaths were

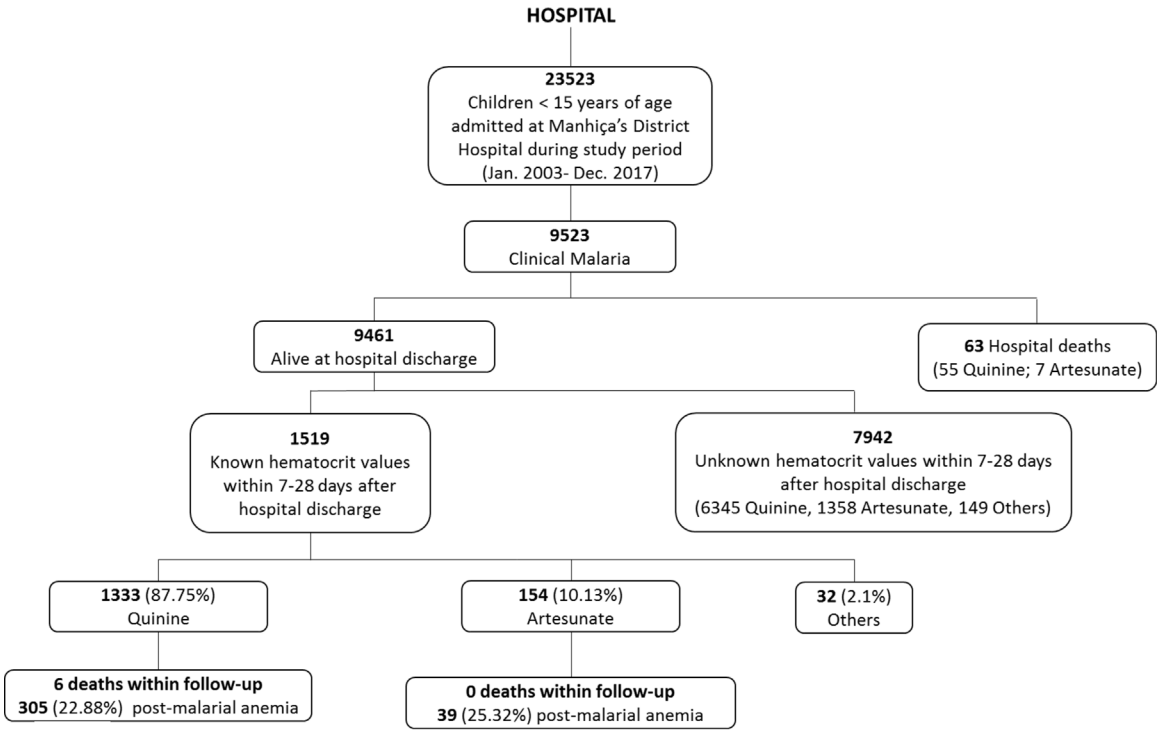

Figure 1. Study profile.

children treated with quinine, and five out of 62 were treated with artesunate (Figure 1). Of those alive, 1519 (16.05%) children had at least one passive case detection contact with the MSS, including at least one hematocrit value detected within the 28 days after hospital discharge. There were 7942 children with unknown values of hematocrit (6345 quinine, 1358 artesunate, 149 others). Children without hematocrit data were excluded from the analysis. Of those with hematocrit data, 1333 (87.75%) had initially received quinine for their malaria treatment, 154 (10.13%) parenteral artesunate, and 32 (2.1%) other treatments.

Baseline characteristics of children by treatment group (univariate analysis)

Table 1 compares the baseline characteristics of admitted children treated with artesunate or quinine. Values of hematocrit during their original admission and proportion of malaria cases with severe anemia were similar in both groups. Children treated with quinine tended to have higher respiratory and heart rates ($p < 0.0001$, both), and they also had a higher percentage of palpable spleens ($p < 0.0001$). Children receiving artesunate were more prone to present with impaired consciousness and deep coma ($p = 0.0115$ and $p = 0.0190$).

Prevalence of post-malarial anemia

Of those children with known hematocrit values within 28 days after hospital discharge, 154/1519 (10.13%) had been treated with artesunate, and 1333/1519 (87.75%) had received quinine (Figure 1). No differences were observed in terms of the prevalence of post-malarial anemia according to the treatment group: 22.8% (305/1333) in the quinine group vs. 25.32% (39/154) in the artesunate group (OR = 1.14, 95% CI = 0.78, 1.68; p-value: 0.4962) (Table 2).

Post-malarial anemia incidence rates

The overall incidence rate of post-malarial anemia episodes throughout the study period was 288.84 episodes/1000 CMAR (CI: 259.88, 321.04). There were no differences in the incidence of anemia between children receiving artesunate and those treated with quinine (285.42 episodes/1000 CMAR vs. 318.8 episodes/1000 CMAR; Hazard ratio (HR): 1.12, 95% CI: 0.81-1.57; p-value: 0.4879) (Table 3). Mean time to post-malarial anemia episode was 16.89 days (95%CI: 16.06-17.72) in the artesunate group and 16.74 days (95%CI: 14.40-19.08) in the quinine group, without statistical difference (p-value: 0.9078). The cumulative incidence curve estimates for time to the first-or-only episode of post-malarial anemia did not show any differences according to treatment received (Weighted log-rank test for the Cumulative Incidence of anemia episode: Chi2(1) = 0.48; p-value = 0.4877) (Figure 2).

Given that this was not a randomized control trial, it would appear essential to adjust the comparison to potential existing confounders. The adjusted analysis further explored the differences between the two treatment groups, identifying splenomegaly as the single factor that remained independently associated with post-malarial anemia during the 28 period days post-discharge (Supplementary material, tables 1 and 2), with no evidence of other significant differences.

Blood transfusions

The overall rate of blood transfusions during the follow-up period was 20.64 episodes/1,000 CMAR (CI: 14.15, 30.09). Artesunate-recipients showed a higher rate when compared to the quinine group (6/154 vs. 21/1133; 44.73 episodes/1,000 CMAR vs. 17.88 episodes/1,000 CMAR, respectively) (HR: 2.50; CI = 1.01–6.19; p-value: 0.0479) (Table 4). This difference was still statistically significant when adjusting for other variables (supplementary material, Table 3).

Table 1
Univariate analysis of clinical variables and diagnosis according to treatment group.

Variable		Treatment		Total (N = 1487)	p-value
		Quinine (N = 1333)	Artesunate (N = 154)		
Sex [1]	Male	701 (53%)	74 (48%)	775 (52%)	0.4438[2]
	Female	631 (47%)	76 (49%)	707 (48%)	
Age at discharge [1]	0-<1y	290 (22%)	15 (10%)	305 (21%)	< 0.0001 [2]
	1y-<5y	922 (69%)	100 (65%)	1022 (69%)	
	5y<-15y	121 (9%)	39 (25%)	160 (11%)	
Parasitemia at admission [1]	Low (1-2 crosses)	126 (9%)	10 (6%)	136 (9%)	< 0.0001 [2]
	Medium (3-4 crosses)	650 (49%)	43 (28%)	693 (47%)	
	High (>4 crosses)	557 (42%)	101 (66%)	658 (44%)	
Length of admission (days) [3]		3.05 (2.94, 3.16) [1333]	2.62 (2.14, 3.10) [154]	3.00; (2.90, 3.11) [1487]	0.0173 [4]
Weight (kg) [3]		11.34 (11.08, 11.61) [1332]	13.83 (13.04,14.63) [154]	11.60; (11.34,11.85) [1486]	< 0.0001 [4]
Height (cm) [3]		83.80 (82.89, 84.72) [1217]	92.63 (89.61, 95.65) [135]	84.69; (83.80, 85.57) [1352]	< 0.0001 [4]
BMI: Body Mass Index (kg/m^2) [3]		15.83 (15.54, 16.13) [1216]	15.64 (15.30, 15.98) [135]	15.82; (15.55, 16.08) [1351]	0.6673 [4]
Weight for age z-score [3]		−0.90 (-0.97, -0.84) [1312]	−0.91 (-1.10, -0.72) [149]	−0.90; (-0.97, -0.84) [1461]	0.9587 [4]
Length/height for age z-score [3]		−0.96 (-1.05, -0.87) [1185]	−0.97 (-1.23, -0.71) [129]	−0.96; (-1.05, -0.88) [1314]	0.9673 [4]
BMI for age z-score [3]		−0.36 (-0.44, -0.28) [1182]	−0.27 (-0.51, -0.03) [130]	−0.35; (-0.42, -0.27) [1312]	0.4979 [4]
Malnutrition as a secondary diagnosis (E40-E46 at ICD-10) [5]		34 / 1333 [3%; (2, 4)]	0 / 154 [0%; (0, 2)]	34 / 1487 [2%; (2, 3)]	0.0450 [2]
Palpable spleen [5]		355 / 1332 [27%; (24, 29)]	17 / 153 [11%; (7, 17)]	372 / 1485 [25%; (23, 27)]	< 0.0001 [2]
Palpable liver [5]		17 / 1332 [1%; (1, 2)]	3 / 153 [2%; (0, 6)]	20 / 1485 [1%; (1, 2)]	0.4523 [6]
Temperature [3]		38.37 (38.29, 38.44) [1328]	38.57 (38.37, 38.77) [153]	38.39; (38.32, 38.46) [1481]	0.0727 [4]
Heart rate [3]		132.75 (131.34, 134.16) [1313]	113.37 (109.58, 117.16) [154]	130.71; (129.36, 132.07) [1467]	< 0.0001 [4]
Respiratory rate [3]		41.06 (40.46, 41.66) [1325]	35.88 (34.29, 37.46) [154]	40.52; (39.95, 41.09) [1479]	< 0.0001 [4]
Glycemia (mmol/L) [3]		6.12 (5.95, 6.29) [1257]	6.29 (5.98, 6.60) [118]	6.13; (5.98, 6.29) [1375]	0.5549 [4]
Hematocrit at admission [3]		27.29 (26.91, 27.68) [1333]	27.98 (26.83, 29.14) [154]	27.36; (27.00, 27.73) [1487]	0.2581 [4]
Severe anemia (PCV < 25% if < 28 days, PCV < 15% if ≥ 28 days) [5]		34 / 1333 [3%; (2, 4)]	5 / 154 [3%; (1, 7)]	39 / 1487 [3%; (2, 4)]	0.5916 [6]
Deep Coma (BCS ≤ 2) [5]		11 / 1332 [1%; (0, 1)]	5 / 154 [3%; (1, 7)]	16 / 1486 [1%; (1, 2)]	0.0190 [6]
Impaired consciousness (BCS < 5) [5]		42 / 1332 [3%; (2, 4)]	11 / 154 [7%; (4, 12)]	53 / 1486 [4%; (3, 5)]	0.0115 [2]
Repeated convulsions (≥2/24 h) [5]		73 / 218 [33%; (27, 40)]	7 / 35 [20%; (8, 37)]	80 / 253 [32%; (26, 38)]	0.1112 [2]
Hypoglycemia (<2.2 mmol/L) [5]		16 / 1260 [1%; (1, 2)]	1 / 120 [1%; (0, 5)]	17 / 1380 [1%; (1, 2)]	1.0000 [6]
Prostration [5]		150 / 1333 [11%; (10, 13)]	18 / 154 [12%; (7, 18)]	168 / 1487 [11%; (10, 13)]	0.8716 [2]
Respiratory distress [5]		98 / 1332 [7%; (6, 9)]	6 / 154 [4%; (1, 8)]	104 / 1486 [7%; (6, 8)]	0.1109 [2]

1: n (Column percentage). 2: Chi-squared test. 3: Arithmetic Mean (95% Confidence Interval) [n]. 4: t-test. 5: n [Column percentage; (95% Confidence Interval)]. 6: Fisher's exact test

Table 2
Prevalence of anemia within 28 days after discharge.

Treatment	Clinical malaria admissions	Anemia episodes	Rate estimations		Model estimations		
			Prevalence (%)	95% ConfidenceInterval	Odds Ratio	95% Confidence Interval	p-value
Quinine	1333	305	22.88	(20.65, 25.23)	1	-	0.4962
Artesunate	154	39	25.32	(18.67, 32.95)	1.14	(0.78, 1.68)	
Total	1487	344	23.13	(21.01, 25.36)	-	-	-

Table 3
Incidence of post-malarial anemia within 28 days after discharge.

Treatment	Clinical malaria admissions	Delayed anemia episodes	Time At Risk (CMAR)	Rate estimations		Model estimations		
				Incidence Rate (Episodes per 1000 CMAR)	95% Confidence Interval	Hazard Ratio	95% Confidence Interval	p-value
Quinine	1333	305	1068.62	285.42	(255.12, 319.31)	1	-	0.4879
Artesunate	154	39	122.34	318.8	(232.92, 436.33)	1.12	(0.81, 1.57)	
Total	1487	344	1190.95	288.84	(259.88, 321.04)	-	-	-

CMAR: children month at risk

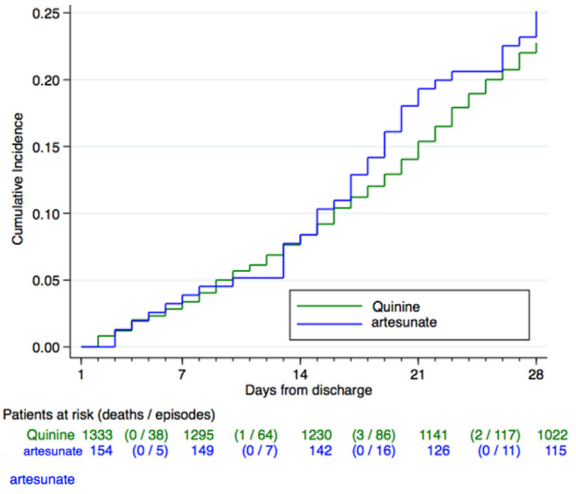

Figure 2. Weighted log-rank test for the Cumulative Incidence of anaemia episodes: Chi2 = 0.48; p-value = 0.4877.

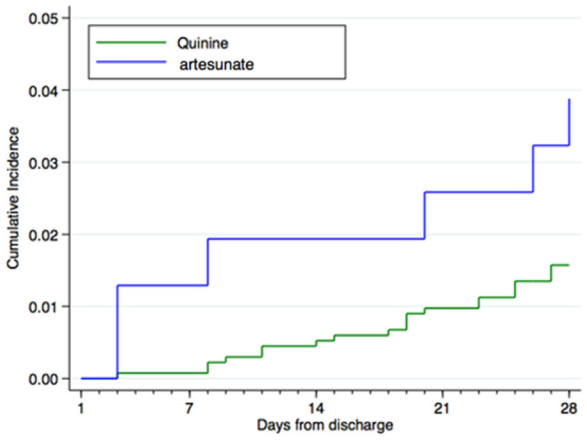

Figure 3. Weighted log-rank test for the Cumulative Incidence of blood transfusions: Chi2(1) = 4.23; p-value = 0.0398.

Figure 3 shows the cumulative incidence curve estimates for blood transfusions by treatment group (Weighted log-rank test for the Cumulative Incidence of blood transfusions: Chi2 = 4.23; p-value = 0.0398).

Discussion

The overall prevalence of post-malarial anemia observed in the study was 23.13%, with an estimated incidence rate of 288.84 episodes/1,000 CMAR in the follow-up period (28 days after discharge). There were no differences between treatment groups, although the study showed a higher association of blood transfusion treatment with artesunate.

Rolling et al. (Rolling et al., 2014) conducted a prospective observational study to investigate delayed hemolysis in African children with severe malaria treated with parenteral artesunate and found a percentage of delayed hemolysis of 7% in a cohort of 72 children (7). Children who presented with delayed hemolysis were younger and had higher mean parasitemia than those without it. In a recent update of this study, Rolling et al. (Scheu et al., 2019) found a slightly lower percentage (5%) and stated that PADH and hyperparasitemia were associated with early malarial anemia (Scheu et al., 2019). However, Rolling et al. used a restrictive definition of delayed hemolysis, taking into account levels of hemoglobin, lactate dehydrogenase, and haptoglobin during an active follow-up. This may explain the differences with the current analysis, which was based on passive detection, whereby only levels of hematocrit were evaluated without using specific markers of hemolysis. A prospective study developed in The Democratic Republic of the Congo (Burri et al., 2014) in patients treated with parenteral artesunate also demonstrated a decrease in hemoglobin levels between days seven and 21 after treatment in 11.4% of patients. Only 1% of cases presented severe anemia during follow-up. All delayed anemia cases were clinically manageable and evolved without complications. Another recent study in 91 Ugandan children with severe malaria treated with parenteral artesunate found that none of those patients met their standardized definition of PADH (Hawkes et al., 2019) and, like others, anemia was prevalent on admission. The abovementioned studies lacked a control group, and the criteria used to investigate anemia hindered the extrapolation and comparison with the results from the current analysis.

An open-label, randomized controlled trial study conducted in The Democratic Republic of Congo by Fanello et al. (Fanello et al., 2017) compared the proportion of children with a $\geq$ 10% reduction in hemoglobin during the six weeks after treatment with quinine or parenteral artesunate and was able to demonstrate that up to 5% of the patients in each group presented a delayed anemia episode. The authors could not show any statistically significant differences between groups of treatment (Fanello et al., 2017). In a meta-analysis performed pooling data from a variety of clinical trials for the treatment of uncomplicated malaria with oral artemisinin derivatives, no association was found between the use of oral artemisinin-based therapies and the incidence of delayed anemia (defined as anemia -severe or not- observed any time of follow-up from day seven to day 28) although a significant drop of haemoglobin on day seven after treatment was detected (Sagara et al., 2014).

This study found a higher association of blood transfusions with the use of artesunate treatment. One possible explanation could be the decreases in malaria transmission and the associated changes in severe malaria presentation with older and less immune children in the artesunate period. Also, a less conservative use of blood products could have influenced these differences.

This study has several important limitations. First, most of the children included in the analysis received quinine in comparison to artesunate. This difference in the number of treatments may be explained for different reasons, including a decrease of malaria

Table 4
Incidence of blood transfusions within 28 days after discharge.

Treatment	Clinical malaria admissions	Blood transfusions	Time at Risk (CMAR)	Rate estimations		Model estimations		
				Incidence Rate (Episodes per 1000 CMAR)	95% ConfidenceInterval	Hazard Ratio	95% Confidence Interval	p-value
Quinine	1333	21	1174.18	17.88	(11.66, 27.43)	1	-	-
Artesunate	154	6	134.14	44.73	(20.09, 99.56)	2.51	(1.01, 6.21)	0.0471
Total	1487	27	1308.32	20.64	(14.15, 30.09)	-	-	-

CMAR: children month at risk

cases, more rational management of those cases -including severe ones- or a more extended period of quinine use.

Second, the study used hematocrit to define anemia, and it is well known that PCV may not accurately represent hemoglobin levels and the degree of anemia (Quinto et al., 2006). Third, more detailed data on other common causes of anemia such as iron deficiency, hemoglobinopathies, bacteremia, viral infections (Parvovirus B19, Epstein Barr Virus, HIV) or intestinal parasitic infections, which could have confounded the relation studied, were not systematically available, and therefore the post-malarial anemia is likely an overestimation (Moraleda et al., 2017). Besides, the absence of systematic follow-up of all discharged alive patients undoubtedly has contributed to missing a significant number of cases. This low follow-up rate is a good reason for caution in the generalization and extrapolation of the results. Finally, the relatively small sample size for the artesunate component (only 129 children treated with artesunate with a measure of their potential anemia available in the following 28 days) and the low number of anemia cases, may have impaired the statistical power of some of the comparisons; larger sample sizes could have helped to provide more conclusive evidence.

Conclusions

This study found a high overall frequency of post-malarial anemia in children in Mozambique but was unable to find differences in children treated with either intravenous quinine or parenteral artesunate. The high burden described in this study irrespective of the treatment received, and the high prevailing risk of multifactorial anemia in this setting, urgently call for the establishment of an active follow-up system to ensure the well-being of the ones who survive their malarial episodes.

Authors' contribution

RV, LM, and QB conceived the study and contributed to the design. LQ, QB, and RV analyzed and interpreted the data. RV wrote the first draft of the manuscript, together with QB. All authors critically revised and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

This study retrospectively assessed data collected in the context of routine clinical practice. The MSS in place at MDH has been approved by the National Bioethics Committee for Health of Mozambican (CNBS-IRB00002657). The analytical plan of this specific analysis was assessed and approved by CISM's Internal Scientific Committee.

Consent for publication

All the authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.05.089.

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Malaria Journal

RESEARCH Open Access

African isolates show a high proportion of multiple copies of the *Plasmodium falciparum plasmepsin-2 gene*, a piperaquine resistance marker

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Abstract

Background: Today, the development of new and well-tolerated anti-malarial drugs is strongly justified by the emergence of *Plasmodium falciparum* resistance. In 2014–2015, a phase 2b clinical study was conducted to evaluate the efficacy of a single oral dose of Artefenomel (OZ439)–piperaquine (PPQ) in Asian and African patients presenting with uncomplicated falciparum malaria.

Methods: Blood samples collected before treatment offered the opportunity to investigate the proportion of multidrug resistant parasite genotypes, including *P. falciparum kelch13* mutations and copy number variation of both *P. falciparum plasmepsin 2* (*Pfpm2*) and *P. falciparum multidrug resistance 1* (*Pfmdr1*) genes.

Results: Validated *kelch13* resistance mutations including C580Y, I543T, P553L and V568G were only detected in parasites from Vietnamese patients. In Africa, isolates with multiple copies of the *Pfmdr1* gene were shown to be more frequent than previously reported (21.1%, range from 12.4% in Burkina Faso to 27.4% in Uganda). More strikingly, high proportions of isolates with multiple copies of the *Pfpm2* gene, associated with piperaquine (PPQ) resistance, were frequently observed in the African sites, especially in Burkina Faso and Uganda (> 30%).

Conclusions: These findings were considered to sharply contrast with the recent description of increased sensitivity to PPQ of Ugandan parasite isolates. This emphasizes the necessity to investigate in vitro susceptibility profiles to PPQ of African isolates with multiple copies of the *Pfpm2* gene and estimate the risk of development of PPQ resistance in Africa.

Trial registration Clinicaltrials.gov reference: NCT02083380. Study title: Phase II efficacy study of artefenomel and piperaquine in adults and children with *P. falciparum* malaria. https://clinicaltrials.gov/ct2/results?cond=&term=NCT02083380&cntry=&state=&city=&dist=. FSFV: 23-Jul-2014; LSLV: 09-Oct-2015

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Background

Emergence of *Plasmodium falciparum* resistance to anti-malarial drugs is currently the primary rationale supporting the development of new and well-tolerated drugs. While the estimated number of malaria cases in the world decreased from 237 million (218–278 million) in 2010, to 211 million (192–257 million) in 2015, the morbidity and the mortality have stabilized in 2016 with estimates of 216 million cases (196–263 million) and 445,000 deaths (compared to 446,000 in 2015) as reported by the World Health Organization (WHO) [1–3].

Globally, the vast majority of deaths (>90%) caused by malaria is due to *P. falciparum* infections, occurring in Africa, in children under 5 years of age. Artemisinin-based combination therapy (ACT) which are currently recommended as first-line treatment of uncomplicated falciparum malaria, is less effective in Southeast Asia, particularly in Cambodia, where high rates of treatment failure associated with artemisinin and piperaquine resistance are currently reported [4–16]. The containment and the elimination of these multidrug resistant parasites in Southeast Asia are a priority for the WHO to avoid their spread to Africa as was the case with previous generations of anti-malarial drugs (e.g. chloroquine, sulfadoxine–pyrimethamine) [17]. Fortunately, molecular markers associated with such resistance are available [10]. In particular, mutations in the propeller domain of a *kelch* gene located on the chromosome 13 (*kelch13*), and amplification of a cluster of genes encoding both *plasmepsin 2* (*Pfpm2*) and *plasmepsin 3* proteins, have been recently shown to be associated with artemisinin and PPQ resistance, respectively [18–20].

According to the latest WHO update on artemisinin resistance [21], to be validated a *kelch13* resistance mutant has to be correlated with delayed parasite clearance in clinical studies and reduced drug in vitro susceptibility with survival rate $\geq$1% expressed by the Ring-stage Survival Assay, (RSA0–3 h) in fresh isolates (ex vivo assays), or culture-adapted field parasites or *kelch13* genome-edited parasites (in vitro assays) [22–25]. To date, only nine *kelch13* mutations have been shown to be validated (C580Y, Y493H, R539T, I543T, N458Y, P533L, M476I, R561H and F446I). The F446I mutant is highly prevalent in Myanmar as recently reported [26]. In Africa, a broad array of rare non-synonymous mutations in the *kelch13* gene have been described in *P. falciparum* isolates, but none of these mutants have been associated with artemisinin resistance [27], attesting that not all non-synonymous *kelch13* mutations confer resistance to artemisinin.

More recently, resistance to PPQ has been associated with an increase of survival rates of parasite exposed to 200 nM PPQ for 48 h in the piperaquine survival assay (PSA) and with the amplification of *plasmepsin 2–3* genes (*Pfpm2–3*) [6, 20]. In Cambodia, where high rates of treatment failure to dihydroartemisinin–piperaquine (DHA–PPQ) are observed (i.e. >60% in some provinces), it has been demonstrated that amplification of *Pfpm2* gene and presence of validated *kelch13* mutations were highly predictive of DHA–PPQ treatment failure [20]. Most of these parasites harbour a single copy of *Pfmdr1* gene leading to the recovery of mefloquine sensitivity [4, 6] and suggesting a natural antagonism between PPQ resistance and mefloquine resistance. However, so far it was not understood whether *Pfmdr1* de-amplification (from multiple copies to single copy *Pfmdr1*) was due to the implementation of DHA–PPQ as first-line treatment or due to the release of mefloquine pressure and an increase in parasite fitness accompanying *Pfmdr1* gene de-amplification. To date, DHA–PPQ resistance was confined to Southeast Asia. So far, only few studies conducted on parasites from Mozambique and Mali have provided evidence of the presence (at low frequency, 1.1%, 10%) of parasites carrying multiple copies of *Pfpm2* [28, 29].

Facing the threat of losing all current artemisinin-based combinations front-line therapies due to resistance, a new generation of endoperoxides with more favourable pharmacokinetic profiles like the ozonide Artefenomel® (OZ439) have been developed [30]. The efficacy of this new chemical entity was evaluated in combination with PPQ in African and Southeast Asian (Vietnam) patients with uncomplicated falciparum malaria infection [30]. The primary objective of this phase 2b clinical study was to determine whether a single oral dose combination of artefenomel/PPQ was efficacious and safe [$\geq$95% of patients cured on the basis of polymerase chain reaction (PCR)-adjusted adequate clinical parasitological response at day 28 (ACPR28)] in adults and children infected by *P. falciparum*. Blood samples collected in 2014–2015 from this clinical trial offered the opportunity to investigate the proportion of multidrug resistant parasites (i.e. *P. falciparum kelch13* mutants and gene copy number of both *Pfpm2* and *Pfmdr1*). Here, the occurrence of such genotypes from these samples is reported and a map of potential risk of emergence of resistance to the main front-line therapies currently used to treat malaria-infected patients and to the next generation of anti-malarial combinations is provided.

Methods

Study design, study sites and population

The study was a randomized, double-blind, single-dose design to investigate the efficacy, safety, tolerability and pharmacokinetics of artefenomel 800 mg in loose combination with three doses of PPQ phosphate (640 mg,

960 mg, 1440 mg) in male and female patients aged ≥6 months to <70 years, with uncomplicated falciparum malaria in Africa and Southeast Asia (Vietnam), as previously described [31]. This study was conducted in 13 sites, including Burkina Faso (three sites, N=127), Uganda (one site, N=124), Benin (one site, N=1), the Democratic Republic of Congo (one site, N=5), Gabon (two sites, N=94), Mozambique (one site, N=14), and Vietnam (four sites, N=83). A total of 448 patients were randomized into each of three treatment arms: artefenomel 800 mg/PPQ 640 mg (N=148), artefenomel 800 mg/PPQ 960 mg (N=151) and artefenomel 800 mg/PPQ 1440 mg (N=149). Patients presenting *P. falciparum* mono-infection confirmed by microscopy to be in the range of 1000 to 100,000 asexual parasites/μL of blood, and with fever (axillary temperature ≥37.5 °C) or reported fever episodes in the preceding 24 h, were included in the study after having submitted their written informed consent/assent. The following important exclusion criteria were considered: presence of severe malaria (WHO definition), haemoglobin below 8 g/dL, known history or evidence of clinically significant cardiac disorder, including QTcF or QTc B>450 ms, or family history of sudden death or clinical conditions known to prolong QTc, clinically significant hepatic dysfunction and prior anti-malarial treatment within a specified time windows. After the drugs administration, patients were followed for 42 days or 63 days at some centres. Patients remained in the clinical unit for a minimum of 48 h (African patients>5 years old) or 72 h (African patients≤5 years old and all Asian patients) and were discharged on the basis of absence of detectable parasites and fever.

DNA extraction

Plasmodium falciparum DNA was extracted from dried blood spots using the QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer's instructions. Samples were screened to confirm the presence of *P. falciparum* DNA using first a qualitative real-time PCR assay targeting the *Plasmodium cytochrome b* gene and secondly on positive samples, four real-time PCR assays specifically amplifying *P. falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* [32].

Detection of *kelch13* mutations

Plasmodium falciparum positive samples were tested for the presence of mutations in the propeller domain of the *kelch13* gene (PF3D7_1343700) that have been associated with artemisinin resistance [19]. Amplification of the Kelch-propeller domain (codons 440–680, 720 bp) was performed as previously described [27]. Cross-contamination was evaluated by adding no template samples (dried blood spots negative for *P. falciparum*) in each PCR run. PCR products were sequenced by Macrogen (Seoul, Korea). Electropherograms were analysed on both strands, using PF3D7_1343700 as the reference sequence. The quality of the procedure was assessed by including dried blood spots with known *kelch13* mutations (wild-type, C580Y, R539T, I543T, Y493H) which were tested blindly in the same batches (each 96-well) with the test samples. Isolates with mixed alleles were considered as mutant. Following WHO recommendations, *kelch13* mutants were classified in three groups: (i) wild-type group (parasites with no synonymous or non-synonymous mutations compared to 3D7 sequence), (ii) *kelch13* validated (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, C580Y) and candidate mutation (P441L, G339A, V568G, P574L, A675V) group, and (iii) other *kelch13* mutants group (parasites with synonymous or non-synonymous mutations not present in the *kelch13* validated and candidate resistance mutation group).

Pfpm2 and *Pfmdr1* gene copy number variation assessment

Pfpm2 (PF3D7_1408000) and *Pfmdr1* (PF3D7_0523000) gene copy number were measured by qPCR using a CFX96 real-time PCR machine (Bio-Rad, France), relative to the single copy of the *β-tubulin* gene (used as reference gene), as previously described [20]. Amplification was carried out in triplicate. In each amplification run, six replicates using DNA from 3D7 parasite reference clone and three replicates without template (water) used as negative controls were included. Copy numbers were calculated using the formula: copy number $=2^{-\Delta\Delta Ct}$; with $\Delta\Delta C_t$ denoting the difference between ΔC_t of the unknown sample and ΔC_t of the reference sample (3D7). Specificities of *Pfpm2* and *Pfmdr1* amplification curves were evaluated by visualizing the melt curves. Multiple copies vs single copy, of both *Pfmdr1* and *PfPm2*, were defined as copy numbers <1.5 and ≥1.5 respectively.

Statistical analysis

Data were recorded and analyzed using Excel software and MedCalc (MedCalc Software, Belgium). Groups were compared using the Chi squared test or the Fisher's exact test. All reported *P*-values are two-sided and were considered statistically significant if <0.05.

Results

The *P. falciparum* samples collected from patients before treatment and yielding a successful result, by country and molecular assay, are presented in Table 1.

Global genotypes overview

Among the 68 Southeast Asian clinical isolates collected in Vietnam with available data, 67.6% (46/68) were found to harbour parasites with validated or candidate *kelch13*

Table 1 Number of isolates collected from each site in Southeast Asia (Vietnam) and Africa and number and proportion of successful molecular tests

Sites	No. isolates	No. of successful tests (%)		
		kelch 13	*Pfpm2*	*Pfmdr1*
Southeast Asia				
Gai Lai	18	13 (72)	18 (100)	18 (100)
Binh Phuoc	30	26 (87)	28 (93)	28 (93)
Quang Tri	1	1 (100)	1 (100)	1 (100)
Khanh Hao	34	28 (82)	32 (94)	32 (94)
Africa				
Benin	1	1 (100)	0 (0)	0 (0)
Burkina Faso	127	114 (90)	105 (83)	105 (83)
DR Congo	5	4 (80)	2 (40)	2 (40)
Gabon	94	83 (88)	71 (76)	72 (77)
Mozambique	14	14 (100)	8 (57)	12 (86)
Uganda	124	116 (94)	112 (90)	113 (91)
Total	448	400 (89)	377 (84)	383 (85)

resistance mutations (Table 2). Details regarding *kelch13* mutants according to the collection sites are presented in Table 3. By contrast, none of the 332 isolates collected from African patients and successfully tested were found to carry validated or candidate *kelch13* resistance mutations.

Significant difference in proportion of isolates with multiple copies *Pfmdr1* were found between Africa (21.1%, 64/304, 95% CI 16.2–26.9%) and Asia (6.3%, 5/79, 95% CI 2.0–14.8%, P = 0.002, Table 2). Parasites with multiple copies of *Pfpm2* were observed in 11 Asian samples (13.9%, 11/79, 95% CI 6.9–24.9%) and unexpectedly at higher proportion in African isolates (26.8%, 80/298, 95% CI 21.3–33.4%, P = 0.02, Table 2). However, multiple copies of *Pfpm2*/single copy *Pfmdr1*, hypothesized to favour resistance to PPQ, were found at similar proportion in a small cohort of 10 Asian isolates (12.7%, 10/79, 95% CI 6.1–23.3%) and 47 African samples (15.8%, 47/298, 95% CI 11.6–21.0%, P = 0.72, Table 2 and Fig. 1).

In Asia, seven isolates (10.8%, 7/65, 95% CI 4.3–22.2%) had genotypes associated with both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate resistance mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*) (Fig. 2a). In Africa, no clinical isolates had mutations conferring both artemisinin and PPQ resistance due to the absence of *kelch13* mutant-type parasites (Fig. 2b).

Southeast Asian (Vietnamese) genotypes

kelch13 validated and candidate mutations were detected in > 60% of the isolates in all sites (from 61.1% in Gai Lai to 73.0% in Binh Phuoc) except Quang Tri (where only one sample was collected) (Table 3). C580Y was the most predominant *kelch13* validated and candidate mutation

Table 2 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected from Southeast Asia and Africa in 2014–2015

Locus	Allele/haplotype	Number of isolates (%) detected in		*P* value
		Asia (N = 82)	Africa (N = 355)	
kelch 13	ART	*46* (*67.6*)	*0* (*0.0*)	$< 10^{-4\pm}$
	OTH	1 (1.5)	10 (3.0)	
	WT	21 (30.9)	322 (97.0)	
Pfpm2	Single copy	68 (86.1)	218 (73.2)	$0.02^{¥}$
	Multiple copies	*11* (*13.9*)	*80* (*26.8*)	
Pfmdr1	Single copy	74 (93.7)	240 (78.9)	$0.002^{¥}$
	Multiple copies	5 (6.3)	64 (21.1)	
Pfpm2/Pfmdr1	Single copy/single copy	64 (81.0)	189 (63.4)	$0.009^{\pm}$
	Single copy/multiple copies	4 (5.1)	29 (9.7)	
	Multiple copies/single copy	*10* (*12.7*)	*47* (*15.8*)	
	Multiple copies/multiple copies	1 (1.3)	33 (11.1)	
kelch 13/Pfpm2/Pfmdr1	ART/multiple copies/single copy	*7* (*10.8*)	*0* (*0.0*)	$< 10^{-4\pm}$
	WT/multiple copies/single copy	2 (3.0)	43 (14.7)	
	ART/others	38 (57.5)	0 (0.0)	
	WT/others	18 (27.3)	241 (82.5)	

ART: validated or candidate *kelch 13* mutations; WT: *kelch 13* Wild type, OTH: *kelch 13* mutations with unknown association with artemisinin resistance (detailed in Tables 3 and 4)

Italic font denotes the allele or haplotype associate with drug resistance. P-value (Chi squared test$^{\pm}$ or Fischer exact test$^{¥}$)

Table 3 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected in four sites located in Southeast Asia in 2014–2015

Locus	Allele/haplotype	Site							
		Gai Lai		Binh Phuoc		Quang Tri		Khanh Hao	
		N	%	N	%	N	%	N	%
kelch 13	ART								
	C580Y	*3*	*23.1*	*16*	*61.5*	*0*		*6*	*21.4*
	C580Y + P553L	*0*		*0*		*0*		*2*	*7.1*
	I543T	*0*		*1*	*3.8*	*0*		*0*	
	P553L	*4*	*30.8*	*2*	*7.7*	*0*		*11*	*39.3*
	V568G	*1*	*7.7*	*0*		*0*		*0*	
	OTH C469P	1	7.7	0		0		0	
	WT	4	30.8	7	26.9	1	100	9	32.1
Pfpm2	Single copy	15	83.3	20	71.4	1	100	32	100
	Multiple copies	*3*	*16.7*	*8*	*28.6*	*0*		*0*	
Pfmdr1	Single copy	16	88.9	27	96.4	1	100	30	93.8
	Multiple copies	2	11.1	1	3.6	0		2	6.3
Pfpm2/Pfmdr1	Single copy/single copy	14	77.8	19	67.9	1	100	30	93.8
	Single copy/multiple copies	1	5.6	1	3.6	0		2	6.3
	Multiple copies/single copy	*2*	*11.1*	*8*	*28.6*	*0*		*0*	
	Multiple copies/multiple copies	1	5.6	0		0		0	
kelch 13/Pfpm2/Pfmdr1	ART								
	Single copy/single copy	7	53.8	10	41.7	0		18	64.3
	Single copy/multiple copies	0		1	4.2	0		1	3.6
	Multiple copies/single copy	*0*		*7*	*29.2*	*0*		*0*	
	Multiple copies/multiple copies	1	7.7	0	–	0		0	
	OTH								
	Single copy/single copy	–		0		0		0	
	Single copy/multiple copies	1	7.7	0		0		0	
	Multiple copies/single copy	–		0		0		0	
	Multiple copies/multiple copies	–		0		0		0	
	WT								
	Single copy/single copy	2	15.4	6	25	1	100	8	28.6
	Single copy/multiple copies	–		0		0		1	3.6
	Multiple copies/single copy	2	15.4	0		0		0	
	Multiple copies/multiple copies	–		0		0		0	

ART: validated or candidate *kelch 13* mutations; WT: *kelch 13* Wild type, OTH: *kelch 13* mutations with unknown association with artemisinin resistance

Italic font denotes the allele or haplotype associate with drug resistance

(54.3%, 25/46, 95% CI 25.2–80.2%) followed by P553L (37.0%, 17/46, 95% CI 21.5–59.2%), I543T (2.2%, 1/46, 95% CI 0.5–12.1%) and G568G (2.2%, 1/46, 95% CI 0.5–12.1%). In Khanh Hao, two isolates were found to have both C580Y and P553L single mutant parasites (likely from a polyclonal infection).

Isolates with multiple copies of *Pfpm2* were detected only in two sites located along the Cambodian border: in Gai Lai (16.7%, 3/18, 95% CI 3.4–48.7%) and in Binh Phuoc (28.6%, 8/28, 95% CI 12.3–56.3%). No parasites with multiple copies were detected out of 32 isolates in Khanh Hao. Parasites with a single copy of *Pfmdr1* were frequent (> 88%) in samples collected from all four study sites (from 88.9% in Gai Lai to 100% in Quang Tri).

Parasites with multiple copies *Pfpm2*/single copy *Pfmdr1* were observed in 10/79 (12.7%, 95% CI 6.1–23.3%) of the isolates collected from Vietnamese patients, representing in Gai Lai 11.1% (2/18, 95% CI 1.4–40.1%) and in Binh Phuoc 28.6% (8/28, 95% CI 12.3–56.3%).

Isolates with genotype conferring both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*)

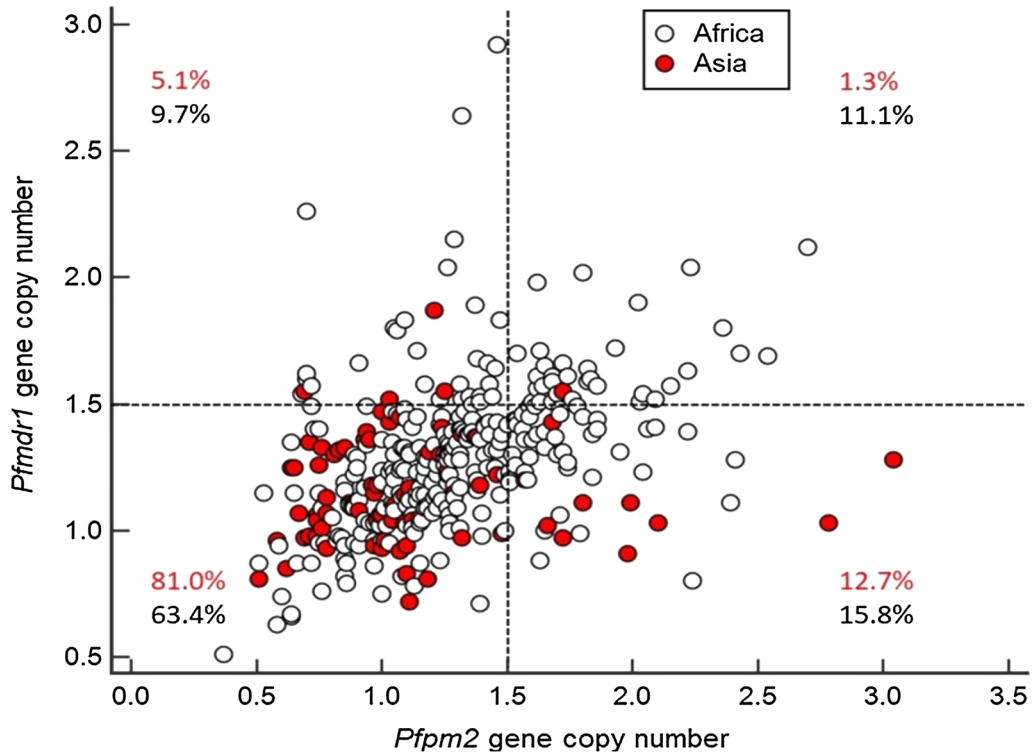

Fig. 1 *Pfmdr1* and *Pfpm2* gene copy numbers of *Plasmodium falciparum* isolates collected from Southeast Asia (in red) and from Africa (in black). Proportion of the isolates from Southeast Asia and African are given for each group: *Pfpm2* single copy/*Pfmdr1* single copy (lower-left quadrant), *Pfpm2* single copy/*Pfmdr1* multiple copies (upper-left), *Pfpm2* multiple copies/*Pfmdr1* single copy (lower-right) and *Pfpm2* multiple copies/*Pfmdr1* multiple copies (upper-right)

were only observed in patients enrolled in Binh Phuoc (29.2%, 7/24, 95% CI 11.7–60.0%).

African genotypes

No *kelch13* validated and candidate mutations was detected at any site. Other non-synonymous mutations were observed: A578S was the most predominant *kelch13* mutation (7/10; 3 in Uganda, 2 in Gabon, 1 in Mozambique and 1 in Burkina Faso) followed by Y541F, M562T and A626V (only detected once in isolates from Burkina Faso) (Table 4).

Isolates from Uganda and Burkina Faso showed an unexpected high frequency of parasites with multiple copies of *Pfpm2* (33.9%, 38/112, 95% CI 24.0–46.6% and 30.5%, 32/105, 95% CI 20.9–43.0%, respectively). Samples from Gabon and Mozambique had a lower frequency of multiple copies of *Pfpm2* estimated at 11.3% (8/71, 95% CI 4.9–22.2%) and 12.5% (1/8, 95% CI 0.3–69.6%), respectively. Of note, in the Democratic Republic of Congo, results from two isolates were available and one isolate was found to carry parasites with multiple copies of *Pfpm2*.

Parasites with single copy *Pfmdr1* were detected in almost all isolates in patients enrolled across the six African sites, therefore, only 13/105 (12.4%, 95% CI 6.6–21.2%) isolates from Burkina Faso, 2/12 (16.7%, 95% CI 2.0–60.2%) from Mozambique, 17/72 (23.6%, 95% CI 13.8–37.8%) from Gabon and 31/113 (27.4%, 95% CI 18.6–38.9%) from Uganda had multiple copies of *Pfmdr1*. One out of two patients harboured parasites with multiple copies of *Pfmdr1* in DRC.

Parasites with multiple copies *Pfpm2*/single copy *Pfmdr1* were observed at a frequency of 20.9% (22/105, 95% CI 13.1–31.7%) in Burkina Faso, 18.8% (21/112, 95% CI 11.6–28.7%) in Uganda, 12.5% (1/8, 95% CI 0.3–69.7%) in Mozambique and 4.3% (3/71, 95% CI 0.9–12.5%) in Gabon. However, isolates with genotype conferring both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*) were not observed in patients enrolled in Africa since there were no *kelch13* validated and candidate mutations.

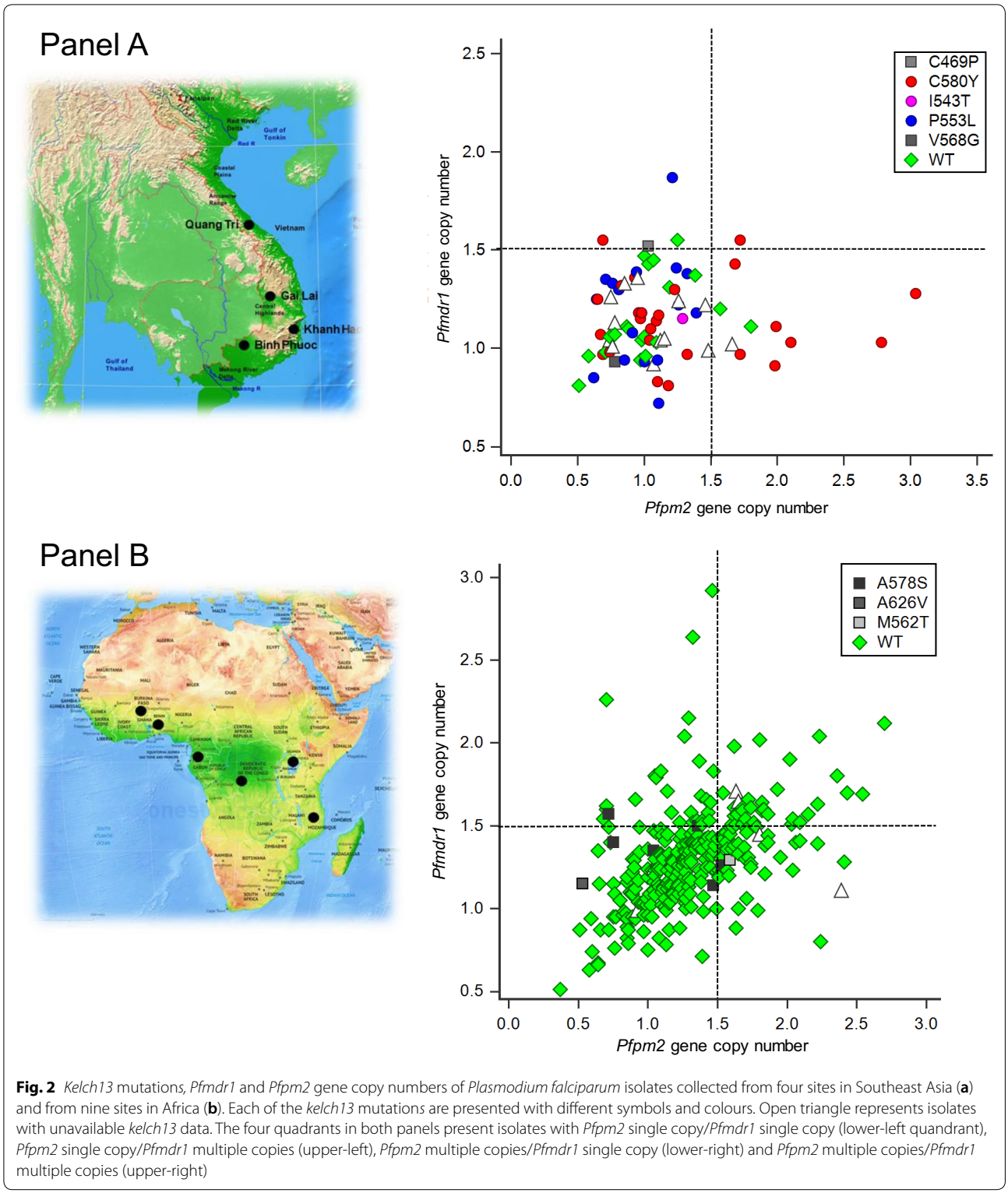

Fig. 2 *Kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers of *Plasmodium falciparum* isolates collected from four sites in Southeast Asia (**a**) and from nine sites in Africa (**b**). Each of the *kelch13* mutations are presented with different symbols and colours. Open triangle represents isolates with unavailable *kelch13* data. The four quadrants in both panels present isolates with *Pfpm2* single copy/*Pfmdr1* single copy (lower-left quandrant), *Pfpm2* single copy/*Pfmdr1* multiple copies (upper-left), *Pfpm2* multiple copies/*Pfmdr1* single copy (lower-right) and *Pfpm2* multiple copies/*Pfmdr1* multiple copies (upper-right)

Discussion

The current phase 2b clinical study of artefenomel, an ozonide showing improved pharmacokinetics properties compared to artemisinins, combined with PPQ was designed to assess the efficacy of single oral doses in patients with uncomplicated falciparum

Table 4 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected in nine sites located in Africa in 2014–2015

Locus	Allele/haplotype	Site											
		BEN		BF		DRC		GAB		MOZ		UGA	
		N	%	N	%	N	%	N	%	N	%	N	%
kelch 13	ART	*0*		*0*		*0*		*0*		*0*		*0*	
	OTH												
	A578S	0		1	0.875	0		2	2.4	1	7.1	3	2.6
	Y541F	0		1	0.875	0		0		0		0	
	M562T	0		1	0.875	0		0		0		0	
	A626V	0		1	0.875	0		0		0		0	
	WT	1	100	110	96.5	4	100	81	97.6	13	92.9	113	97.4
Pfpm2	Single copy	0		73	69.5	1	50	63	88.7	7	87.5	74	66.1
	Multiple copies	0		*32*	*30.5*	*1*	*50*	*8*	*11.3*	*1*	*12.5*	*38*	*33.9*
Pfmdr1	Single copy	0		92	87.6	1	50	55	76.4	10	83.3	82	72.6
	Multiple copies	0		13	12.4	1	50	17	23.6	2	16.7	31	27.4
Pfpm2/Pfmdr1	Single copy/single copy	0		70	66.7	1	50	51	71.8	7	87.5	60	53.6
	Single copy/multiple copies	0		3	2.9	0		12	16.9	0		14	12.5
	Multiple copies/single copy	*0*		*22*	*20.9*	*0*		*3*	*4.3*	*1*	*12.5*	*21*	*18.8*
	Multiple copies/multiple copies	0		10	9.4	1	50	5	7	0		17	15.1
kelch 13/Pfpm2/Pfmdr1	ART												
	Single copy/single copy	0		0		0		0		0			
	Single copy/multiple copies	0		0		0		0		0			
	Multiple copies/single copy	*0*		*0*		*0*		*0*		*0*			
	Multiple copies/multiple copies	0		0		0		0		0			
	OTH												
	Single copy/single copy	0		0		0		0		1	12.5	2	1.9
	Single copy/multiple copies	0		1	1	0		1	1.5	0		1	0.9
	Multiple copies/single copy	0		2	1.9	0		0		0			
	Multiple copies/multiple copies	0		0		0		0		0			
	WT												
	Single copy/single copy	0		69	66.3	1	50	51	71.8	6	75	56	52.3
	Single copy/multiple copies	0		3	2.9	0		11	15.5	0		13	12.1
	Multiple copies/single copy	0		20	19.2	0		3	4.2	1	12.5	19	17.8
	Multiple copies/multiple copies	0		9	8.7	1	50	5	7	0		16	15

Countries: BEN: Benin; BF: Burkina Faso; DRC: Democratic Republic of Congo; GAB: Gabon; MOZ: Mozambique; UGA: Uganda. ART: validated or candidate *kelch13* mutations; WT: *kelch 13* Wild type, OTH: *kelch13* mutations with unknown association with artemisinin resistance

Italic font denotes the allele or haplotype associate with drug resistance

malaria in Southeast Asia (Vietnam) and Africa. In addition to the clinical outcome assessment, three molecular markers associated with drug resistance for mapping the potential risks of future treatment failures were investigated in isolates collected before treatment. The frequency of k*Kelch13* mutations associated with artemisinin resistance, and *Pfmdr1* and *Pfpm2* genes copy number were measured in available isolates collected from all clinical sites. Artemisinin resistance was confirmed to be still confined in Southeast Asia. A high proportion of *kelch13* validated and candidate resistance mutations were observed as well as a new unreported one (C469P) in Vietnamese parasites and the complete absence of these mutants in African isolates. As previously reported [27, 33, 34], a low proportion of *kelch13* mutations was detected in African samples and all these mutations have not been shown to be associated to artemisinin resistance [27].

However, a higher proportion (threefold) of parasites with multiple copies of *Pfmdr1*, a gene encoding a drug

efflux pump, was observed in African samples compared to Southeast Asian isolates. This observation contrasts with previous reports showing high frequency of parasites with multiple copies of *Pfmdr1* in Asia [35–37] compared to Africa [38–40]. These finding likely reflect the profiles of evolution of *P. falciparum* populations linked to anti-malarial drug pressure in both continents. Especially, the prevalence of high *Pfmdr1* amplification observed in Africa might be linked with the routine use of artemether–lumefantrine as first-line treatment for more than a decade. Indeed, increased *pfmdr1* copy number is known to modulate parasite responses to a wide range of drugs including lumefantrine [38, 41, 42]. Supporting this expectation, it seems feasible that such parasites exposed to lumefantrine as monotherapy for several days following clearance of artemether have been selected, while parasites with a single copy have been eliminated. In contrast, the low prevalence of *Pfmdr1* multiple copies observed in Southeast Asia could be due to the recent implementation of DHA–PPQ, the removal of the mefloquine drug pressure or both, as the case in Cambodia [18, 20, 43]. Resistance to piperaquine and resistance to mefloquine have been shown to be mutually exclusive in South East Asia. A possible inverse susceptibility mechanism could be at the origin of this observation.

High frequency of isolates with multiple copies of the *Pfpm2* has already been reported in recent studies conducted in Cambodia [18, 20, 43]. As the Vietnamese clinical sites (Gai Lai and Binh Phuoc) are located alongside the Cambodian border (Fig. 2), data from this study might reflect an evolving situation where the amplification of *Pfpm2* is spreading beyond Cambodia, as described recently [5, 7]. To date, frequencies observed in Vietnamese isolates are not yet as high as the ones observed in Cambodia but might continue to increase in the future.

Unexpectedly, in African isolates, amplification of *Pfpm2* gene was shown to occur at a much higher frequency (~27% on average across clinical sites in Africa, reaching 30.5% in Burkina Faso and 33.9% in Uganda) than was recently described (from 11.1 to 13.8% in Uganda, 10% in Mali and 1.1% in Mozambique) [28, 29, 44]. Considering the geographical extent and the diversity of the clinical sites in Africa, the high frequency reported at sites distant to each other suggests that amplification of *Pfpm2* gene occurred independently in each site. More importantly, since in Southeast Asia most parasites with multiple copies of *Pfpm2* also display *kelch13* resistance mutations, which is not the case in African samples, it is likely that *Pfpm2* amplification originated in Africa, independently of Southeast Asia.

Unfortunately, in vitro or ex vivo drug susceptibility assays were not possible and association between *Pfpm2* amplification and clinical resistance to PPQ was not verifiable in the current study. An evaluation is currently ongoing to see whether, and if so to what extent, these markers of artemisinin and PPQ resistance affected the parasite clearance half-life (PCT1/2) and PCR-adjusted 28 days follow-up in patients treated with artefenomel/PPQ (study MMV OZ439 13,003). However, it was recently reported that compared to drug sensitivities measured on Ugandan isolates from 2010 to 2013 (from the same site, namely Tororo), those measured in 2016 to chloroquine, amodiaquine, and PPQ were increased by 7.4, 5.2 and 2.5-fold, respectively [44]. This longitudinal study showed that rather than drug resistance developing to these three anti-malarial drugs, an increase in sensitivity was observed that was correlated with low prevalence of the polymorphisms recently associated with resistance to artemisinins or PPQ. Indeed, clinical resistance to DHA-PPQ has not yet been reported in Africa [45]. Although, the possibility that parasites showing amplification of *Pfpm2* observed in the current study are resistant to PPQ without confirmation of in vitro or ex vivo phenotypes cannot be excluded, data reported by Rasmussen et al. [44] suggest that significant occurrence of clinical resistance to PPQ is unlikely. In other words, in Africa it is unclear whether the amplification of *Pfpm2* is necessary and/or sufficient for the development of resistance to PPQ. The ongoing analysis relating the markers of resistance to clinical outcome may provide some insights regarding this question. Until very recently, it was still debated whether additional genetic modifications in the *P. falciparum chloroquine resistance transporter* (*Pfcrt*) gene are required to confer such resistance [46, 47]. Recent genomic and biological investigations have revealed a rapid increase in the prevalence of novel *Pfcrt* mutations in Cambodia (H97Y, F145I, M343L, and G353V). These mutants (from culture-adapted Cambodian field isolates or Dd2 gene-edited clones) were confirmed to confer PPQ resistance as determined using the PSA^{0-3h} [6, 48].

Conclusions

At present, several artemisinin-based combinations are used in Africa and Asia to treat patients with uncomplicated malaria. Artemether–lumefantrine (AL), artesunate–amodiaquine (AS–AQ), artesunate–mefloquine (AS–MQ), artesunate–sulfadoxine–pyrimethamine (AS–SP), dihydroartemisinin–piperaquine (DHA–PPQ) and pyronaridine–artesunate (PA) achieve more than 95% efficacy in clinical trials based on PCR-adjusted Day 28 ACPR. Due to the long post treatment prophylaxis of the well-tolerated PPQ, DHA–PPQ is currently under evaluation in a number

of interventions, such as intermittent preventive treatment in pregnant women or in infants (IPTp, IPTi) and mass drug administration campaigns (MDA) in Africa. As a key surveillance goal, it is therefore of particular importance to continue following the evolution of *Pfpm2* amplification along with mutations in the *Pfcrt* gene and to investigate whether these genetic signatures are associated with PPQ resistance in Africa.

Authors' contributions

FM, DL, BL, RK, NK, EL and DM substantially contributed to analysis and interpretation of data. DM, RK, NK, and EL substantially contributed to acquisition of data. YA, SO, AB, GMN, JMNN, RV, YD, AKT, TTD and PBQ were responsible for the prospective collection of biological samples for this study. FM substantially contributed to conception and design. DL and DM drafted the manuscript. All authors contributed to critically reviewing of the manuscript. The authors agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Competing interests

FM and DL are employees of Medicines for Malaria Venture. None of the other authors declare competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Consent for publication

No details relating to individual participants are presented in this manuscript.

Ethics approval and consent to participate

The study (MMV OZ439 13 003) conformed to the Declaration of Helsinki and Standard Operating Procedures that meet current regulatory requirements and guidelines laid down by the International Conference on Harmonization for Good Clinical Practice in Clinical Studies, and approved by the relevant Independent Ethics Committees (IEC), National Institutional Review Boards and where relevant, local regulatory authorities at each of the participating sites. Patients and parents or guardians of participants provided written informed consent prior to enrollment. Study related information was provided in the participants' local languages. The study protocol was registered and the study results are reported on clinicaltrials.gov (NCT02083380).

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TITLE: Host Biomarkers are associated with severe malaria in Mozambican children: a case–control study

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ABSTRACT

Introduction: Laboratory parameters easily measurable and associated with a higher severity risk in malaria infection would allow for early screening, risk stratification and better management of this life-threatening disease. The primary objective of this study was to identify biomarkers of inflammation and endothelial activation differentially expressed in cases of severe malaria compared to uncomplicated malaria cases.

Methods: We conducted a case-control study (2014-2016) in a rural hospital recruiting as cases pediatric patients with severe malaria (defined by World Health Organization criteria) and as controls pediatric patients with uncomplicated malaria matched by age, sex, and *Plasmodium falciparum* parasitaemia. We compared the levels of biomarkers associated with total parasite mass (plasma levels of the *P. falciparum* histidine-rich protein 2 (HRP-2)) and host response to infection: Angiopoietin 1 and 2 (Ang-1, Ang-2); ratio Ang-2:Ang-1, soluble Tie2 (sTie2), brain-derived neurotrophic factor (BDNF), Cystatine C (Cys-C), soluble FMS-like tyrosine kinase-1 (sFt-1), Interleukin (IL-6), Interleukin (IL-8), 10 kDa interferon γ-induced protein (IP-10), soluble tumor necrosis factor receptor 1 (sTNFR-1) and soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) between both groups. We also compared those levels between children with different severe clinical manifestations and scores using the Lambaréné Organ Dysfunction Score (LODS).

Results: The levels of Ang-2, Ang-2: Ang-1 ratio, sTie-2, sFlt-1, IL-6, IL-8, IP-10, TFNR1, sTrem-1 were significantly higher in children with SM when compared with matched controls with uncomplicated malaria. After application of Bonferroni correction for multiple-comparisons Ang-2, sFlt-1 and IL-8 levels were still significantly higher in children with SM. sFlt-1, IL-6 and IL-8 levels were higher among those children with higher LODS scores. HRP-2 levels were not significantly different between severe cases and their matched controls although HRP-2 levels were strongly correlated with levels of Ang-2.

Conclusions: Host biomarkers associated with endothelial activation and inflammation can reliably identify those patients with a greater severity. Ang-2 is the most promising candidate for future clinical applications as it can be used to guide malaria diagnosis and tailor supportive treatment.

Keywords: severe malaria, risk-stratification, pediatric, endothelial activation, angiopoietin-2; biomarkers.

INTRODUCTION

Plasmodium (P.) falciparum malaria is the parasitic disease with a major impact in the world's health. In 2018, it caused 228 million cases and 405,000 deaths, most of them occurring in children in sub-Saharan Africa (SSA) [1]. Severe malaria (SM), accounting for around 2-4 million cases per year [2], is a complex multi-system disease which may present with many manifestations, although most patients may be identified by three overlapping syndromes with different biological, clinical and epidemiological characteristics: severe anemia (SA), acidosis and cerebral malaria (CM) [2]. The onset and evolution of the disease depends on the intricate interaction between parasite, host and socio-geographic factors which will determine the range of expression from asymptomatic patients to death [3]. SM is characterized by high parasite burden, cytoadherence of parasitized erythrocytes to the microvasculature; impaired tissue perfusion; dysregulated inflammatory responses; and activation of the complement system, mononuclear cells, and endothelium [4]. Understanding the differential characteristics of uncomplicated and severe cases and the factors which determine poor outcomes is essential to identify those children at higher risk of SM and death; and to find new diagnostics and therapeutic tools to reduce the burden of disease. High parasite biomass is thought to trigger the pathological interaction between endothelial dysfunction, inflammation and red blood cells (RBCs) sequestration leading to SM [4]. Histidine-rich protein-2 (HRP-2) is a water-soluble protein produced by *P. falciparum* (and not other plasmodium species) and released from erythrocytes that translates the total body parasite biomass better than the direct measurement of peripheral blood parasitaemia [5, 6]. Higher concentrations of HRP-2 have been associated with specific severity syndromes, disease progression, and mortality and high plasmatic levels of this parasite-based marker seems to be a good predictor of SM in both children and adults [7-15]. In addition, different markers of endothelial activation have also been linked to disease severity such as those of the Angiopoietin (Ang)-Tie axis [16]. Different studies have shown that dysregulation of the Ang-Tie2 axis can be quantified to differentiate between uncomplicated and SM, and is associated with poor disease outcomes. Indeed, low levels of Ang-1 and high levels of Ang-2 and soluble Tie-2 (sTie-2) seem to be associated with an adverse prognosis in malaria [17-24]. Changes in plasma levels of different inflammation biomarkers such as Interleukin (IL-6), Interleukin (IL-8), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), 10 kDa interferon γ-induced protein (IP-10), soluble FMS-like tyrosine kinase-1 (sFt-1), soluble tumor necrosis factor receptor 1 (sTNFR-1) or brain-derived neurotrophic factor

(BDNF) have also been associated with SM and specific manifestations [20, 25-31]. Laboratory markers of organ failure have also been explored in recent years as independent predictors of adverse outcomes. Notably, renal impairment also appears as an independent predictor of poor outcome in children with SM [32] and, contrarily to what had been previously considered, seems to be frequent in children [33, 34]. Levels of Cystatin C (Cys C), a biomarker of kidney functional status, have been recently associated to SM and increased mortality [34]. Acidosis is a defining criterion of SM and hyperlactatemia is perhaps the most well-known and widely studied parameter related to disease severity and poor prognosis in SM [35-46].

Some clinical scoring systems have been developed to predict mortality in patients affected by infectious diseases in low-resource settings [47]. Among them, the Lambaréné Organ Dysfunction Score (LODS), which combines three simple features (coma, prostration, and deep breathing), has demonstrated to be highly sensitive and specific to predict survival of SM [47, 48]. Furthermore, adding specific biomarkers such as Ang-2 and sFlt-1 to LODS has shown to improve the model's predictive power [29]. Overall, this supports that new approaches, combining the identification of certain clinical features/syndromes and the measurement of plasma biomarkers have an enormous potential to risk stratify and anticipate the patient's evolution, acting as simple and useful diagnostic and prognostic tools.

This study aimed, with a matched case-control approach, to investigate clinical factors, host and parasite biomarkers previously associated with prognosis, so as to derive a comprehensive understanding of the utility of these markers to reliably identify children with different clinical manifestations and severity. The main objectives of the study were 1) to identify parasite and host molecules in plasma differentially expressed in children with severe and uncomplicated malaria; and 2) to describe the relationship between parasite biomass and host biomarkers with clinical manifestations of disease. The underlying hypothesis was that levels of biomarkers would be differently expressed in severe than uncomplicated malaria cases, and could therefore become the basis of future risk stratification point of care tools.

METHODS

Study area and population

The study took place in Manhiça¸ a district in rural southern Mozambique, where the *Centro de Investigação em Saúde de Manhiça* (CISM; Manhiça Health Research Centre) has been running a continuous demographic surveillance system since 1996. A full description of the Manhiça demographic surveillance system area and methods has been reported elsewhere [49]. The area is a flat savannah with moderate vegetation. There are 2 seasons, a hot and wet season (October–May) and a dry and cold season during the rest of the year. Two reference district hospitals in Manhiça and Xinavane, as well as 11 peripheral health facilities constitute the government's health network within the Manhiça district. Malaria transmission is perennial in this district of Mozambique with some marked seasonality, with highest incidence typically peaking between November and April. *P. falciparum* accounts for over 98% of all malaria cases [50]. In 2003–2005, malaria accounted for 30.5% of all pediatric outpatient visits [50] and nearly half (49%) of all pediatric admissions [51], 27% of which fulfilled the World Health Organization (WHO) criteria for being considered SM cases. Almost 19% of all in-hospital pediatric deaths were due to malaria [51]. Among all SM admissions, prostration (55.0%), respiratory distress (41.1%) and severe anemia (17.3%) were the 3 most prevalent clinical presentations [51]. Recent changes in the epidemiology of malaria in SSA have encompassed a steady decline in malaria incidence, coupled with a decrease in severe disease, which reached its nadir in 2010, with only 3 deaths related to malaria occurring in hospital in the whole year (Guinovart et al, submitted). From that year onwards, malaria incidence is on the rise again, and severe cases have again become frequent in the Manhiça Hospital wards, with however, important differences in their mean age (shifted to older ages) and syndromic presentation (more cerebral malaria cases, which were rare before).

Study design and patients

This was a sex, age (+/- 3 months in children less than one year old and +/- 6 months in children more than one year old) and parasitemia (same or +/- one cross level) matched case–control study in children under 10 years of age presenting at CISM, with severe and uncomplicated malaria. The children were recruited between September 2014 and May 2016. Children with a clinical diagnosis of *P. falciparum* malaria were recruited after written informed consent was given by their parents or guardians. SM cases were defined as patients with a clinical diagnosis of malaria, an asexual *P.*

falciparum parasitaemia > 0 parasites/μL by microscopic examination of Giemsa-stained blood smears and fulfilling at least one of the following criteria: Cerebral Malaria (CM), Severe Anemia (SA) (packed cell volume <15% or hemoglobin <5 g/dL), Acute Respiratory distress (ARD) (chest indrawing and/or deep breathing), hyperlactatemia (lactate >5 mM), prostration (inability to sit or breastfeed in children old enough to do so), hypoglycaemia (blood glucose <2.2 mM) and Multiple seizures (MS) (≥2 convulsions in the preceding 24 h). Uncomplicated malaria cases were defined as a child admitted (or not) in Manhiça District Hospital (MDH) with a clinical diagnosis of malaria with a *P. falciparum* asexual parasitaemia > 0 parasites/μL and not fulfilling the criteria for SM. Patients were assessed by the study clinician to confirm the patient's eligibility to participate in the study and that malaria was the sole or principal cause of the disease. LODS score was calculated, as previously described, combining three variables (coma, prostration, and deep breathing) to obtain a value between 0 and 3 [47, 48]. Considering the potential effect on the biomarkers levels, patients were excluded from the study if they had a history of blood transfusion or use of antimalarials drugs in the 15 days before attending the hospital. They were also excluded if they had participated in any other study including the administration of antimalarial drugs or vaccines within the previous 6 months. Patients were treated following the Mozambican national guidelines for malaria management [52]. Patients with severe disease were admitted at Manhiça District Hospital (MDH) and treated with parenteral artesunate until able to receive oral antimalarial medication. Patients with uncomplicated malaria were treated according to the Mozambican first line policy with artemether-lumefantrine [52].

Clinical evaluation and data collection

The procedures for identifying children with SM were not different from those used in routine clinical practice at CISM and MDH. Capillary glycaemia and hematocrit were determined on admission to identify patients with hypoglycemia and quantify anaemia, as it is done for all children currently admitted. A questionnaire about the demographic data, together with a thorough clinical history of each child included in the study, were completed. Clinical data including basic demography, consciousness state, and history of convulsions, age, hematocrit, and outcome of infection were recorded for all patients. For each admitted SM case, a summary sheet of information regarding the clinical evolution was collected at the end of the admission, independently of the outcome. For uncomplicated cases, the same demographic and clinical

information (with the exception of the clinical evolution in hospital) was collected and completed before discharge from the outpatient department. The use of antimalarial drugs before attending the hospital was actively enquired.

Sample collection and processing

Whole blood was collected by venipuncture and anticoagulated using acid citrate dextrose (5 ml in children <5 years and 10 ml in children between 5-10 years). Two drops of the blood were spotted onto a Whatmann filter paper. Blood was centrifuged within 4 h of sample collection and plasma was aliquoted, frozen and stored at -80^{o}C without thawing until analyzed. Samples were thawed overnight at 4^{o}C and aliquoted at room temperature immediately prior to assay performance.

Laboratory methods

Hematological and biochemical parameters were performed for each patient using Vitros DT60 and Sysmex Kx21 analyzers. Thick and thin blood films for malaria diagnosis were processed as previously detailed [53, 54]. The Lambaréné method [55] was used to calculate peripheral parasitaemia. Additionally, and as part of the routine clinical management at CISM, a semiquantitative "cross" system is in place, classifying parasitaemia levels from 0 (no malaria infection) to +++++ ("5 crosses", high parasitaemia infection) [56].

PCR and multiplicity of infection

Total genomic DNA (gDNA) was extracted from a blood drop spotted onto filter paper using QIAmp DNA Mini Kit (Qiagen) and tested in triplicate to measure the parasite density by real-time quantitative PCR (qPCR) targeting the *P. falciparum* 18S ribosomal RNA gene [57]. Parasitemia was quantified by extrapolation of cycle thresholds (Ct) from a standard curve of *Pf* ring infected erythrocytes. Samples without amplification (no Ct detected) were considered negative. A negative control with no template DNA was run in all reactions.

Quantification of HRP2

A commercial HRP2 ELISA kit (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia) was used to estimate HRP2 levels. One hundred µLof each plasma sample were

transferred to the ELISA plates in duplicate along with necessary controls and a standard curve, and the plates were incubated at room temperature for 1h in a humid chamber followed by 5 washing steps with the washing solution provided in the kit. 100 µl of the diluted antibody conjugate was added to each well after completion of 1h incubation followed by 5 washing steps as stated above, 100 µL of the chromogen substrate (tetramethylbenzidine) was added to each well. Plates were incubated for 15min in the dark, followed by addition of 50 µl of the stop solution. Spectrophotometric analysis was performed at 450nm.

Quantification of biomarkers in plasma

Luminex ® Panel 1 including 3 high-abundance biomarkers, tested at a dilution of 1:20: BDNF, TNFR1 and CysC. Luminex ® Panel 2 included 7 low-abundance biomarkers, tested at a dilution of 1:2: IL-6, IL-8, IP-10, Ang-2, Ang-1, Flt-1 and Trem-1. Tie2 was performed by ELISA (R&D Systems, Minneapolis, MN, and Cellabs, Sydney, Australia) at a dilution of 1:20. Unfiltered plasma was diluted in assay diluents provided by the manufacturer. Each 96 well plate included a 7-point serial dilution of standards, in duplicate and 72 patient samples, 8 of which were tested in duplicate. Assays were performed according to manufacturer's magnetic Luminex ® screening assay or ELISA protocols. The dynamic rangefor each assay were as follows: Ang-1 (84-61,380 ng/mL), sFlt-1 (48-35,060 ng/mL), IL-8 (3-2280 pg/mL), IP-10 (2-1380 ng/mL), sTREM-1 (87-63,560 ng/mL), Ang-2 (171-125,260 ng/mL), IL-6 (3-2360 pg/mL), BDNF (73-530,000 ng/mL), TNFR1 (1477-1,076,400 ng/mL), CysC (4387-3,198,000 ng/mL), and Tie2 (412-200,000 ng/mL). Values outside the lower limit of quantification were assigned a value of ½ of the lower limit of the standard curve. The coefficient of variance (CV%) between replicates were between 1.8 – 7.7.

Statistical analysis

Data were analyzed using Stata® 16 (Stata corp., College Station, Texas, USA). For clinical and demographic variables, differences between groups were assessed using the Exact McNemar´s test for categorical variables or Wilcoxon matched-pairs signed rank test for continuous variables. Wilcoxon matched-pairs signed rank test was used to compare biomarker levels between uncomplicated and severe malaria cases. For children with severe malaria, biomarker levels were compared between LODS score groups using Wilcoxon rank sum tests. Correlations between different biomarkers were assessed by Pearson´s correlation coefficient and linear regression after

logarithmic scale transformation. P-values inferior to 0.05 were generally considered as statistically significant, but Bonferroni correction was used for multiple biomarker comparisons.

Ethical considerations

This study was reviewed and approved by the Mozambican National Bioethics Committee (CNBS) (Ref. 71/CNBS/2014) and the Clinical Research Ethics Committee of the Hospital Clínic, Barcelona, Spain (Ref. HCB/2013/8749). All participants and their parents/legal guardians were given detailed oral and written information about the study, and children were recruited only after a written informed consent was signed by their parents/legal guardians.

RESULTS

Characteristics of study participants

A total of 163 children were enrolled between September 2014 and May 2016. Of these, 79 presented with UM and 84 with SM. After pairing by sex, age and parasitemia 112 children were included in the study (56 pairs of children with UM and SM) (Table 1).

Table 1: characteristics of children with uncomplicated malaria (UM) and severe malaria (SM)

Variable		**UM** (n=56)	**SM** (n=56)	**p-value**
Sex N (%) [1]	**Male**	35/56 (62.5)	35/56 (62.5)	NA (matched)
	Female	21 (37.5)	21/56 (37.5)	
Age[1]	**0-<1y**	2/56 (3.5)	2/56 (3.5)	NA (matched)
	1y-<5y	38/56 (67.8)	40/56 (71.4)	
	5y<-10y	16/56 (28.5)	14/56 (25)	
Parasitaemia at admission [1]	+	0	1/56 (1.7)	NA (matched)
	++	0	4/56 (7.1)	
	+++	5/56 (8.9)	5/56 (8.9)	
	++++	14/56 (25)	13/56 (23.2)	
	+++++	36/56 (64.2)	33/56 (58.9)	
Previous episodes of malaria[1]		3/53 (5.6)	6/51 (11.7)	0.69
Treatment before recruitment	**Any type**	12/56 (21.4)	12/56 (21.4)	1.00
	Traditional	0	3 (5.3)	
	Antipyretic	9 (16)	5 (8.9)	
	Antimalarial	3 (5.3)	3 (5.3)	
	Antibiotic	0	1 (1.7)	
Fever upon recruitment[1]		48/56 (85.7)	53/56 (94.6)	0.23
Previous days of fever[1]	**1**	32/53 (60.3)	27/54 (50)	
	2	11/53 (20.7)	14/54 (25.9)	
	3	10/53 (18.8)	10/54 (18.5)	
	≥4	0/53	3/54 (5.5)	
Vomiting at recruitment[1]		9/53 (16.9)	16/52 (30.7)	0.17
Previous days of vomiting[1]	**0**	44/53 (83)	38/54 (70.3)	
	1	8/53 (15)	12/54 (22.2)	
	2	1/53 (1.8)	1/54 (1.8)	
	3	0/53	1/54 (1.8)	
	≥4	0/53	2/54 (3.7)	
Weight (kg)[2]		14.5 (5) [55]	14.35 (6.1) [56]	0.45
MUAC (cm)[2]		16 (2) [56]	15 (2) [56]	0.001
Weight for age z-score[2]		-0.81 (1.13) [54]	-0.78 (1.52) [56]	0.26
Total leukocytes (x10^9/L)[2]		8370 (5685) [52]	8510 (5,290) [55]	0.41
Proportion of lymphocytes (%)[2]		27.1 (28.6) [53]	26.7 (18.4) [52]	0.24
Proportion of neutrophils (%)[2]		59.95 (37) [52]	63.15 (20.8) [50]	0.36
Platelets(x/L)[2]		128000 (171000) [52]	91000 (91000) [56]	0.002
Hemoglobin (g/L)[2]		9.81 (1.59) [53]	8.10 (2.63) [56]	0.0003
Lactate (mmol/L)[2]		2.35 (1.50) [56]	3.30 (2.9) [56]	0.0008
Glucose (mmol/L)[2]		6.7 (2.35) [56]	5.85 (2.70) [56]	0.21

1: n (Column percentage). 2: Median (Interquartile range) [n]. UM: uncomplicated malaria; SM: severe malaria; MUAC: Mid-Upper Arm Circumference

Characteristics of children with SM

Table 2 shows the characteristics of children admitted with SM. Eight children (14.2%) presented with CM while thirty-three (58.9%) and forty-five (80.36) had MS and prostration, respectively. Twenty children (35.7%) had ARD and fourteen (25%) hyperlactatemia. Eight children (14.3%) presented with SMA and two (3.5%) with hypoglycemia. According to the total number of inclusion severity criteria, fifteen children (26.7%) presented a single one, 21 (37.5%) had two, nine (16%) had three and eleven (19.6%) had four or more. The number of children with a LODS score of 0, 1, 2, and 3, were eleven (19.6%), thirty-six (64.3%), eight (14.3%) and one (1.8%), respectively (see also figure 1). Fifty-one children survived, one child died, one child absconded and three children were transferred to a higher-level facility. In addition, among children with SM, 18 out 54 children (33.3%) had splenomegaly and 8 out of 54 (14.81%) presented hepatomegaly. Five of the children with SM (8.93%) were HIV positive.

Table 2: clinical characteristics and outcomes of children presenting with severe malaria

Severe malaria characteristics (n=56)	N (%)
Type of inclusion severity criteria	
Cerebral malaria	8 (14.29)
Severe anaemia	8 (14.29)
Acute respiratory distress	20 (35.71)
Hypoglycaemia	2 (3.57)
Multiple seizures	33 (58.93)
Prostration	45 (80.36)
Hyperlactatemia	14 (25.00)
Number of inclusion severity criteria	
1	15 (26.79)
2	21 (37.50)
3	9 (16.07)
≥4	11 (19.64)
LODS score	
0	11 (19.64)
1	36 (64.29)
2	8 (14.29)
3	1 (1.79)
Clinical outcome	
Death	1 (1.79)
Absconded	1 (1.79)
Transfer	3 (5.36)

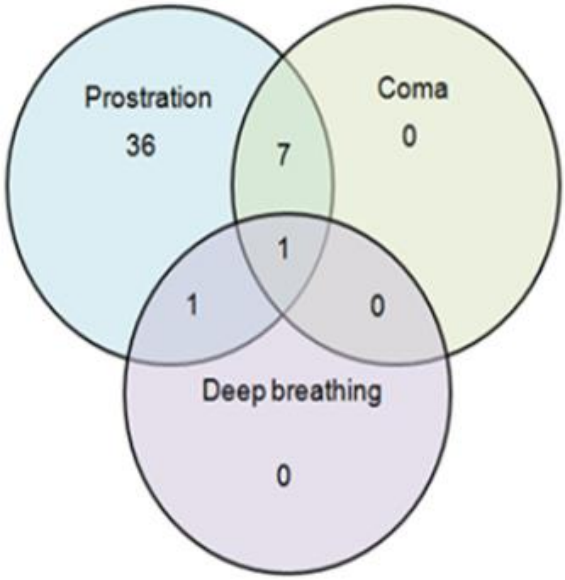

Figure 1: Venn diagram depicting the number of clinical signs included as criteria for LODS score.

Biomarker levels in uncomplicated vs. severe malaria patients

Biomarkers of endothelial activation and inflammation were assayed from plasma samples obtained at presentation (Table 3). Ang-2, sTie-2, TNRF, sFlt-1, IL-6, IL-8, IP-10 and sTREM-1 were significantly increased in SM compared to UM ($p \leq 0.05$), while Ang-1, BDNF and CysC did not differ between groups ($p > 0.05$) (figure 2). When considering the Bonferroni correction for multiple comparisons the levels of Ang-2, sFlt-1 and IL-8 in children with SM had significant differences compared to UM ($p \leq 0.0042$).

Table 3. Biomarker levels in Mozambican children with uncomplicated malaria (UM) and severe malaria (SM)

Biomarkers	**UM (n=56)** Median (IQR)	**SM (n=56)** Median (IQR)	**p-value**
Ang-1 (ng/mL)	1243.6 (478.2-3178.0)	857.3 (336.1-2477.6)	0.62
Ang-2(ng/mL)	2973.5 (2182.3-3900.2)	3861.7 (3041.0-5649.5)	0.001
Ang-2: Ang-1	2.5 (1.0-7.2)	4.3 (1.7- 11.1)	0.01
sTie2 (ng/mL)	31080 (20670-45900)	41630 (31580-56070)	0.05
BDNF (ng/mL)	577.7 (107.2-1273.7)	363.1(135.8 -787.9)	0.45
CysC (ng/mL)	603904.7 (471176.3-704183.8)	563055.4 (409462.3-705625.1)	0.78
sFlt-1 (ng/mL)	293.2 (170.2-440.1)	430.6 (277.0-899.0)	0.0004
IL-6 (ng/mL)	25.4 (8.1-190.2)	125.8 (37.2- 349.4)	0.02
IL-8 (ng/mL)	21.9 (13.4-38.5)	45.6 (19.8-112.8)	0.003
IP-10 (ng/mL)	513.6 (174.3-1151.3)	816.5 (494.1- 4140)	0.007
TNFR (ng/mL)	11195.1 (6643.8-14585.1)	16295.4 (11233.7-21023.0)	0.004
sTREM-1 (ng/mL)	321.0 (239.0-457.4)	407.0 (299.1-755.3)	0.01

Biomarker levels and LODS score

Biomarkers of endothelial activation and inflammation were compared between children with SM presenting with different scores in the LODS score (Figure 3). Ang-1, Ang-2, Ang-2:Ang-1, sTie-2, BDNF, CysC, IP-10 and sTREM-1 were not significantly different when comparing children with different scores. On the other hand levels of IL-6 were higher in children with LODS score of 1 when compared to LODS score of 0 (p =0.01). There were also significant differences between children with LODS score of 0 and LODS score of ≥2 (p= 0.002). The relationship between IL-8 and LODS score showed a similar trend (0 vs1, p= 0.01; 0 vs ≥2, p = 0.006). Levels of sFlt-1 were also significant different between children with LODS score of 0 and LODS score of ≥2 (p= 0.004).

qPCR and HRP-2 levels in uncomplicated vs. severe malaria patients

The median parasitemia measured by microscopy or by qPCR in children with SM was not significantly different in comparison to their parasitaemia matched UM controls (table 4). Likewise, there were no significant differences for plasma PfHRP-2 levels between children with UM and children with SM (124 ng/mL [IQR 24.3–747.5 ng/mL] and 494.21 ng/mL [IQR 79.9-983.2ng/mL], respectively, p = 0.0645) (Table 4). When comparing HRP-2 levels with LODS score, there were no statistical differences (Figure 4a). HRP-2 levels significantly correlated with parasitemia measured by microscopy or by qPCR in children with uncomplicated and SM (Figure 4b and 4 c). We correlated levels of HRP-2 with those biomarkers significantly higher in children with SM considering Bonferroni correction (Figure 5). sFlt1 and IL-8 lacked correlation although levels of Ang-2 where strongly correlated with HRP-2 levels (Pearson's correlation coefficient (r) = 0.20, p=0.04).

Table 4: *P. falciparum* parasitaemia by optic microscopy, quantitative PCR (qPCR) and Histidine rich protein 2 (HRP2) levels in Mozambican children with uncomplicated malaria (UM) and severe malaria (SM)

	UM (n=50) Median (IQR)	**SM (n=50)** Median (IQR)	**p-value**
Optic microscopy (parasites/µL)	53449 (21593 – 107422)	34608 (16316 – 109376)	0.5606
qPCR (parasites/µL)	88594.05 (35899-170814)	106062.9 (38193.4-291887)	0.4486
HRP2 (ng/mL)	124 (24.3-747.5)	494.21 (79.9-983.2)	0.0645

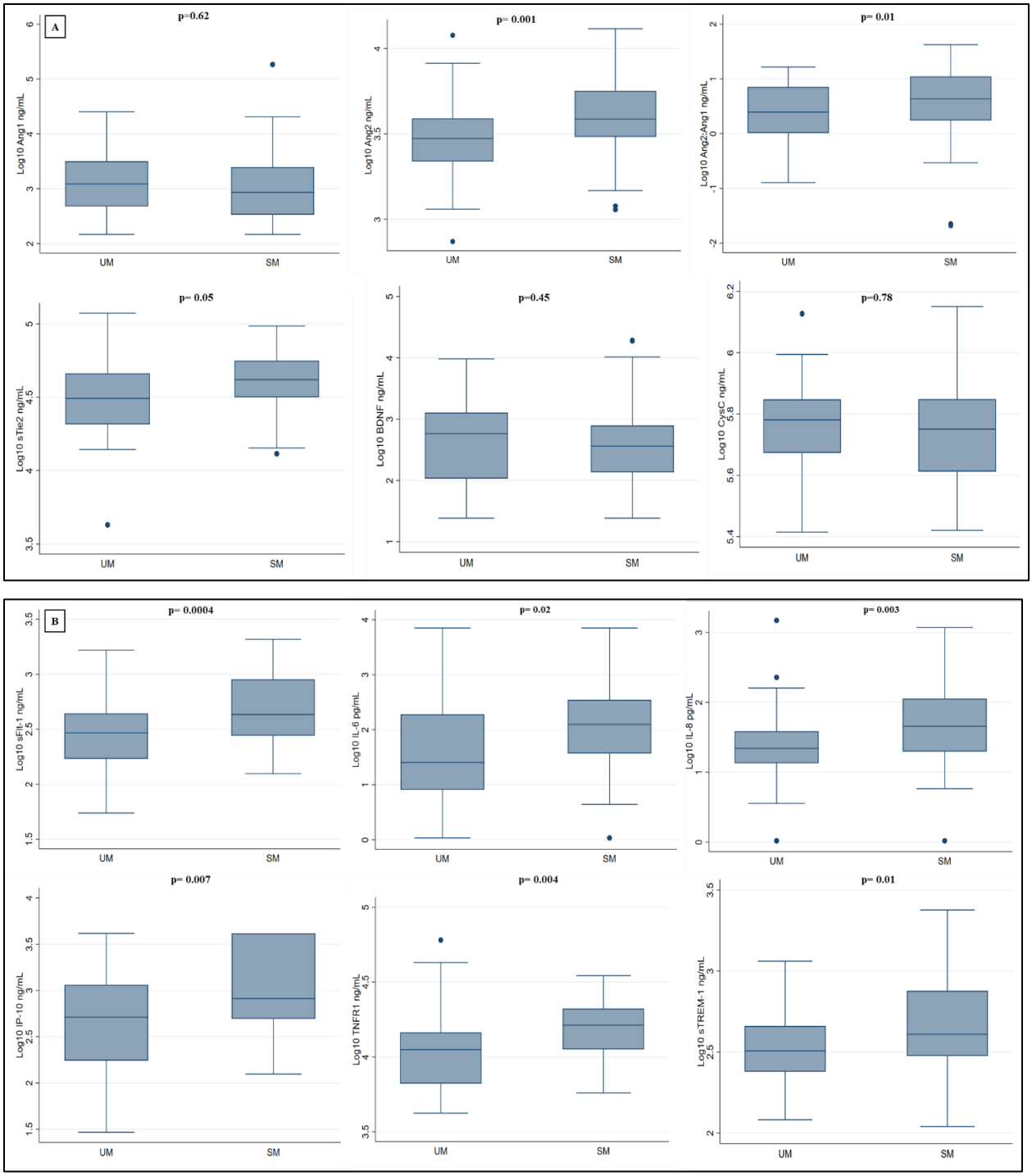

Figure 2. Biomarkers in children with uncomplicated malaria (UM) and severe malaria (SM). Graphs showing the median and scatter of (A) Ang-1(ng/mL), Ang-2(ng/mL), Ang-2:Ang-1, sTie-2 (ng/mL), BDNF (ng/mL), Cys C (ng/mL) and (B) sFlt-1 (ng/mL), IL-6 (pg/mL), IL-8 (pg/mL), IP-10 (ng/mL), TFNR1 (ng/mL), Strem-1(ng/mL) levels in UM and SM as measured by ELISA (Wilcoxon matched-pairs signed rank test). Dot plots showing plasma biomarkers concentration value; box plots showing median, interquartile range, maximum and minimum for plasma biomarkers.

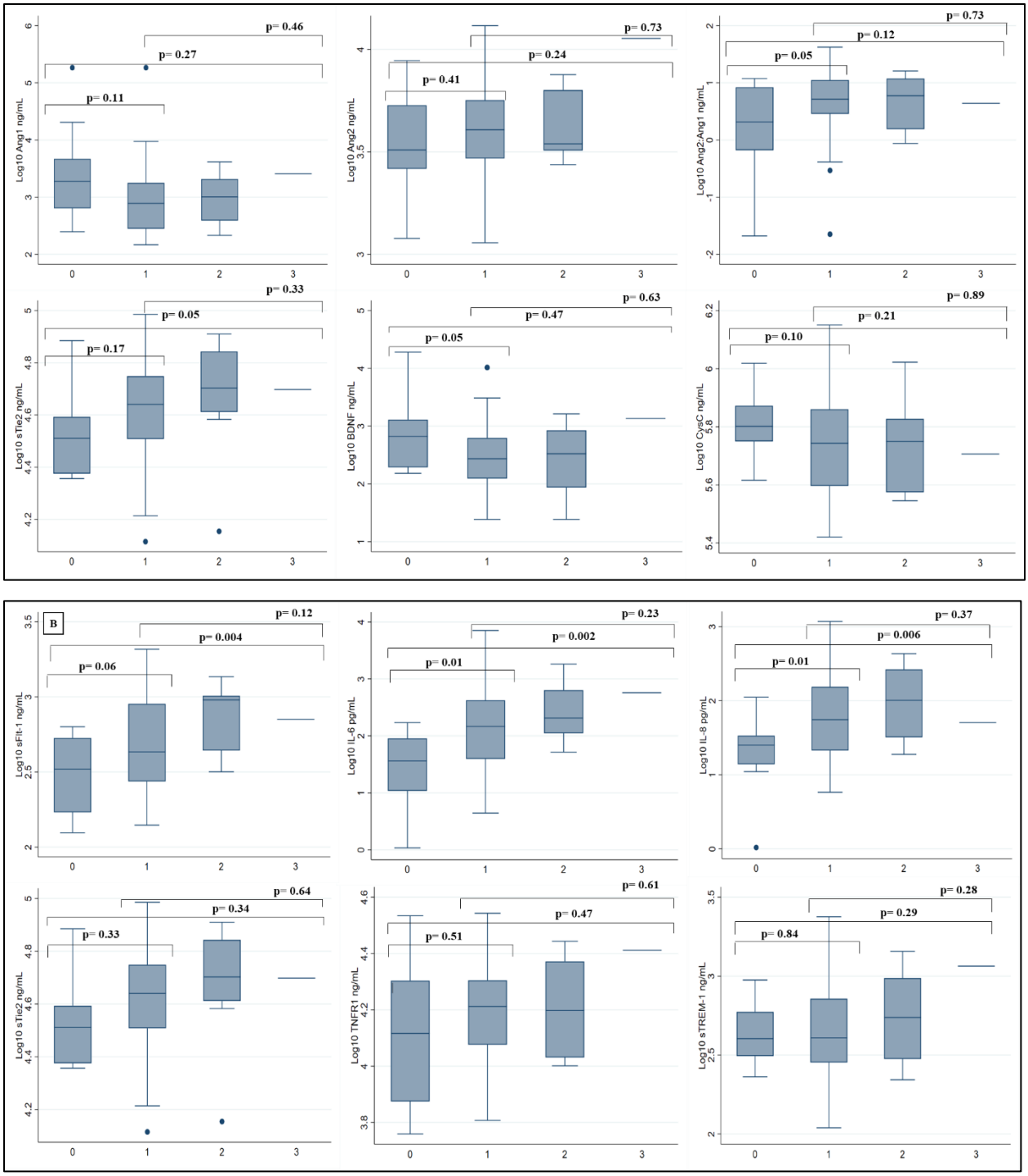

Figure 3. Biomarkers in children with SM according to varying LODS score. Graphs showing the median and scatter of (A) Ang-1(ng/mL), Ang-2(ng/mL), Ang-2:Ang-1, sTie-2 (ng/mL), BDNF (ng/mL), Cys C (ng/mL) and (B) sFlt-1 (ng/mL), IL-6 (pg/mL), IL-8 (pg/mL), IP-10 (ng/mL), TFNR1 (ng/mL), Strem-1(ng/mL) levels in SM as measured by ELISA (Wilcoxon Rank Sum Test). Dot plots showing plasma biomarkers concentration value; box plots showing median, interquartile range, maximum and minimum for plasma biomarkers. ns (not significant, p.0.05), *statistically significant ($p < 0.05$)

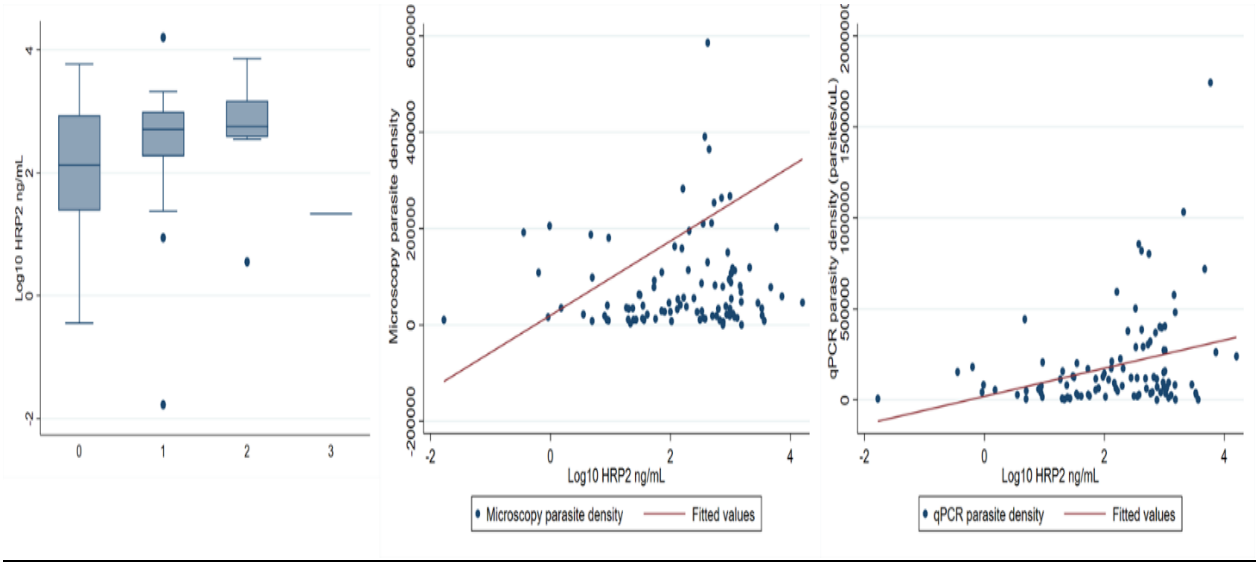

Figure 4 (A) Box plots showing Plasma histidine-rich protein-2 (HRP-2) concentration in children (50 pairs analyzed) with different LODS scores Wilcoxon Rank Sum Tests: 0 vs.1, p = 0.52; 0 vs. 2/3, p = 0.52; 1 vs. 2/3, p = 0.97. (B) Correlation between plasma PfHRP-2 and blood parasitemia measured by microscopy in children with uncomplicated and severe malaria (Pearson's correlation coefficient (r) = 0.09; P=0.40). (C) Strong correlation between plasma PfHRP-2 and blood parasitemia measured by qPCR in children with uncomplicated and severe malaria (Pearson's correlation coefficient (r) = 0.31; P=0.001)

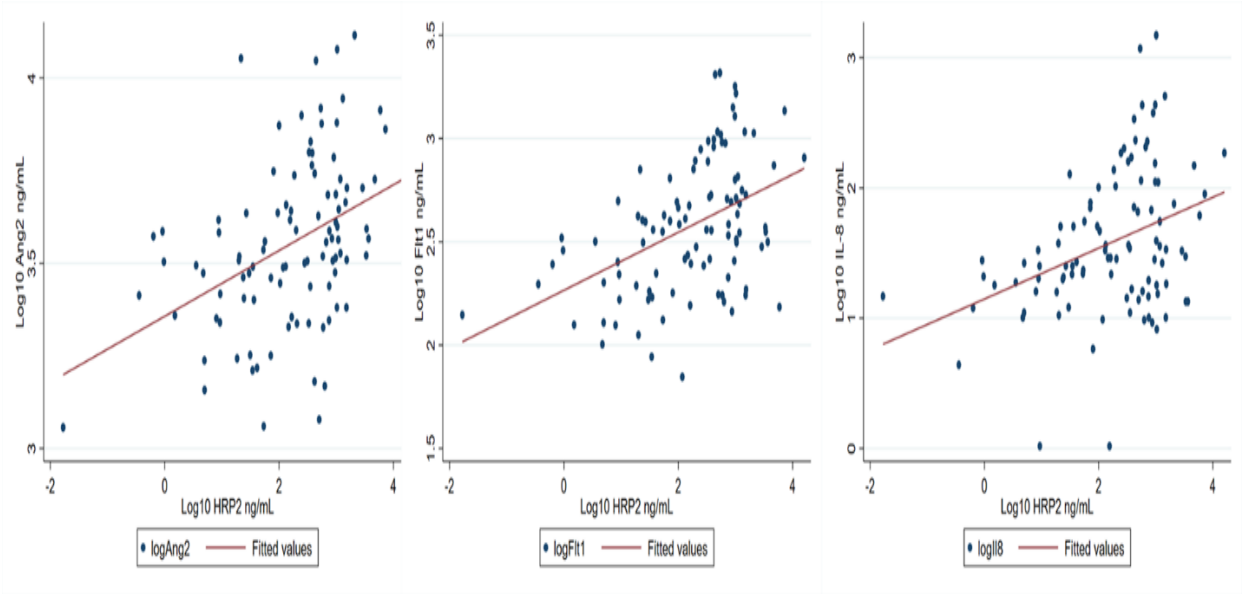

Figure 5. A. Significant correlation between Plasma histidine-rich protein-2 (HRP-2) concentration and Ang -2 levels (Pearson's correlation coefficient (r) = 0.41, p=<0.0001). B, C. Correlation between plasma PfHRP-2 and IL-8 (Pearson's correlation coefficient (r) = 0.37, p=0.0002) and sFlt-1 (Pearson's correlation coefficient (r) = 0.45, p=<0.0001)

DISCUSSION

This matched case-control study investigated the different associations between parasite and host (clinical and biomarkers) factors and disease severity and prognosis, in Mozambican children with uncomplicated and SM. The levels of Ang-2, Ang-2:Ang-1 ratio, sTie-2, sFlt-1, IL-6, IL-8, IP-10, TFNR1, sTrem-1 were significantly higher in children with SM when compared with children with uncomplicated malaria. After application of Bonferroni correction for multiple-comparisons Ang-2, sFlt-1 and IL-8 levels remained significantly higher in children with SM. Levels of IL-6 and IL-8 were higher in in children with LODS score of 1 when compared to LODS score of 0. They were also significantly higher in patients with a LODS score of ≥2 as well as levels of sFlt-1. HRP-2 levels were not different in children with UM and SM and there were not significant association between HRP-2 levels and LODS score, although the fact that matching was conducted in this study also by peripheral parasitaemia may have hindered the evaluation of such differences. HRP-2 levels were significantly correlated with levels of Ang-2. These data show that host biomarkers of inflammation and endothelial activation are associated with SM, and they may have interesting predictive potential when trying to identify those children with a poorer prognosis.

Members of the Ang-Tie axis have been associated with the pathophysiology of SM [16]. Tie-2 is the receptor of both Ang-1 and Ang-2. When Ang-1 bounds to Tie-2, it promotes endothelial stability and vascular quiescence and, moreover, have anti-inflammatory and anti-apoptotic effects [16]. However, Ang-2 antagonizes these actions and, when released from the endothelial cells, triggers a pro-inflammatory and pro-coagulant state. In our study, we confirm previous reports describing high levels of Ang-2 in both adults and children as a biomarker of severity in malaria infection [17-24]. Ang-2 levels were elevated in children with SM although we did not find statistical differences in those children with higher LODS scores. In addition, the ratio Ang-2:Ang-1 was also significantly different between the UM and SM groups. These data show that dysregulation in the levels of Angiopoietins are directly involved with the endothelial activation presented in the pathophysiology of SM. These data confirm the robustness of Ang-2 as a risk stratifier molecule, and its potential to be used as diagnostic tool and therapeutic target for those children with SM. Importantly, we found a strong correlation between the levels of Ang-2 and HRP-2 (Figure 5a). It is thought that the physiological interaction between endothelial dysfunction and other pathways of severity may be triggered by a high parasite biomass [4]. There have not been previous studies studying the relationship between parasite and host-biomarkers but

these data show the relevance of that interaction. It will be important to take into account this fact to design strategies which can act simultaneously in both pathways.

Tie-2 receptor is essential for the stability of endothelium and vascular quiescence as a part of the Ang-Tie2 axis [16]. High levels of sTie-2 have consistently been described in SM in comparison to UM [19, 20]. This study confirms this and further supports the essential role of this axis in the pathophysiology of SM.

BDNF is the most important and more abundant neurotrophic factor in the central nervous system and low circulating levels have been associated with disease severity and poor clinical outcomes in children with SM [31]. This study has failed to demonstrate a relationship between this marker and SM, but the low number of children with CM (n=8) may have limited the potential to observe significant differences. Cys C may reflect kidney impairment and it has been seen that elevated levels are associated to SM and increased mortality [34]. However, the study failed to provide evidence for such an effect, although the absence of measures of kidney damage hinders the adequate assessment of this association.

SM, including SMA and CM, have all been co-related with a deregulated pro-inflammatory state [19, 20, 25, 26, 58]. sFt-1 is related to the vascular endothelial growth factor receptor (VEGFR)-1 and is expressed in monocytes and endothelium. Its expression is induced by hypoxia and VEGF, and is thought to contribute to vascular dysregulation. How sFt-1 exactly contributes to malarial severe disease is unknown but high levels of this biomarker have been associated to SM [20, 29]. These data confirm such an association but further research is needed to clarify its role in the pathological process. The individual role of interleukins in SM is not completely understood but IL-6 and IL-8 have been described as elevated in children with SM [25-27, 58]**.** The results of this study further support that trend and, interestingly, evidenced that children with higher LODS score had elevated levels of sFt-1 and both interleukins compared to lower LODS score. This could be explained by the initiation of the pro-inflammatory state in severe children that could help to identify those who are at higher risk of progress to more severe forms of the disease. Although these are promising results they need to be further evaluated to explore their clinical relevance in the management of those children. IP-10, a pro-inflammatory chemokine, is associated with CM and can discriminate well those children with prolonged clinical recovery times and higher mortality [20, 29]. Again, this study confirmed those findings.

Tumour necrosis factor alpha (TNF) is another pro-inflammatory cytokine which is elevated in different severe diseases, including malaria, in which it has been involved with neurological sequelae in children affected by CM [59]. Such a finding has triggered some attempts to find anti-TNF therapies for SM although without positive results [60]. In concordance with those data, the apoptotic factor sTNFR-1 was shown to be elevated in CSF in children with CM [30]. This study also showed increased sTNFR-1 levels in children with SM and this should be taken into account for designing new and successful anti-TNF therapies.

The activation of Triggering receptor expressed on myeloid cells 1 (TREM-1), expressed in monocytes and neutrophils, is involved in pro-inflammatory responses and anti-apoptotic mechanisms. sTREM-1 negatively regulates TREM-1 and both molecules maintain a physiological balance [61]. The dysregulation of these molecules may result in the trigger of an apoptotic state of different immune cells and the consequent immunosuppression [62]. Higher levels of sTREM-1 have been observed in children with SM when compared with uncomplicated malaria [28, 63] and those elevated levels correlated well with poor prognosis [20, 29]. Accordingly, these results confirm that sTREM-1 levels were higher in children with SM and confirm the potential of sTREM-1 as a malaria severity biomarker.

Although high concentrations of HRP-2 have been associated with SM [7-15] the fact that children with UM and SM were paired by parasitaemia hindered to explore the differences between both groups. The most relevant finding (commented before) is the correlation between levels of HRP-2 and Ang-2 which shows the close interaction between these markers in the pathophysiology of SM. As HRP-2 is the main antigen used in malaria rapid diagnostic tests it could be plausible to think about incorporating both biomarkers with quantitative measures to manage children with SM and improve its outcomes. Further research is needed to better investigate these promising findings.

Apart from the difficulties previously explained this study has some other limitations. First, the cross-sectional single encounter (at hospital first encounter) approach, prevented the evaluation of the evolution and dynamics of biomarker levels in response to infection and antimalarial treatment. In addition, the study of biomarkers and cytokines in peripheral blood may not accurately reflect the course of the infection and the response in specific organs where the parasite is most commonly sequestered. Furthermore, the low numbers of fatal malaria episodes hinders exploring variations in biomarker levels in relation to mortality.

CONCLUSIONS

Host biomarkers associated with endothelial activation and inflammation are able to robustly and reproducibly identify on first encounter those patients with greater severity. Their future use as part of a rapid, point-of-care, low-cost diagnostic test could revolution the screening and management of this disease whose impact in poor countries remains so devastating. Among all of them, Ang-2 appears as the most promising candidate as it represents an enzymatic pathway for which interventions already exist, and measuring its levels could both inform of prognosis and define interventional management.

ABBREVIATIONS

Ang-1: angiopoietin 1

Ang-2: angiopoietin 2

ARD: acute respiratory distress

BDNF: brain-derived neurotrophic factor

CI: confidence interval

CISM: Centro de Investigação em Saúde de Manhiça (Manhiça Health Research Centre)

CM: Cerebral Malaria

Cystatine C: Cys C

DSS: demographic surveillance system

HIV: Human immunodeficiency virus

IL-6: Interleukin 6

IL-8: Interleukin 8

IP-10: 10 kDa interferon γ-induced protein

LODS: Lambaréné Organ Dysfunction Score

MDH: Manhiça District Hospital

MS: Multiple seizures

MSS: morbidity surveillance system

OR: odds ratio

PCV: packed cell volume

PfHRP-2: Plasmodium falciparum histidine-rich protein-2

RBCs: Red blood cells

RDT: Rapid diagnostic test

SA: Severe Anaemia

SD: Standard deviation

sFt-1: soluble FMS-like tyrosine kinase-1

sTNFR-1: soluble tumor necrosis factor receptor 1

sTREM-1: soluble triggering receptor expressed on myeloid cells 1

SSA: sub-Saharan Africa

qPCR: real-time quantitative PCR

WHO: World Health Organization

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RESEARCH

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Safety and tolerability of adjunctive rosiglitazone treatment for children with uncomplicated malaria

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Abstract

Background: Despite the widespread use and availability of rapidly acting anti-malarials, the fatality rate of severe malaria in sub-Saharan Africa remains high. Adjunctive therapies that target the host response to malaria infection may further decrease mortality over that of anti-malarial agents alone. Peroxisome proliferator-activated receptor-gamma agonists (e.g. rosiglitazone) have been shown to act on several pathways implicated in the pathogenesis of severe malaria and may improve clinical outcome as an adjunctive intervention.

Methods: In this study, the safety and tolerability of adjunctive rosiglitazone in paediatric uncomplicated malaria infection was evaluated in Mozambique, as a prelude to its evaluation in a randomized controlled trial in paediatric severe malaria. The study was a prospective, randomized, double-blind, placebo-controlled, phase IIa trial of rosiglitazone (0.045 mg/kg/dose) twice daily for 4 days versus placebo as adjunctive treatment in addition to Mozambican standard of care (artemisinin combination therapy Coartem®) in children with uncomplicated malaria. The primary outcomes were tolerability and safety, including clinical, haematological, biochemical, and electrocardiographic evaluations.

Results: Thirty children were enrolled: 20 were assigned to rosiglitazone and 10 to placebo. Rosiglitazone treatment did not induce hypoglycaemia nor significantly alter clinical, biochemical, haematological, or electrocardiographic parameters.

Conclusions: Adjunctive rosiglitazone was safe and well-tolerated in children with uncomplicated malaria, permitting the extension of its evaluation as adjunctive therapy for severe malaria.

The trial is registered with Clinicaltrials.gov, NCT02694874

Background

Malaria causes an estimated 212 million infections and 429,000 deaths annually [1]. Following the demonstration of the superiority of intravenous artesunate compared to quinine, artesunate has become the standard of care for severe malaria in both adults and children [2, 3]. However, in spite of its improved efficacy over quinine, case fatality rates for severe malaria remain high, ranging from 8.5 to 30% [2, 3]. In addition, substantial post-severe malaria morbidity persists with long-term neurocognitive impairments, such as deficits in attention, memory, speech, and language reported in up to one-third of children surviving severe malaria [4–14]. Both parasite and host determinants contribute to the pathobiology of severe malaria. The host immune response plays a central role in the onset, severity and outcome of malaria infections, and this has accelerated the search for immunomodulatory adjunctive therapies that could improve

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clinical outcome. To date, several putative adjunctive strategies have been tested in severe malaria, however with disappointing results [15, 16].

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a member of the family of nuclear hormone receptors that function as ligand-activated transcription factors via their heterodimerization with another nuclear receptor, retinoic X receptor (RXR) [17–19]. PPARγ agonists are promising candidates for adjunctive malaria treatment as they have been reported to have anti-inflammatory, anti-oxidant, and neuroprotective properties [19–24]. The PPARγ agonist rosiglitazone is in the thiazolidinedione (TZD) class of drugs and is approved for the treatment of type II diabetes [25]. Rosiglitazone acts by increasing insulin sensitivity rather than increasing insulin levels, does not induce hypoglycaemia, and has an established safety profile in adults [25–29].

Rosiglitazone has been shown to enhance macrophage phagocytosis of *Plasmodium falciparum* parasitized erythrocytes, and to reduce parasite-induced pro-inflammatory cytokine secretion from monocytes and macrophages in vitro [30]. In a pre-clinical in vivo model of experimental cerebral malaria (ECM), rosiglitazone improved survival over artesunate alone, enhanced parasite clearance, reduced systemic inflammation and endothelial activation, prevented vascular leak, enhanced neuroprotective pathways, and protected mice from malaria-induced cognition and motor impairments [22, 31]. In light of these promising pre-clinical results, a randomized double-blind placebo controlled trial was conducted in young adults with uncomplicated malaria on the Thai–Cambodian border [32]. In this randomized trial, rosiglitazone was safe and well tolerated, and led to significantly improved parasite clearance times, lower levels of pro-inflammatory mediators, evidence of enhanced endothelial quiescence, and increased levels of the neuroprotective mediator brain-derived neurotropic factor (BDNF) [22, 32].

Together, these results support the hypothesis that adjunctive rosiglitazone may improve outcomes in patients with severe malaria. Since the majority of severe malaria and associated deaths occur in children under 5 years of age in sub-Saharan Africa, we conducted a phase IIa safety and tolerability trial in Mozambican children with uncomplicated malaria, as a prelude to undertaking a randomized trial in children with severe malaria.

Methods

Study design and participants

This was a prospective, parallel arm, unequally randomized, placebo-controlled, double-blind trial of rosiglitazone versus placebo, in 30 Mozambican children with uncomplicated malaria. All children received the Mozambican standard of care for uncomplicated malaria (Coartem® Dispersible; artemether–lumefantrine 20 mg/120 mg, Novartis) with dosage determined by body weight, twice daily as recommended by National guidelines [33]. An unequal randomization list (2:1, in favour of rosiglitazone) was generated using blocks of 3, using the free online randomization software Sealed Envelope™ (https://www.sealedenvelope.com/). Randomization codes were placed inside individual sealed envelopes that were opened only by the nursing staff responsible for the administration of the drug. The remaining investigators were blind to the allocated intervention. All laboratory tests and statistical analyses were performed blinded to treatment group. Enrollment took place between February and March 2016.

Ethical considerations

This study was reviewed and approved by the Mozambican National Bioethics Committee (CNBS) (Ref. 230/CNBS/15), the pharmaceutical department of the Mozambican Ministry of Health (Ref. 374/380/DF2016), the Clinical Research Ethics Committee of the Hospital Clínic, Barcelona, Spain (Ref. HCB/2015/0981), and the University Health Network Research Ethics Committee, Toronto, Canada (UHN REB Number 15-9013-AE). All research was conducted according to the principles expressed in the Declaration of Helsinki. The trial was registered with ClinicalTrials.gov on 9 December 2015, NCT02694874. All participants and their parents/legal guardians were given detailed oral and written information about the trial, and children were recruited only after a written informed consent was signed by their parents/legal guardians. Verbal assent was obtained from children over the age of 8.

Study setting, inclusion and exclusion criteria

The trial was conducted by the *Centro de Investigação em Saúde de Manhiça* (CISM) at the Manhiça District Hospital (MDH), in southern Mozambique. A detailed description of CISM may be found elsewhere [34]. In Mozambique, malaria transmission is perennial, with a seasonal peak from November to April [35]. Parents/caregivers of children presenting to MDH were asked to participate in the trial and were screened for eligibility. Children, aged 1–12 years, were included in the study if they were positive for *P. falciparum* by microscopy, whereby a thick blood film confirmed malaria infection with parasitaemia >2500 parasites/μL. Children were excluded if they were known to have any known pre-existing illness (including neurological or neurodegenerative disorders, cardiac, renal or hepatic disease, diabetes, epilepsy, cerebral palsy), presented any reason for hospitalization, or if they had clinical or laboratory

evidence of severe malaria (including severe anaemia, hypoglycaemia, acidosis, repeated seizures, prostration, impaired consciousness, respiratory distress, or age-adjusted tachypnea). Children receiving any therapy with potential anti-malarial activity (including cotrimoxazole), or treatment with a TZD were also excluded. Patients were approached after voluntarily presenting to MDH as part of routine care, and no financial incentives were provided.

Intervention

Rosiglitazone (Avandia®, GlaxoSmithKline) and an identical looking placebo manufactured at the Hospital Clínic's pharmacology department in Barcelona, Spain, were packaged and labelled to ensure blinding of study staff and hospital personnel. Children received either rosiglitazone (0.045 mg/kg/dose) or placebo twice daily for 4 days [36]. This dose was based on the maximal dose used by the manufacturer in the pediatric evaluation of rosiglitazone in children 10–17 [36]. The study medication was administered at the hospital, within the Clinical Trials Unit, by authorized members of the study team only. The study intervention (rosiglitazone or placebo) was started together with the first dose of artemether–lumefantrine. The interventions were administered orally. If any patient vomited or otherwise expelled the medication within 5 min of administration, the patient would be retreated. Rosiglitazone and placebo tablets were crushed and administrated as a powder mixed in water.

Treatment follow-up and laboratory procedures

Following documentation of informed consent, participants had an initial targeted physical examination performed by the study physician. Anthropomorphic measures were calculated upon admission using the WHO AnthroPlus Software version 1.0.4 for children 0–19 years old [37]. A blood sample was taken at baseline and prior to the administration of the study intervention, for malaria diagnosis by microscopy, and haematological (haemoglobin, haematocrit, platelets, white cell full blood count) and biochemical (renal and liver function, glucose and lactate) evaluations. For a strict monitoring of glycaemia, finger-prick samples for glucose monitoring were obtained on admission, every 6 h for the first 48 h, and then every 24 h until discharge, and again at the day 7 and day 14 follow-up visits. Hypoglycaemia was defined as blood glucose <2.5 mmol/L (45 mg/dL) in an adequately-nourished child according to WHO definition [37]. Lactate was monitored on admission, every 12 h for the first 24 h, and then every 24 h until discharge, and again at the day 7 and day 14 follow-up visits. Biochemistry, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, lactate dehydrogenase (LDH), and indirect and direct bilirubin, were assessed in venous blood every 24 h from admission until discharge and once again on day 7 follow-up. Venous blood extraction for haematology was performed every 24 h from admission until discharge and again on day 7 and 14 follow-up. Finally, venous blood extraction for biomarker analysis was performed on admission, 12, 24, 36, 48, 60, 72, and 84 h after admission, and again at the day 7 and 14 follow-up visits. Electrocardiographic monitoring was performed using a portable 12 lead electrocardiogram (ECG) machine (Cardioline ECG100+; AB Medica Group SA) at screening (before administration of study interventions), on day 1 (24 h after admission and after the second dose of study intervention), and on day 4 (after the last dose of study intervention). An additional ECG was conducted on day 7, only if abnormalities were recorded on day 4. The study clinicians reviewed all ECG tracings immediately after they were obtained, paying special attention to the QT segment length and potential prolongations from baseline. All children were kept at the health facility for the 4-day dosing period, despite being uncomplicated malaria cases. The mother/guardian was asked to return with the child for scheduled visits on day 7 and 14 post-treatment, or if any symptoms occurred. On each visit, a physical examination was performed by the study clinicians, vital signs were recorded, and body temperature measured.

Outcomes

The primary outcome was safety and tolerability over the first 84 h of hospital admission as determined by clinical, biochemical, haematological, and electrocardiographic observations according to specific pre-defined local reference ranges. Adverse events (AEs) and serious adverse events (SAEs) were recorded and monitored throughout the study.

Statistical analyses

Statistical analyses were performed with SPSS v.24 and Graph Pad Prism v.7. Differences between groups were assessed using the Fisher's exact test for categorical demographic values, and by t test or Mann–Whitney U test (two-tailed) for clinical laboratory data based on the distribution of the data. Glucose measures at each time point were compared using a Mann–Whitney U test (two-tailed).

Mann–Whitney U tests were used to compare the median changes from baseline for glucose, AST, ALT, haemoglobin and haematocrit between the two treatment arms. Mean haematocrit and haemoglobin values were compared using ANOVA. A p value <0.05 was considered as statistically significant.

Results

The trial's flow diagram is shown in Fig. 1. Baseline characteristics were similar between the two treatment groups (Table 1). Clinical monitoring of vital signs, including respiratory rate, heart rate, blood pressure, and oxygen saturation levels did not differ between groups. All study patients, irrespective of randomization group, had an eventless clinical course. No adverse drug reactions were observed, and no patient vomited in either group. No patient enrolled in this study had a SAE or died.

Treatment with rosiglitazone did not induce hypoglycaemia, and all glucose were above the lower blood glucose threshold of 2.5 mmol/L (Fig. 2). Median changes in glucose levels between baseline and day 14 did not significantly differ between the placebo and rosiglitazone arms (1.2 vs. 0.3 mmol/L, p = 0.52). Only one isolated lactate value out of the normal range (0–5 mmol/L) was observed in a rosiglitazone-treated patient at 24 h post recruitment (5.5 mmol/L) that stabilized without any additional treatment.

Only two values of AST were out of range (0–100 U/L). These were observed at baseline in two children receiving placebo. They spontaneously normalized and were not associated with any clinical symptoms or signs. Median changes in AST levels between baseline and day 7 did not significantly differ between placebo and treatment arms (3.5 vs. 7 U, p = 0.78). Nor was there a significant difference in median changes in ALT between placebo and rosiglitazone treated participants (3.5 vs. 11 U, p = 0.15) between baseline and day 7. For the remaining biochemical parameters, including urea, creatinine, LDH, direct or indirect bilirubin, no significant differences or trending abnormalities were observed.

Table 1 Patient characteristics on admission

	Placebo, N = 10	Rosiglitazone, N = 20	p
Female sex (%)	7 (70%)	11 (55%)	0.70
Age (years)[a]	8 (4.6, 9.1)	6.9 (4.7, 9.8)	0.92
Weight (kg)	21.5 [15.0, 24.8]	20.2 [16.1, 2.7]	0.87
Height (cm)	124.0 [101.0, 132.5]	115.0 [106.0, 131.5]	0.75
BMI/age	−1.13 [−1.40, −0.37]	−0.15 [−3.2, 0.24]	0.11
Weight-for-age Z score (WAZ)	−1.1 [−1.4, −0.73]	−0.67 [−1.5, 0.81]	0.34
Temperature (°C)	38.3 [36.6, 39.1]	36.6 [36.1, 37.5]	0.07
Heart rate (bpm)	123.2 (15.58)	120.1 (14.6)	0.60
Glucose (mmol/L)	7.2 [5.3, 8.3]	5.9 [5.1, 7.3]	0.40
Lactate (mmol/L)	2.5 [2.1, 2.9]	1.9 [1.8, 3.2]	0.23
Haemoglobin (g/L)	10.1 (0.8)	10.1 (1.5)	0.99
Haematocrit (%)	30.1 (2.3)	30.4 (4.0)	0.83
Leukocytes ($\times 10^9$/L)	8.3 (3.0)	7.4 (2.5)	0.41
Platelets ($\times 10^9$/L)	96.3 (34.3)	131.8 (59.4)	0.09
AST (U/L)	53.8 (41.7)	36.4 (10.5)	0.08
ALT (U/L)	27.5 [5.3, 41.0]	33 [26, 36.8]	0.54
Urea (mg/dL)	12.5 [12.0, 17.3]	14.0 [13.0, 15.8]	0.67
Creatinine (μmol/L)	35.3 [30.7, 39.0]	31.2 [29.8, 42.8]	0.81
Parasite density (parasites/μL)	23,499 {4166, 55,491}	24,622 {3346, 91,869}	0.91

Median [IQR] for non-normally distributed variables

Number (%) for categorical variables

Parasite density is represented as geometric mean {range}

[a] Mean (SD) for normally distributed variables

Haematological adverse events were uncommon. Three children had a haemoglobin decrease >2 g/dL from their baseline values, and further two had a haemoglobin value

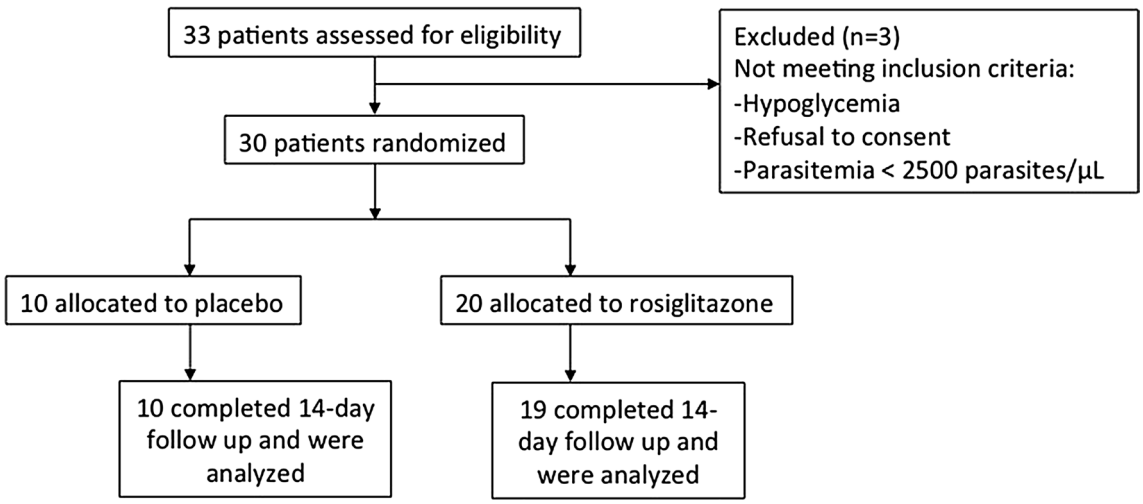

Fig. 1 Study profile of 33 patients screened for eligibility for a randomized, placebo-controlled, phase IIa trial of adjunctive rosiglitazone for the treatment of uncomplicated malaria in children

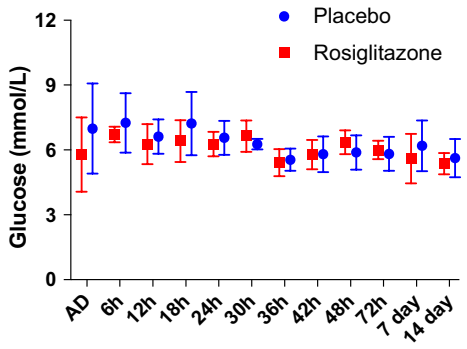

Fig. 2 Median glucose levels of study participants on admission (AD) and at each measured time point. *Bars* represent interquartile range

below the 7 g/dL threshold; none were associated with clinical manifestations. Median changes in haemoglobin levels between baseline and day 14 did not significantly differ between the placebo and rosiglitazone arms (0.4 vs. 0.7 g/L, p = 0.56). Median changes in haematocrit levels between baseline and day 14 did not significantly differ between the placebo and rosiglitazone arms (0.4 vs. 0.7 g/L, p = 0.56). There was no significant difference between the treatment groups on haematocrit or haemoglobin levels (p = 0.13 and p = 0.12, respectively, Fig. 3). No differences in haematological parameters, including leukocytes, platelets or other components of the complete blood cell count, were observed between groups.

Table 2 summarizes all ECG abnormalities found during the study follow up. None of these events were associated with clinical findings or with the study intervention. No additional medication was administered to these patients. No patient had a QTc of more than 500 ms at any of the measured time points.

Mean parasite densities at baseline and throughout the study were similar between groups, (Fig. 4). Median time to parasite clearance was 33 h in both the rosiglitazone and placebo groups (p = 0.88).

Discussion

In this study, the safety and tolerability of rosiglitazone as an adjunctive therapy for the treatment of uncomplicated malaria was investigated, as a first step before

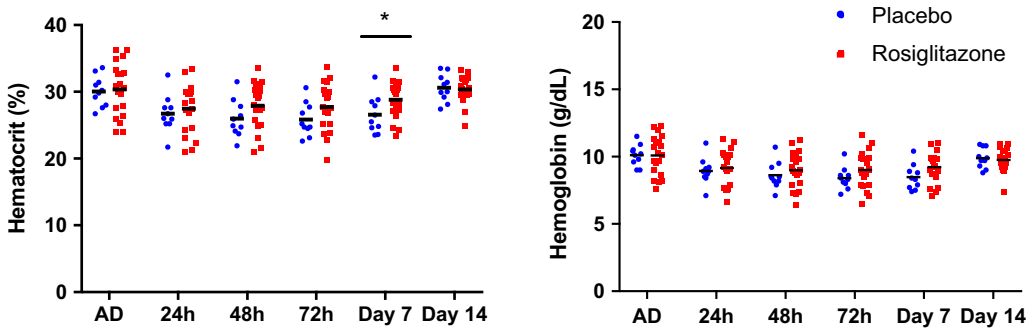

Fig. 3 Mean hematocrit and hemoglobin levels according to study group on admission (AD) and at each measured time point

Table 2 ECG abnormalities

Patient	Treatment	QTc baseline (ms)	QTc maximum (ms)	ECG findings
ROSI-002	Rosiglitazone	352	407	Increase of QtcF >50 ms on day 4, NCS
ROSI-007	Rosiglitazone	396	427	Increase of QtcF >50 ms on day 2, NCS. Finished on day 4
ROSI-008	Rosiglitazone	356	424	Increase of QtcF >50 ms on day 2, NCS. Continue on day 7, NCS
ROSI-009	Placebo	342	425	Increase of QtcF >50 ms on day 4 with associated bradycardia, NCS Increase of QtcF >50 ms on day 7, NCS and without bradycardia
ROSI-010	Placebo	336	409	Increase of QtcF >50 ms on day 4, NCS. Finished on day 7
ROSI-012	Rosiglitazone	323	404	Increase of QtcF >50 ms on day 2, NCS. Finished on day 4
ROSI-014	Placebo	364	425	Left bundle branch block from screening, NCS Increase of QtcF >50 ms on day 4, NCS. Finished on day 14 Bradycardia on day 4, NCS
ROSI-021	Placebo	361	414	Increase of QtcF >50 ms on day 4, NCS. Finished on day 7
ROSI-022	Placebo	349	402	Increase of QtcF >50 ms on day 4, NCS. Finished on day 7

These abnormalities were not clinically significant (NCS). QT corrected for heart rate using Fridericia's method

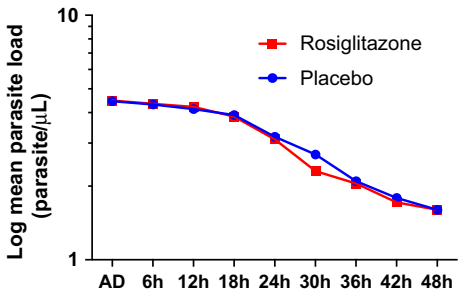

Fig. 4 Log mean parasite load in Mozambican children with uncomplicated malaria according to study group

evaluating its efficacy in the treatment of severe paediatric malaria and prevention of malaria-associated neurocognitive complications. Although rosiglitazone has a well-established safety profile, and millions of doses have been administered to adults with type II diabetes mellitus [38], this was the first time that the drug was used (off-label) as an adjunct to standard malaria treatment in African children. For this reason, and in concordance with recommendations issued by the CNBS, 30 children with uncomplicated malaria were carefully followed, and assessed for a variety of haematological, biochemical, clinical, and electrocardiographic safety variables, to ensure that the drug was not associated with adverse events. Although rosiglitazone was found to be safe and well-tolerated in children with uncomplicated malaria it cannot be assumed that a similar safety profile would be observed in children with severe malaria. Any future studies investigating the use of rosiglitazone for this purpose will be required to include additional safety evaluations.

Hypoglycaemia is a common and life-threatening complication of malaria [39], and drugs with negative effects on glycaemia levels would be problematic as adjuvant malaria therapies. Thus, particular attention was placed in assessing whether rosiglitazone would be associated with any glycaemia abnormalities in this population. Rosiglitazone acts by increasing insulin sensitivity rather than increasing insulin levels, and therefore, it is unlikely to induce hypoglycaemia. Although our sample size was small, it was reassuring that none of the malaria infected participants displayed glycaemia levels below 2.5 mmol/L, and glycaemia levels were similar between the treatment and placebo groups.

A second important aspect of this trial was to assess potential adverse cardiac events. Initial case–control studies of long-term use in elderly high-risk diabetic patients reported a potential increase in acute cardiac events [28]; however these studies had methodological problems and recent reports from the USA Food and Drug Administration (FDA) have concluded that rosiglitazone is safe and was not associated with excess cardiovascular risk [28]. Importantly, as of June 2013, an FDA expert panel relaxed restrictions on this drug [29]. In agreement with these findings, no cardiac adverse events or increased risk over placebo were observed in a previous randomized trial in adults with uncomplicated malaria [32]. Similarly, and as anticipated for a paediatric non-diabetic patient population treated with a short course (4 days) of rosiglitazone, no cardiovascular adverse events were observed in this study. Additionally, some oral artemisinin-based combinations have been reported to induce prolongation of the electrocardiogram's QT interval, while malaria infection itself can increase the sympathetic tone of the heart, which manifests as a shortening of the QT interval in ECG traces [40]. Repeated ECG measurements detected only a few electrocardiographic abnormalities, none of which were deemed related to the investigational drug. The incidence and clinical significance of these electrocardiographic abnormalities were equally distributed between the placebo and rosiglitazone groups.

Rosiglitazone has been reported to decrease mean haemoglobin and haematocrit in a dose-related fashion in adults, particularly when it is taken on a daily basis [41]. This potential effect needed to be evaluated in the context of malaria, a disease often associated with decreases in haemoglobin levels. No major declines in haemoglobin or haematocrit were observed in either study arm, and no significant interaction between the study arm and sampling time were observed for either haemoglobin or haematocrit.

Rosiglitazone, malaria, and many anti-malarial drugs can be associated with increases in hepatic transaminases [41–44]. In this trial, transaminase levels were monitored in addition to direct and indirect bilirubin, and no patient receiving rosiglitazone had levels outside of the normal range.

This study was not powered to evaluate efficacy endpoints of rosiglitazone. As such, the outcomes of the uncomplicated malaria participants recruited to evaluate the safety and tolerability of the drug, may not be sufficiently informative of the potential that the drug has to impact the course of disease. Although increase parasite clearance times were previously observed in adult patients randomized to rosiglitazone in a previous randomized control trial these patients received atovaquone/proguanil [32]. Even with a study powered to study efficacy we may not see improved parasite clearance times due to the fast clearance of early rings by artemisinins. There is limited pharmacokinetic–pharmacodynamic data of rosiglitazone in children under the age of 10. These data would have added more reassurance regarding the safety of rosiglitazone in children and is a

limitation of the study. Further evaluation of rosiglitazone, when used as an adjunctive therapy in the context of severe malaria, will be required to explore its impact on pathways implicated in the pathogenesis and outcome of severe malaria.

Conclusion

This is the first report of rosiglitazone use in African children with an acute uncomplicated malaria infection. The safety and tolerability results, including no vomiting, no idiosyncratic drug reactions, and no serious adverse events support its continued evaluation as an adjuvant therapy in the treatment of severe paediatric malaria.

Abbreviations

ACT: artemisinin-based combination therapy; AE: adverse events; ALT: alanine aminotransferase; Ang-2: angiopoeitin-2; AST: aspartate aminotransferase; BDNF: brain-derived neurotropic factor; CISM: Centro de Investigação em Saúde de Manhiça; CNBS: Mozambican National Bioethics Committee; ECM: experimental cerebral malaria; LDH: lactate dehydrogenase; MDH: Manhiça's District Hospital; ms: milliseconds; PPARγ: peroxisome proliferator-activated receptor-gamma; RXR: retinoic X receptor; SAE: serious adverse event; TZD: thiazolidinedione.

Authors' contributions

KCK, LS and QB conceived of the study and KCK, QB, LS, RV and VMC contributed to study design. RV and AS acquired the data. KCK, QB, AM, RV, VMC and LS analysed and interpreted the data. KCK, QB, RV, VMC, LM, AM and LS drafted the manuscript. AM, LS, LM, QB, KK, VMC, RV, HM and RB critically revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors do not hold a patent for this indication of rosiglitazone. Quique Bassat had during the duration of the study a fellowship from the programme Miguel Servet of the ISCIII (Plan Nacional de I+D+I 2008–2011, Grant Number: CP11/00269). Lola Madrid had a fellowship from the programme Río Hortega of the ISCIII (CM13/00260) while the study was conducted.

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Clinical trials to assess adjuvant therapeutics for severe malaria

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Abstract

Despite potent anti-malarial treatment, mortality rates associated with severe falciparum malaria remain high. To attempt to improve outcome, several trials have assessed a variety of potential adjunctive therapeutics, however none to date has been shown to be beneficial. This may be due, at least partly, to the therapeutics chosen and clinical trial design used. Here, we highlight three themes that could facilitate the choice and evaluation of putative adjuvant interventions for severe malaria, paving the way for their assessment in randomized controlled trials. Most clinical trials of adjunctive therapy to date have been underpowered due to the large number of participants required to reach mortality endpoints, rendering these study designs challenging and expensive to conduct. These limitations may be mitigated by the use of risk-stratification of participants and application of surrogate endpoints. Appropriate surrogate endpoints include direct measures of pathways causally involved in the pathobiology of severe and fatal malaria, including markers of host immune and endothelial activation and microcirculatory dysfunction. We propose using circulating markers of these pathways to identify high-risk participants that would be most likely to benefit from adjunctive therapy, and further by adopting these biomarkers as surrogate endpoints; moreover, choosing interventions that target deleterious host immune responses that directly contribute to microcirculatory dysfunction, multi-organ dysfunction and death; and, finally, prioritizing where possible, drugs that act on these pathways that are already approved by the FDA, or other regulators, for other indications, and are known to be safe in target populations, including children. An emerging understanding of the critical role of the host response in severe malaria pathogenesis may facilitate both clinical trial design and the search of effective adjunctive therapeutics.

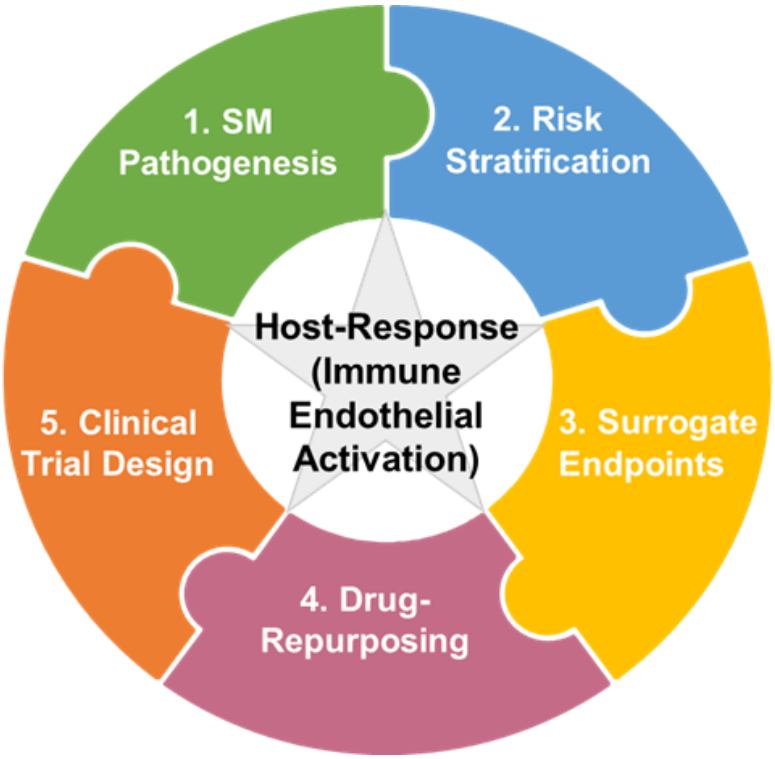
Keywords: Severe malaria, Angiopoietin-2, Immune and endothelial activation, Microvascular dysfunction, Host-biomarkers, Surrogate endpoints, Drug-repurposing.

Background

Mortality and morbidity rates associated with falciparum malaria infection remain high. The World Health Organization (WHO) estimated that malaria accounted for 405,000 deaths in 2018 [1], mostly affecting sub-Saharan African (SSA) children [1]. Despite effective treatment with artesunate, between 8.5 and 18% of patients diagnosed with severe malaria (SM) die [2] and up to 50% of cerebral malaria (CM) survivors may develop long-term neurological sequelae [3-5]. The Global Technical Strategy for Malaria 2016-2030 Report calls for at least a 90% reduction in malaria incidence and mortality by 2030 [6]. However, without new and accelerated interventions this goal will not be achieved. Thus, there is an urgent need to develop adjuvant therapy to be used concurrently with anti-malarial drugs to improve clinical outcomes.

SM is a multi-organ syndrome resulting from a complex interaction between both pathogen and host determinants, and its pathophysiology is yet to be fully understood [7]. However, it is becoming increasingly clear that endothelial and immune mediators play key roles in determining disease severity and outcome and thus represent attractive targets for host-directed interventions [8, 9]. There have been multiple efforts to identify adjunctive therapeutics, although to date none of these has been successful [10]. This likely reflects both our limited understanding of malaria physiopathology, as well as the challenges, cost and feasibility of conducting suitably powered randomized controlled trials (RCT) to evaluate mortality outcomes [11]. Most RCTs have relied on specific population sub-groups and were largely underpowered. In addition, study design/characteristics diverge widely between RCTs making it difficult to compare and extrapolate results from the available data [10]. Here, we outline three areas that may help to address limitations of previous efforts to identify effective adjunctive therapeutics (Figure 1).

Figure 1. Dysregulated host immune and endothelial activation as the rationale to enhance clinical trial design and identify adjunctive therapeutics for severe malaria The host-response plays a central role in the pathogenesis and outcome of severe malaria. Therefore, measuring levels of biomarkers of immune and endothelial activation, could be used both to identify patients that would benefit most from randomized control trials and as surrogate endpoints. FDA-approved drugs that protect and/or stabilize the host microvasculature and/or that are immunomodulatory could be repurposed as adjunctive therapeutics for severe malaria. These candidate therapeutics should be paired with the enhanced design of clinical trials.



Risk-stratification of patients with malaria

In SSA, there are challenges in the early recognition and triage of SM, with as few as 10% of malaria cases appropriately triaged for care and <30% of SM cases diagnosed and treated promptly, resulting in increased mortality and brain injury in survivors [12, 13]. WHO criteria for SM are commonly used to recruit patients for RCTs [14]. However, these criteria, which are a mixture of clinical and laboratory parameters, are broad, have widely variable prognosis [15], may overlap and can present with other co-morbidities, making it

difficult to assess and classify children [16]. Taylor *et al.* showed, in a *post-mortem* study, that 23% of children clinically diagnosed with CM, had died from other causes [16]. A recent meta-analysis highlighted the variability between SM-defining criteria and fatal outcomes. Some criteria, such as impaired consciousness and prostration, are weakly associated, while others, such as renal failure and deep breathing, are strongly correlated with death/outcome [15]. Additionally, the changing epidemiology of SM has caused a shift in its clinical characteristics (e.g., children that develop SM are no longer primarily restricted to <5 years of age) [17, 18].

It is important to re-evaluate WHO criteria to include emerging insights of SM pathogenesis and new aspects of SM epidemiology. Additionally, complementing WHO criteria with prognostic biomarkers could help identify high-risk patients that would most benefit from RCTs. Histidine-rich protein-2 (HRP-2), lactate, C-reactive protein (CRP) and procalcitonin (PCT), have all shown to be associated with poor outcomes in patients with SM, and have been considered for risk-stratification of children with malaria [19-24]. More recently, host-biomarkers of endothelial and immune activation, which may better reflect the pathological pathways underlying SM, have been identified as independent and quantitative markers of disease severity and outcome in both children and adults with malaria, both in Africa and Asia [25]. The most promising candidates are those that may be involved in casual pathways leading to death such as Angiopoietin-2 (Ang-2), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), soluble FMS-like tyrosine kinase-1 (sFt-1) and soluble tumour necrosis factor receptor 1 (sTNFR-1) and others [26-28]. Additional prospective studies to evaluate their predictive accuracy are required to define their potential clinical utility in triage and risk stratification. The available evidence to date supports Ang-2 as one marker that best addresses the priorities in this article and is also associated with disease severity in *Plasmodium vivax* and *Plasmodium knowlesi* infections [29, 30].

Ang-2, an integral member of the Ang/Tie axis, is a promising candidate for risk stratification and triage. During normal physiological states, the Ang/Tie axis is involved in maintaining endothelial integrity through the binding of Angiopoietin-1 (Ang-1) to its

receptor Tie-2. SM triggers a pro-inflammatory environment which promotes the expression and release of Ang-2, the antagonist of Ang-1, which competes for binding to Tie-2 and destabilizes the microvasculature [31]. Preclinical studies in mice have shown a casual and mechanistic link of the Ang/Tie axis in the pathogenesis of SM [32]. Data from human studies strongly support Ang-2 as an excellent biomarker for malaria disease severity and related multi-organ dysfunction and death; consequently, Ang-2 is a valuable new option for identifying high-risk patients for RCTs [26, 27, 33-35]. Ang-2 plasma concentrations are higher in children with SM compared to those with uncomplicated malaria (UM) [27, 34, 36, 37], and have also been linked to CM with retinopathy [36]. Importantly, the identification of retinal changes in children with CM has been a major advance in the risk-stratification of those patients [38].

Searching for surrogate endpoints of mortality

Conducting RCTs can be costly and time-consuming and in low-and middle-income countries the challenges are even greater [11]. To demonstrate efficacy of adjunctive therapeutics in reducing mortality, requires the enrolment of very large numbers of participants, which may be untenable due to cost and/or logistics. Power calculations indicate that at least 30,000 participants would have to be enrolled in order to observe a 10% change (parting from a 9% mortality rate) [11]. In an effort to address this problem the Severe Malaria African Children: A Clinical Network (SMAC) was created [39]. This was a multicentre pan-African effort to coordinate RCTs with mortality endpoints. Still, with such a network in place, it may take 3-4 years to enrol the required participants, meaning only a limited number of interventions can be assessed [11, 39]. Ultimately, underpowered studies can result in the inappropriate rejection of novel therapeutics because of their failure to show beneficial effects [11]. The identification of new surrogate endpoints, such as biomarker levels, might help address these problems. However, it is important to note that mortality should always be measured as a secondary endpoint in these RCTs, to allow a better characterization of the trends and relationships between levels of biomarkers and groups of treatment.

An appropriate surrogate endpoint should be able to predict/measure a clinical outcome for a specific intervention and be part of the casual pathway of the disease. This is particularly true when considering biomarkers, as if they are not direct readouts of the underlying pathobiology of SM, but rather just correlated to disease outcome, they may lead to confounding findings. Moreover, biomarkers used as surrogate endpoints and the intervention being assessed should also converge on the same pathways [40]. To date, the only proposed surrogate endpoint that has been validated for SM is plasma lactate. A secondary analysis on three datasets from clinical studies looking at anti-malarial efficacies, showed that measuring changes in plasma lactate concentration at 8 or 12 hours after intervention, is a valid surrogate endpoint for mortality for treatments aiming to improve microcirculation [11]. However, lactate has a number of limitations discussed in detail by Jeeyapant *et al.* [11]. Briefly these include that only a proportion of patients with SM will present with metabolic acidosis and that patients have poor outcomes related to multiple organ dysfunction (e.g., coma or acute kidney injury). Therefore, adjunctive therapy could improve survival through mechanisms that do not involve lactate clearance, and interventions that reduce lactate may not be effective adjunctive therapy.

In contrast to lactate, the Ang/Tie2 axis has been shown to have a causal relationship to severity and death for malaria [32] and Ang-2 concentrations are associated with multi-organ dysfunction leading to death, including acute kidney injury and coma [26, 41]. High Ang-2 concentrations have been linked to multi-organ dysfunction and mortality for multiple causes of sepsis, including malaria [27, 42-45]. Specifically, Ang-2 has been demonstrated to be elevated in patients with SM and to be an independent and quantitative predictor of mortality [27, 33]. Importantly, Ang-2 levels at admission are higher in children who die in hospital, as well as being associated with longer recovery times in survivors and post-discharge mortality [26]. Reduction in plasma levels of Ang-2 has already been used as a primary outcome in a RCT assessing inhaled nitric oxide as adjunctive therapy for paediatric SM [46]. Moreover, interventions targeting this pathway improve outcome in preclinical models [32, 47]. Taking into consideration the central role that endothelial activation and microcirculatory dysfunction play in SM pathogenesis and the mechanistic link that the Ang/Tie axis plays, we propose Ang-2 as another possible

surrogate endpoint candidate, either alone or in conjunction with other markers such as lactate. Furthermore, lactate can already be measured using a point-of-care (POC) test and there is ongoing research trying to design similar POC devices for Ang-2 and other markers. This could facilitate the implementation and impact of marker-based risk-stratification in resource-constrained settings.

Drug repurposing

Identification of novel therapeutics is expensive, time consuming and risky, with many promising new chemical entities never reaching or showing efficacy in Phase III trials. In the field of cancer research, it has been estimated that *de novo* therapeutic development takes between 10-17 years with cost estimates of 1-2 billion USD [8]. However, this can be de-risked, at least in part, by drug repurposing, which involves the search of new therapeutic indications for already marketed drugs with known safety profiles [48]. With this strategy, success rates may be enhanced with dramatically reduced costs and timelines to RCTs [8, 49, 50]. Therefore, drug repurposing is an attractive avenue for therapeutic development in common and rare diseases, including SM [8, 49, 50].

The primary hurdle in drug repurposing is the identification of appropriate drugs to test. A multitude of databases, data mining tools and compound libraries are emerging to help the scientific community sift through the plethora of potential candidates [50]. For example, Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME), is an open access screening library of 12,000 compounds compiled from commercial drug competitive intelligence databases [51]. Such tools could be used towards identifying adjunctive therapeutics for SM that target either deleterious host immune responses and/or protect/stabilize the microvasculature. A recent review explores the advantages and challenges of using licensed pharmaceuticals, developed originally as therapy for cancer and neurological disease, as possible candidates for CM. Furthermore, they emphasize the importance of targeting pathways of microvascular stability and blood brain barrier (BBB) function [52]. However, an accelerated strategy will still require that any promising candidate be prospectively evaluated in Phase II RCTs and then, if proven to be effective,

further assessed in larger Phase III trials evaluating adverse events and mortality before they can be more widely implemented.

A direct example of drug repurposing used in the context of SM is rosiglitazone [53, 54]. Rosiglitazone, a peroxisome proliferator-activated receptor (PPARγ) agonist, with immunomodulatory activity and capacity to promote endothelial integrity, was originally developed to treat type II diabetes. PPARγ-agonists were initially investigated because they were predicted to act on similar gene response elements as vitamin A metabolites (e.g., 9-cis retinoic acid), which were associated with protection in malaria preclinical models and in vitamin A malaria studies [55, 56]. Current evidence supports its utility to modulate multiple pathways in malaria pathogenesis. Preclinical models have shown that rosiglitazone reduces levels of Ang-2, increases levels of Ang-1, stabilizes the BBB and is neuroprotective [47, 57]. Adjunctive treatment with rosiglitazone has been shown to decrease inflammatory biomarkers associated with adverse outcomes, and reduce parasite burdens in adults [54]. In addition, rosiglitazone has been demonstrated to be safe and well tolerated in children with UM [53]. Cumulatively, this has led to its assessment as an adjuvant therapy in children with SM in an ongoing Phase II clinical trial (clinicaltrials.gov: NCT02694874). The primary endpoint of which is to determine whether rosiglitazone, in addition to parenteral artesunate (standard of care anti-malarial treatment), accelerates the rate of decline in Ang-2 from admission levels, compared to standard of care plus placebo. Atorvastatin is another FDA-approved drug that has been suggested as a possible adjuvant therapy due to its anti-inflammatory and neuroprotective effects [9].

Current barriers for biomarker implementation

The future use of Ang-2 and other biomarkers in RCTs has some important limitations that need to be considered. Although these molecules are independent and quantitative markers of severity and outcome, it is unlikely that any single clinical or laboratory measurement will be uniformly predictive. Therefore, algorithms that combine predictive clinical (e.g., LODs [58] or qSOFA [59]) and marker data may ultimately be most predictive. Importantly, these algorithms still need to be developed and validated. Moreover, evaluation of baseline malaria mortality (irrespective of being recruited to a trial using

biomarkers for risk-stratification) in the study population will need to be conducted, and would allow a better understanding of 'real mortality risk' in those not captured by biomarker levels. In addition, there is a clear variability in the thresholds/cut-offs and confidence intervals (CI) currently reported for biomarkers (including lactate and Ang-2) in association with mortality endpoints. There are many technical and methodological issues that may contribute to this variability and that currently preclude providing specific data on cut-offs/ranges. These include: the sample source (finger-prick *vs* venipuncture) and matrix used (whole blood, plasma (EDTA, heparin, etc.), serum); fresh *versus* frozen samples; the platform used to detect and quantitate the marker(s) (e.g., ELISA, Luminex™, ELLA™, etc.); patient population (adult, paediatric, underlying disease, HIV-1 infection).

What is clear is that there is an urgent need for rigorous prospective evaluation of candidate markers head-to-head under standardized protocols to first determine, and then validate cut-offs and CIs in further multi-site prospective studies. These studies have not yet been rigorously conducted and these issues will remain major barriers to the use of surrogate markers as endpoints of studies.

Conclusions

Our improved understanding of the pathobiology of SM should facilitate enhanced clinical trial design. Specifically: by decreasing required sample sizes by using biomarkers (e.g., Ang-2) to risk-stratify children and adults into RCTs; through the use of validated surrogate endpoints of mortality; and, via the search for safe FDA-approved drugs that modulate these underlying causal pathways (Fig.1).

Abbreviations

Ang: Angiopoietin

BBB: Blood brain barrier

CI: Confidence intervals

CM: Cerebral malaria

CRP: C-reactive protein

HRP-2: Histidine-rich protein-2

PCT: Procalcitonin

PPARγ: Peroxisome proliferator-activated receptor

POC: Point-of-care

RCT: Randomized controlled trial

SM: Severe malaria

SSA: Sub-Saharan Africa

UM: Uncomplicated malaria

WHO: World Health Organization

Declarations

Authors' contributions

RV and CE contributed equally and share first co-authorship. QB and KCK share senior co-authorship. The manuscript was prepared with input from RV, CE, SJ, QB and KCK. All authors read and approved the final manuscript.

Competing interests

KCK is a named inventor on a patent "Biomarkers for early determination of a critical or life-threatening response to illness and/or treatment response" held by the University Health Network. Remaining authors declare that they have no competing interests.

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5. SUMMARY OF RESULTS AND DISCUSSION

The studies included in this thesis provide significant results and underline knowledge gaps that, if correctly addressed, could contribute to improve our understanding of the pathophysiology and clinical manifestations of malaria in African children. Such knowledge could provide the evidence base for new interventions focusing on improving its adverse outcomes, which should be tested and eventually implemented in those areas of the world most affected by malaria, including Mozambique, where the greatest part of the work of this thesis took place.

During the last two decades there has been an unprecedented scale up in effective malaria control strategies such as, for example, LLINs and IRS, which have contributed to the important decline in the malaria burden in large parts of the world (213). In parallel, the wide deployment of new diagnostic and therapeutic tools such as RDTs and ACTs have considerably improved the devastating consequences of the disease (4). Nonetheless, malaria remains a stubborn public health problem, mainly in SSA. Nowadays, almost two out of three Africans live in moderate-to-high transmission areas, and ten countries include up to 87% of the people globally exposed to the highest malaria transmission intensities (214). More strikingly, 90% of the cases and 91% of the fatalities occur in SSA, mainly in children, who also account for two thirds (67%) of the total burden of deaths (215). Malaria is the third cause of death in children under five years of age and is still the most frequent cause of hospital admission in many areas of SSA (216-218). In fact, the reduction in malaria burden has not been homogeneous across the continent and some areas have even seen an increasing rate of malaria associated hospitalizations (219). It is also important to recall that, despite of the improved efficacy of artesunate in comparison to quinine, CFR for SM (8.5% in children and 15% in adults) and in particular CM (18 and 30%, respectively) remains unacceptably high (151, 152). This situation may worsen in areas with poor resources where individual case management is difficult and good clinical care remains often inaccessible.

In the year 2015, the WHO launched its Global Technical Strategy (GTS) for Malaria 2016–2030 (220). One of the main objective of this initiative is to reduce malaria mortality rates

and global incidence by at least 90% by 2030 (220). However, to achieve these ambitious goals there are some difficult challenges to face, from vector control to malaria case management. For instance, changes on mosquito behaviour or the emergence of insecticide resistance may threaten the current vector control measures (221). Climate change also appears as a potential significant threat to malaria control as it could alter the ecology of the mosquito vectors (222). The emergence and spread of artemisinin-resistance from the GMS is another major concern (223), moreover in the absence of new alternative effective antimalarial drugs to replace ACTs. In this situation, not only will it be difficult to attain the objectives of the GTS, but it is possible that all the previous gains could be seriously menaced. A deeper understanding of the interaction between the parasite, the host and their interaction could help, for example, to develop new tools to stratify children at higher risk of poor outcomes and to establish more effective treatment for SM. Furthermore, it could help to find adjuvant therapies to reduce the associated morbidity and mortality of the disease (224, 225).

The first article reviewed the current evidence in this field, not only with a focus on RCTs but also in pre-clinical studies. There have been numerous attempts to reduce malaria associated mortality and long-term *sequelae*, such as neurocognitive outcomes in SM survivors but, unfortunately, the majority of those efforts to enhance the efficacy of antimalarial drugs have failed. Extensive research has been conducted in experimental murine models and this still represents an useful and necessary platform to start investigating novel adjunctive therapies (226). Currently there are strategies based on different mechanism such as immunomodulation or neuroprotection which have given some interesting results. Examples of these are encapsulated glucocorticosteroids, curcumin, lithium chloride and nimodipine which, in different degrees, have improved survival and neurocognitive outcomes (227-236). Other studies have focused -with some success- on compounds for delivering gaseous signalling molecules (Glyceryl trinitrate (234)) or improving endothelial function, like recombinant human Ang-1, Vitamin D or atorvastatin (235-237) . Of these drugs, atorvastatin

seems to be the more attractive option to test in future human studies. These studies in murine models may give a more comprehensive insight of the pathophysiology of severe and CM, and how it can shift in different syndromes and type of patients. Consequently, therapies tested in preclinical models of SM are still a valuable resource of information and research. To improve their efficiency, they should employ scenarios as similar as possible to clinical practice, targeting the onset of clinical malaria symptoms and prevention of long-term *sequelae*.

The first article also examines the adjunctive therapies for SM tested in RCTs. Since 1980 there have been around 40 clinical trials which, considering the magnitude of the malaria problem, seems to be a clearly insufficient number. In addition, less than 50% of them involved patients < 14 years of age and only 11 trials specifically focused on CM. Unfortunately, none of these have shown a clear benefit. Multicentre studies involving a large number of patients or Phase III RCTs (118, 238) have been only a handful, and again, have failed to show clear beneficial results. Most of the studies performed have been Phase I or II RCTs, have been developed in one single centre and only included a small number of participants. In addition, some of them had to stop in advance because of harmful effects of the intervention tested. One big challenge in this area of knowledge is that, when comparing studies, it is difficult to extrapolate conclusions and consider future research considering the heterogeneity of the RCTs in terms of antimalarial used, type of malaria (severe and /or CM, coma), or study characteristics (limited number of patients per study, different and no comparable age of the populations, different treatment doses, studies not designed to identify differences in clinical outcomes or mortality).

The problem is that the global panorama will likely not change as soon as it would be desirable. In the clinical trial registration site ISCTRN (June 2020) there is only a single-centre Phase I trial ongoing (NOVICE_M trial: Evaluating the combined use of non-invasive respiratory support alongside preventative anticonvulsant treatment in children presenting to hospital

with cerebral malaria and convulsions; ISRCTN76942974). Furthermore, a search on ClinicalTrials.gov (June 2020) only identified one Phase III trial (PROTECtS- Effect of Paracetamol on Kidney Function in Severe Malaria; NCT04251351). Beyond this we can report that, only recently, we finalized the recruitment of a Phase II clinical trial performed in Mozambique in which the candidate of this thesis has been heavily involved (ROSI: NCT02694874). The primary objective of this study was to determine whether supplemental rosiglitazone (0.045mg/kg/dose) twice daily for 4 days in addition to standard of care anti-malarial treatment accelerates the rate of decline in Ang-2 from admission levels (as a proxy for a better prognosis) in children with SM compared to standard of care anti-malarial treatment plus placebo.

In conclusion, it seems that there will be little opportunity to reduce mortality burden with the currently ongoing research. Nonetheless, it is not only necessary to expand the number of studies to improve the situation, but also, to modify the current research model (224). Further research, with promising candidates that surpass previous constraints of earlier studies, is urgently needed in order to accelerate the identification of new adjunctive therapies for the treatment of SM. The use of drugs which target multiple pathways instead of a single one or the identification of host biomarkers as therapeutic target could help. Furthermore, the parallel and sustained surveillance of the safety of current available antimalarials and the emergence of resistance to those drugs is of paramount importance.

The second article is a retrospective analysis aiming to compare the use of intravenous artesunate and intravenous quinine for the treatment of malaria in children in terms of subsequent mid-term decrease on haematocrit values and higher need for blood transfusions. After the introduction of artesunate as first-line therapy for SM, some reports, mainly in returning travellers from non-endemic countries, raised awareness about PADH as a potential side effect that seemed to have been previously overlooked. The haemolysis of once-

iRBCs is now an expected consequence of the treatment with artesunate, which typically appears 2-4 weeks after treatment initiation in around 15-30% patients, usually requiring blood transfusions in most severely ill patients (157, 159-165, 239, 240). The main mechanism involved is the removal of pyknotic ring from parasites through splenic clearance of erythrocytes by pitting (166, 167) although the actual underlying cause has not been fully elucidated (see figure 11).

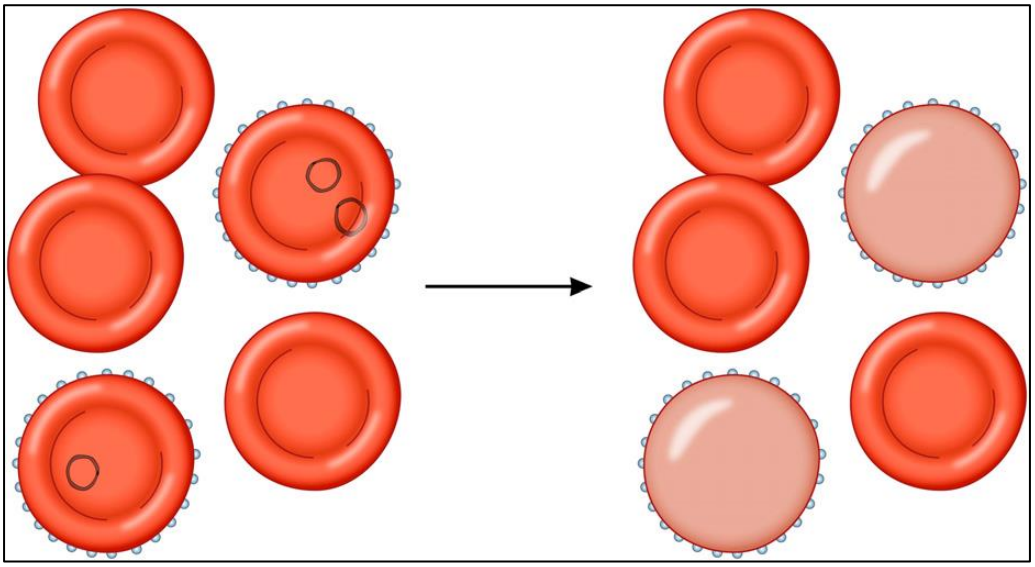

Figure 11: illustration of the process of pitting in the spleen. (Left) Red blood cell-infected. (Right) Parasites killed by artesunate have been removed by the spleen, resulting in a population of once-infected red blood cell with a shorter life span (7-21 days). Adapted from: *Arguin PM. Case definition: postartemisinin delayed hemolysis. Blood. 2014;124(2):157-8.*

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However, data regarding this complication cannot be directly generalized to endemic countries where epidemiological context, immunity, vulnerable age groups, patient characteristics, clinical manifestations, prevalence of anaemia, availability of blood products or quality of care may substantially differ. Contrary to the evidence from non-endemic countries, some studies developed in children in SSA have not reported PADH as a relevant complication of artemisinin treatment, but data are still scarce to understand the current situation. In different African countries like DRC, Uganda, Gabon, Ghana or Mali the

prevalence of haemolytic anaemia after artesunate treatment has ranged between 1% to 7% (156, 169, 241-244), although there are important differences in the study designs that may blur possible comparisons. This may be the case of our retrospective 15-year study in which we included 154 children (10.13%) who received parenteral artesunate and 1333 (87.75%) who received parenteral quinine, for which we had documented anaemia measurements in the weeks after the episode. 25.32% of children treated with artesunate presented an episode of anaemia compared to 22.8% of children treated with quinine without significant differences between both groups (OR=1.14, 95% CI =0.78, 1.68; p-value: 0.4962). Similarly, there were no differences in the incidence of anaemia between children receiving artesunate and those treated with quinine (285.42 episodes/1000 CMAR vs 318.8 episodes/1000 CMAR; HR: 1.12, 95% CI: 0.81-1.57; p-value: 0.4879). In this study, the overall rate of blood transfusions was 20.64 episodes/1,000 CMAR (CI: 14.15, 30.09), with the artesunate-recipients (6 out of 154) showing a higher rate when compared to the quinine group (21 out of 1133) ((44.73 episodes/1,000 CMAR vs 17.88 episodes/1,000 CMAR, respectively), (HR: 2.51; CI = 1.01–6.21; p-value: 0.0471).

In comparison to available data from other African settings, it appears that the anaemia associated to the treatment of SM is, in Mozambique, a more relevant problem. However, our results must be interpreted in the light of the context and limitations of our study. First, the study opted to evaluate the prevalence and incidence of anaemia (irrespective of whether confirmed to be of haemolytic nature or not) as an acceptable proxy for PADH, and its potential occurrence during routine practice. However, we did not use specific biomarkers of haemolysis in a population where a high prevalence of anaemia from other different aetiologies such as iron deficiency, haemoglobinopathies and β-thalassaemia, bacteraemia, viral infections (Parvovirus B19, Epstein Barr Virus, HIV) or intestinal parasites infections has also been described (116). This limitation may have resulted in an overestimation of the anaemia prevalence. Other limitations of this study include the big differences in the number

of participants in each group; the use of haematocrit instead of haemoglobin as marker of anaemia; the absence of active follow-up; and the long time period of analysis during which important epidemiological and case management variations may have occurred. Altogether, our data show that after its introduction as first-line regimen for the treatment of SM in Mozambique, artesunate has not worsened (nor improved) the problem of post-malarial anaemia. However, considering the high prevalence of anaemia irrespective of the treatment received (23.13%), it is necessary to call for active measures of follow-up in patients suffering from SM.

In Manhiça, a setting with a high HIV prevalence and, as many other African settings, with a chronic scarcity of available blood products, transfusions may pose an important problem in the management of SM. Importantly, our study showed that those children treated with artesunate received a higher number of blood transfusions than those treated with quinine. This is difficult to justify with the design of this study, but some hypothesis can be proposed. The epidemiological changes in Manhiça, shifting from a high to a low transmission area, may have provoked some modifications in the clinical presentation of SM cases and, consequently, in its management with, for example, a less conservative use of blood products. Whatever the real cause, it is important to highlight the need of constant surveillance of the antimalarial's safety profile and their effect on a dynamic epidemiological scenario with changing clinical presentation patterns. In Manhiça, for example, data show that SM cases present now in older ages and that CM is now more frequent (Guinovart, personal communication) than only a few years ago, where severe malarial anaemia was clearly the predominant phenotype of SM. A good characterization of all those changes is critical to assess and understand the real impact of new supportive interventions for the treatment of malaria. In addition to the physiological and clinical responses of the host to the infection, it is also important to advance in the description of certain characteristics of the *P. falciparum* parasite including its resistance profile to widely used antimalarials.

The third article investigated the proportion of multidrug resistant parasites (*P. falciparum Kelch13* mutants and gene copy number of both *Pfmdr1*and *Pfpm*2) in samples from patients with uncomplicated malaria infection participating in a RCT (MMV_OZ439_13_003). This Phase IIb clinical trial aimed to see the efficacy of a single dose of OZ439 combined with PPQ in adults and children infected by *P. falciparum* (245) (**Annex 6** of this thesis). The study enrolled 448 participants from 13 sites in Africa and Asia. One of the sites in this multicentre study was CISM from where the candidate participated in the recruitment of patients for this multicentre study.

As explained before (see Antimalarial drug resistance section) the emergence and spread of ACTs resistance from SEA is a major global concern, as this is the area where most resistance to others antimalarials (including piperaquine, chloroquine, SP, mefloquine and primaquine) seems to have initially emerged (131, 134-145). In Africa, no evidence to date supports the presence and circulation of parasites with the *Kelch13* phenotype associated with ACTs resistance, and clinical resistance to DHA-PPQ has not yet been reported (147, 246). The results of this study confirm the current situation because no *Kelch13* validated and candidate mutations were detected in any of the participating African sites (none of the 332 isolates from Burkina Faso, Uganda, Benin, the Democratic Republic of Congo (DRC), Gabon and Mozambique) while, in Vietnam, there was a high proportion of those mutations (67.6%). Having considered these data we should highlight that some *Kelch13* mutations still not validated were identified in Africa. The relevance of these mutations warrants further investigations.

In contrast with previous reports (247-249) there were 3-fold more parasites with multiple copies of *Pfmdr1* in Africa compared to SEA (21.1% vs 6.3%, p=0.002) (in Mozambique the proportion was 16.7 %). The presence of multiple copies of *Pfmdr1* is known to modulate parasite responses to a wide range of drugs including lumefantrine (250-252). As lumefantrine

has a long half-life, and the artemisinin component of the ACT combination disappears rapidly from the blood, lumefantrine ends up being present for a long time in the blood as if it had been effectively been used as monotherapy. Such high proportion of mutations may be explained by the routine use of artemether-lumefantrine as first line treatment in Africa. In Asia the low prevalence may be justified by the introduction of DHA-PPQ and the removal of mefloquine because the resistance of piperaquine and mefloquine seem to be mutually exclusive (148, 150, 253).

Concerning the isolates with multiple copies of *Pfpm2* there was a high frequency of them in Vietnam (13.9%), which might reflect the spread of the amplification of *Pfpm2* beyond the borders of Cambodia, where it also was previously reported in a high proportion of circulating parasites (148, 150, 253). Surprisingly, amplification of *Pfpm2* gene occurred at a much higher proportion (26.8 % on average across clinical sites in Africa with 12.5% in Mozambique and reaching 30.5% in Burkina Faso and 33.9% in Uganda) than was recently described (from 11.1% to 13.8% in Uganda, 10% in Mali and 1.1% in Mozambique) (254, 255). Finally, multiple copies of *Pfpm2*/single copy *Pfmdr1*, hypothesized to favour resistance to PPQ, were found at similar proportion in Africa and Asia (15.8.7 vs 12.7 %, p=0.72). In this region 10.8% of isolates had genotypes associated with both artemisinin and PPQ resistance. Sadly, i*n vitro* or *ex-vivo* drug susceptibility assays were not done and association between *Pfpm2* amplification and clinical resistance to PPQ was not verifiable in the current study. At this moment, in Asia and Africa, ACTs are the treatment of choice in any of their available formulations, including DHA-PPQ which is also being tested for prevention and control strategies such IPTp, IPTi or mass drug administration. The presence of validated *Kelch 13* mutations are associated with a slower parasite clearance but the clinical significance of other mutations like *Pfpm2* has to be further evaluated. In any case, this study shows the importance of closely monitoring the evolution across the world of parasite mutations because resistance

to artemisinin and its partner drugs can jeopardize malaria case management and lead to worse outcomes (225).

The fourth article is a case-control study which aimed to investigate the differential expression of host biomarkers in children with SM compared to children with uncomplicated malaria. We recruited children under the age of 10 who came to the Manhiça District Hospital in Mozambique (from September 2014 to May 2016). Cases and controls were nested by sex, age (+/- 6 months) and parasitaemia (in crosses) with 56 pairs finally included in the analysis. We found that the levels of Ang-2, Ang-2:Ang-1 ratio, sTie-2, sFlt-1, IL-6, IL-8, IP-10, TFNR1, sTrem-1 were significantly higher in children with SM when compared with children with uncomplicated malaria. After application of Bonferroni correction for multiple-comparisons Ang-2, sFlt-1 and IL-8 levels were still significantly higher in children with SM. Levels of IL-6 and IL-8 were higher in in children with LODS score of 1 when compared to LODS score of 0. They were also significantly higher in patients with a LODS score of ≥2 as well as levels of sFlt-1. HRP-2 levels were not different in children with UM and SM and there were no significant associations between HRP-2 levels and LODS score. HRP-2 levels were however significantly correlated with levels of Ang-2. These data show that host biomarkers of inflammation and endothelial activation are associated with SM, and they may have some important utility in differentiating those children with poorer prognosis. Importantly we confirm with our study the key importance of the Angiopoietin-Tie2 axis in the pathophysiology of SM. Angiopoietins 1 and 2 are directly involved in endothelial activation and low levels of Ang-1 and high levels of Ang-2 have been described in severe disease and associated with poor prognosis and mortality (38, 42, 51). Our data also show that Ang-2 levels are higher in children with SM and may help to differentiate those children with higher risk for a poorer outcome. Furthermore, we found a strong correlation between levels of Ang-2 and HRP-2 and this is an important finding which show the interaction between the parasite biomass and its influence in the process of endothelial activation (11).

As far as we can tell, no previous evidence regarding this relationship existed, and this study may open a way for further investigations to describe the ways in which this phenomenon occurs. It would be helpful to typify the parasite to see in which circumstances and what specific characteristics can trigger endothelial activation and dysregulate the Ang-Axis. Those investigations could help to design adjuvant therapies more targeted and effective. Our study also confirmed that there is a deregulated pro-inflammatory state (41, 42, 46-48) as part of the pathophysiological events which lead to SM. Interestingly, IL-6 and IL-8 seemed to be associated with higher LODS scores and this, could help to use them as potential markers of most severe cases. We did not perform statistical analysis to see if the combination of clinical symptoms, HRP-2 and host biomarkers of endothelial activation and inflammation can help better distinguishing the severe cases, so further analyses will bring more light into the importance of the sum of each of those components. In the future, these biomarkers will have to demonstrate their clinical utility and research must focus in finding ways to incorporate them in POC technologies to allow poor-resource areas to benefit from their use as screening tools. The research on those combinations and the strength of the different relationships should also help to find alternative therapies seeking to act in different levels of the pathophysiological chain of events leading to SM and its different manifestations.

In this specific context, the fifth article presents the results of a RCT aiming to determine the safety and tolerability of rosiglitazone (0.045mg/kg/dose) twice daily in children in addition to standard of care antimalarial treatment with uncomplicated malaria versus placebo plus standard of care antimalarial treatment for four days. Rosiglitazone, a PPARγ agonist (see PPAR-γ agonists and Rosiglitazone section), is a member of the thiazolidinediones which has been approved and is normally used for the treatment of type 2 diabetes. It acts by increasing insulin sensitivity rather than increasing insulin levels and it does not induce hypoglycaemia. The cellular mechanism of action of rosiglitazone is mediated by binding to an activation of the PPARγ. Previous research has demonstrated in pre-clinical studies the capacity of

rosiglitazone to reduce reduced systemic inflammation and endothelial activation and its neuroprotective action (175, 185). These results were confirmed in adults with uncomplicated malaria where rosiglitazone improved parasite clearance, and reduced biomarkers of inflammation (IL-6 and monocyte chemotactic protein (MCP)-1) and endothelial activation (Ang-2 to Ang-1 ratio), and increases neuro-protective pathways (BDNF) (186). However, rosiglitazone had never been used before in children < 10 years of age with malaria. The majority of information about safety and tolerability derives from adults, namely patients with diabetes and other cardiovascular or metabolic syndromes. Only a few studies performed in children confirmed in this population its safety profile. Consequently, as a request from the Mozambican Ethics Committee (CNBS) we conducted this trial as a first necessary step prior to further evaluate rosiglitazone in children with SM.

Thirty children, aged 1-12 years, were recruited, 20 of whom received rosiglitazone and 10, placebo. They were all closely monitored with periodical clinical examinations and evaluations of blood glucose levels, biochemical and haematological parameters (e.g. AST, ALT, creatinine, complete blood count) and electrocardiographic follow-up. Adverse events (AEs) and serious adverse events (SAEs) were recorded and assessed throughout the study. No vomiting, no drug reaction and no SAEs were observed. Importantly, there was not a single episode of hypoglycaemia determined among the participants. There were no significant changes in haemoglobin, haematocrit or transaminases in any of the two groups. Finally, there were no relevant alterations recorded in the ECG. This study has been the first report of safe and well-tolerated rosiglitazone use in children under 10 with uncomplicated malaria. Although the study was not powered to evaluate efficacy endpoints of rosiglitazone (no changes in biomarkers of disease severity in both groups), these data allowed to proceed to a Phase IIb trial with 180 Mozambican children to assess the efficacy of rosiglitazone as adjunctive therapy for children with SM (ROSI: NCT02694874). This trial will also allow to complete the safety profile of the drug and explore its real impact on pathways implicated in

the pathogenesis and outcome of severe malaria. Interestingly, the primary endpoint of this trial will be to assess the rate of decline of Ang-2, an endothelial activation biomarker.

The sixth article is a comment on the current challenges to perform RCTs in SM and it proposes some solutions to the limitations of the current model. This model is hampered by the lack of power of the studies to see an impact on mortality rates provoked both by the reduction of incidence and the decrease of CFR. This situation calls for innovative solutions and search of new surrogate markers of mortality. These markers must be involved in the pathophysiological pathways of SM which will allow them to be used as targets for diagnostic and therapeutic tools. We believe that Ang-2, due to its characteristics as host biomarker (see The Pathobiology of Severe and Cerebral Malaria section) would be an ideal candidate to fulfil all those functions and play a central role in the design of future RCTs. First, because Ang-2 could serve as a prognostic biomarker for risk-stratification of patients with SM, allowing to discriminate those with a higher risk for fatal outcomes (38). In addition, as Ang-2 has been proven to be a good independent predictor of mortality in SM (42, 256) this would support Ang-2 as an excellent alternative to be used as surrogate maker. At last but not least, the Ang-2/Tie axis can be used as a therapeutic target. Some drugs which have been already approved by FDA and other regulatory agencies, known to be safe in high interest populations such as children, have demonstrated their impact on affecting the axis and reducing levels of this biomarker. This is the case of rosiglitazone but also of others like atorvastatin. The repurposing of pre-approved and extensively used products may additionally and drastically reduce the associated cost of searching for new products and performing RCTs. New insights in the chain of pathophysiological events leading to SM will bring new options for improving the search of new and urgently needed adjunctive therapies for SM. At the current state of knowledge, Ang-2 seems to be a secured alternative for the present and a very promising solution for the future.

6. CONCLUSIONS

1. There are no currently available adjunctive therapies to improve the clinical and long-term outcomes of severe malaria which, in spite of prompt and effective treatment with antimalarials, still carries an unacceptably high associated mortality. In the absence of accelerated efforts to find new therapeutic tools, the objectives of The Global Technical Malaria Strategy 2016-2030 for a reduction of at least 90 per cent in malaria mortality by 2030 will not be achieved.

2. In a typical representative malaria-endemic setting such as Manhiça, the introduction of artesunate replacing quinine for the treatment of severe malaria is not associated with an increase in the frequency of post-artesunate related haematological disorders.

3. The use of artesunate and its relationship with the need of blood transfusions need to be further evaluated in an area, where the use of blood products is severely restricted by the high endemicity of other important infections transmissible through blood and other important logistical problems

4. Contrary to the situation in Southeast Asia, in Africa resistance to ACTs does not currently appear to be a problem. No evidence supports the circulation in Africa of parasites with the *P. falciparum Kelch 13* phenotype associated with artemisinin resistance.

5. The presence in Africa of the *Pfpm2* piperaquine resistance markers is higher than previously reported and could potentially compromise the clinical response to combinations using this drug, such as for example, DHA-PPQ.

6. Host biomarker levels detectable in the plasma of patients, and related with inflammation and endothelial activation, are differentially affected in severe malaria patients

compared to those with uncomplicated malaria and could help to risk stratify sick patients on arrival by reliably distinguishing between both syndromes.

7. Rosiglitazone as adjunctive therapy is safe and well-tolerated in children with uncomplicated malaria. The use of rosiglitazone is not associated with a higher frequency of vomiting, idiosyncratic drug reactions, or serious adverse events when compared with placebo.

8. Ang-2 as a biomarker appears as a good marker of endothelial activation in SM and could therefore be a promising candidate to be used in randomized control trials as a tool for identifying children with high risk of fatal outcomes secondary to SM, and as a surrogate marker of risk of mortality. Ang-2 merits further evaluation not only as a prognostic tool, but also as endpoint for the evaluation of therapeutic interventions targeting this specific metabolic pathway.

7. RECOMMENDATIONS

1. The current model of randomized control trials for the treatment of severe malaria is inefficient. This model must shift from the typical single-base centre, Phase I/II and low number of participants to a more collaborative model including networks of research centres to increase recruitment capacities, and the power and impact of the trials.

2. For maximizing the possibilities of success in the search of effective adjunctive therapies for severe malaria it is necessary to better characterize malaria epidemiology, pathophysiology and clinical manifestations of the disease. This will facilitate the risk-stratification of participants, the search of new surrogate markers of mortality and the use of new therapeutic targets.

3. The safety profile of currently available antimalarial drugs for the treatment of severe malaria must be well established and monitored in malaria endemic areas

4. It is important to establish surveillance systems to closely monitor the emergence and spread of parasite resistance to currently available drugs, in particular, artemisinin derivatives.

5. The complex pathophysiology of severe malaria must be further investigated to draw a complete picture of the interaction between parasite, host and environment.

6. Efficacy of rosiglitazone as adjunctive therapy for the treatment of severe malaria must be further evaluated in randomized control trials with children with severe malaria.

7. The clinical utility and cost effectiveness of Ang-2 measurements as a risk stratification and severity classifier tool needs further validation in randomized clinical trials.

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9. ANNEXES

TITLE: Update on malaria

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Abstract

Despite recent successful efforts to reduce the global malaria burden, this disease remains a significant global health problem. Only in 2018, malaria caused 228 million clinical episodes, 2-4 million of which were severe malaria cases, and 405,000 were fatal. Most of the malaria attributable mortality occurred among children in sub-Saharan Africa. Nowadays, rapid diagnostic tests and artemisinin derivatives are two of the main pillars for the management of malaria. However, considering the current situation, these strategies are not sufficient to maintain a reducing trend on malaria incidence and mortality. New insights on the pathophysiology of malaria have highlighted the importance of the host response to infection. Understanding this response would help to develop new diagnostic and therapeutic tools. Vector and parasite drug resistance are two major challenges for malaria control that require special attention. The most advanced malaria vaccine (RTS,S) is currently being piloted in 3 African countries

Introduction

Malaria is a protozoan disease transmitted by *Anopheles* female mosquitoes and results from the infection of a vulnerable host by *Plasmodium* parasites. Of the more than 120 *Plasmodium* species known to exist, only five cause malarial infections in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. *P. falciparum* accounts for the overwhelming majority of mortality, accounting for over 99% of all malaria-associated deaths globally. Although *P. vivax* has been traditionally considered to cause uncomplicated malaria, there is evidence of its potential to cause severe disease (1). *P. knowlesi* is a parasite transmitted from primates to humans that can also cause severe manifestations. *P. malariae and P. ovale* cause uncomplicated malaria, although rarely can associate other complications.

Epidemiology

Nearly half of the world's population is at risk of malaria which is currently endemic in 86 tropical and subtropical countries, encompassing all of sub-Saharan Africa (SSA) as well as large areas of South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas. (2).

Malaria caused an estimated 228 million clinical cases and 405,000 deaths in 2018 (2). 94% of all deaths occurred in Sub-Saharan Africa. The greatest burden of severe disease is borne by children under 5 years of age which represent 67% of global deaths (2).

The first 15 years of the millennium showed a stable and consistent reduction in the malaria burden, with important changes in its geographic distribution, and an overall reduction of around 60% in terms of malaria mortality. This led to a renewed enthusiasm, endorsed by the Global health community, towards a second push for malaria eradication. During the last 5 years, however, progress in terms of the malaria burden seems to have stalled, with a set of countries adequately progressing towards malaria elimination, but other countries showing an alarming increasing incidence of their malaria burden. *P. falciparum* accounts for the vast majority of malaria cases in the WHO African region (>99%), but is also a problem in regions of the Western Pacific (71.9%), the Eastern Mediterranean (69%) and South-East Asia (62.8%). *P. vivax* is the parasite driving the

burden in in the Americas and South-east Asia, and, apart from some specific regions of the Horn of Africa, is very uncommon across the rest of Africa due to the absence of Duffy antigen in human populations (3). *P. malariae* and *P. ovale* are globally distributed but their overall prevalence is low with *P. ovale* mainly present in Southeast Asia and West Africa. *P. knowlesi* is a zoonotic malaria, currently only transmitted to humans from macaques, and highly circumscribed in a small geographical region around Borneo in Malaysia. Malaria can be transmitted occasionally through other mechanisms that do not include the triad vector-parasite-human, namely through blood transfusions, organ transplant, or congenitally, but the relative contribution of these mechanisms to the overall burden is almost negligible.

Biology

The lifecycle of *P. falciparum* is summarized in Figure 1. The female *Anopheles* mosquito requires protein for egg development and inoculates the infective form of the parasite (*sporozoites*) when feeding upon humans. The sporozoites circulate for a few minutes in the bloodstream and invade hepatocytes where they remain, replicating for 7-14 days. This is called the pre-erythrocytic stage, and equals to the incubation period, as no symptoms are yet present. For species like *P. vivax* or *P. ovale* this stage may last weeks, months and even years as some parasites can remain dormant in the liver as *hypnozoites* (4), to subsequently relapse. After emerging from the liver as *merozoites*, the parasite starts its erythrocytic stage, whereby leading to the appearance of clinical symptomatology. Each merozoite entering the bloodstream will try to invade a red blood cell (RBC) and multiply into what is known as an erythrocytic schizont which will burst and release merozoites which can re-invade other RBCs and perpetuate the blood stage of the cycle. A small percentage of merozoites will differentiate into a different and parallel pathway and develop into the sexual stages or gametocytes. In order to ensure transmission to the next human being, the female and male gametocytes will need to be absorbed by a second mosquito where they will be able to complete the sexual reproduction in the vector's midgut. After a period of 9-14 days, the mosquito cycle ends up with the *sporozoite*

migrating to the salivary glands, from where they will be ready for a new bloodstream inoculation during a subsequent blood meal, thus completing the transmission cycle.

Pathogenesis

Traditionally, malaria has been defined in relation to periodic fever paroxysms which coincide with the parasite's intraerythrocytic cycles of each species (24 hours for *P. knowlesi* infection; 48 hours for *P. falciparum, P. vivax* and *P. ovale*; and 72 hours for *P. malariae*).

P. falciparum is the unique species able to cause multiple infections on a single RBC as well as to invade RBCs of any age, thus translating into a greater virulence and faster multiplication causing more severe disease. Both parasite and host determinants contribute to the onset and outcome of severe and cerebral malaria (CM) although why some individuals develop severe disease is still unknown (11). RBCs sequestration, inflammation and endothelial dysfunction are key components of the so called pathological triumvirate which leads to severe malaria (SM) (5).

First, sequestration is suggested to be mediated through the adherence of mature forms of infected RBCs to host receptors expressed on the endothelium lining host capillaries, on uninfected RBCs to form rosettes (6) and on platelets to form platelet-mediated clumps (7). Cytoadhesion, a key feature of the pathogenesis of *P. falciparum* associated infections, is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (8), which binds to numerous host receptors. Secondly, SM, including SMA and CM, has been also co-related with an excessive host immune response and, consequently, a deregulated inflammatory state. In third place, endothelial dysfunction is gaining importance as a key component of SM pathogenesis linking sequestration and inflammation (9).

High parasite biomass is also directly related to SM and the phenomena of RBC sequestration, inflammation and endothelial dysfunction (5). *P. falciparum* histidine-rich protein-2 (HRP2) is a water soluble protein produced by the parasite and released from RBCs, the measure of which provides a more robust estimate of the total body parasite

biomass, particularly when comparing it to peripheral parasitaemia (10, 11). High concentrations of HRP-2 are associated with SM and higher mortality (12, 13) .

Immunity

There is no sterilizing immunity to malaria infection. However, repeated exposure to infective mosquito bites leads to the progressive acquisition of clinical immunity and protects against severe disease and death. This is the case of children born in high-transmission areas, who after repeated exposure to infected bites, will acquire immunity to SM if they survive the first years of life (14). This does not mean that they will not suffer infections throughout their childhood or adult life, but the likelihood of those infections becoming clinically evident and severe will be drastically reduced. On the other hand, when transmission is low, and exposure less frequent, severe disease may be present at any age because of the lack of development of clinical immunity. In a context where there is a significant reduction of the malaria incidence, this should be taken into consideration, because reduced transmission may affect the acquisition of natural immune responses and therefore lead to changes in the clinical spectrum of the disease (15) . Those responses and the mechanisms associated are far from being completely understood (16).

Clinical features of malaria

The vast majority of malaria infections do only cause mild disease with only ~1% of *P. falciparum* infections causing severe clinical manifestations. An uncomplicated malaria case is defined as a patient with a clinical diagnosis of malaria with a *Plasmodium* asexual parasitaemia > 0 parasites/μL and not fulfilling the criteria for SM. The cardinal sign of malaria is fever, an abnormally elevated body temperature. In addition to this, the first symptoms of disease are non-specific including general malaise, fatigue, arthralgia, myalgia, headache, abdominal discomfort, nausea, vomiting or orthostatic hypotension. In malaria-endemic areas, malaria is the most common cause of fever and most patients will only have few abnormal physical findings. In non-endemic areas, malaria needs to be suspected in patients with a travel history to endemic countries.

SM is a complex multi-system disease that may be differently defined according to the age group it affects. Most children with SM can be identified by a combination of just three overlapping syndromes which differ in biological, clinical and epidemiological characteristics: Cerebral malaria (CM), Severe malarial anaemia (SMA) and acidosis/hyperlactatemia (clinically manifested as respiratory distress). Other manifestations include hypoglycaemia, acute kidney injury, jaundice, repeated convulsions, pulmonary oedema, significant bleeding, hyperparasitaemia, or shock (Table 1) (17). Clinical manifestations of malaria differ between adults and children, with multiorgan failure and shock being more frequent in the former. New insights, such as the demonstration of high prevalence of acute kidney injury in children (18), are however confirming that some clinical features considered more typical of adults are also frequent in children. Irrespective of the age, neurological involvement, acidosis and renal impairment are associated with poor outcomes and the combination of them may worsen the prognosis (19-21).

CM is characterized by severe impairment of consciousness (deep coma) in the absence of other alternative explanations or diagnoses. CM can also present with repeated seizures or other neurological abnormalities and is associated with different long-term cognitive and neurological deficits in up to one-third of survivors (22-27). CM, both in adults and children, may reach case fatality rates (CFR) of 20%, being brain swelling a key pathogenetic event to explain these high percentages (28-31). Respiratory distress is a common manifestation of SM which develops in up to 25% of adults and 40% of children with severe falciparum malaria (32). It usually presents with deep (acidotic) and labored breathing, tachypnea, low chest indrawing and sustained nasal flaring. Although SM is mainly caused by *P. falciparum*, SMA may present in all types of malaria (33). Prevalence of anemia in malarial endemic areas is very high, particularly in children, and has a multifactorial etiology (34). Malaria associated anaemia decreases with age and increases with exposure (5). Albeit its lower associated CFR, but due to its high incidence, SMA is the principal cause of malaria attributable mortality globally.

In the last decade, the pathogenic potential of other species such as *P. vivax* or *P. knowlesi* has also become evident. These two species should no longer be considered benign, and should be approached more vigorously in terms of their management.

Diagnosis

Malaria manifestations are non-specific and difficult to distinguish from other illnesses only based on a clinical approach. Current guidelines recommend to confirm the parasite presence in all suspected malaria cases before starting early, specific and appropriate treatment. Table 2 summarizes the current available tools for malaria diagnosis. Thick and blood films are still the gold standard for malaria diagnosis. Thick films are highly sensitive to define the presence or absence of infection, and thin films allow differentiation of species and quantification of malaria parasites with a limit of detection between 50-500 parasites/μL which can reach 5 parasites/μL with expert microscopists. However, rapid diagnostic tests (RDTs) are now the most widely available option and often the first-line investigation method, as they provide simple, sensitive and specific diagnosis based on the detection of HRP-2, pan-malaria or species-specific lactate dehydrogenase (LDH), or aldolase antigens in finger-prick collected blood samples (4). They are an affordable, cost-effective and easy to use technology with minimal training required and limited or no need of instrumentation; they can be stored without refrigeration and provide rapid results. Indeed, the RDTs have stirred a diagnostic revolution, allowing the endemic areas to escape from empirical treatment based on suspected malaria diagnosis. Altogether, these characteristics make RDTs a great option to improve the management of malaria cases, especially in areas with limited laboratory resources. They are also a valuable option in epidemic investigations and surveys. However, these tests are mainly qualitative and some of them do not differentiate between species giving less information than microscopy for an adequate management of the disease. RDTs may remain positive for several weeks after acute infection and complete parasite clearance, thereby presenting the possibility of them providing false positive results which merely reflect a recent infection. Another important problem has been recently identified regarding HRP2-based diagnostic tests, whereby, the test may produce negative results in infections caused by *P. Falciparum* parasites lacking the PfHRP-2/3 genes, a genetic evolution of the parasite that renders these RDTs useless. Such a phenomenon is being increasingly evidenced, and in countries such as Eritrea or Peru, up to 80% of the circulating falciparum strains may be HRP2/3 deficient (2), thus triggering the need to include non-HRP2 based RDTs in their diagnostic arsenal. Additionally, some

RDTs may also fail to work in the presence of very high *P. falciparum* parasitaemias (prozone effect), or be unable to detect low parasitaemias (threshold of detection is around 100 parasites/μL) (3). Nucleic acid amplification based-tests can detect low density malaria infections but in endemic countries their use is restricted for epidemiological research and surveys mapping and they do not have a practical role in the clinical management of malaria (35). In high income countries they might be used for accurate species diagnosis of imported malaria.

Case management

Effective case management is based on early and accurate diagnosis and treatment. The discovery of the artemisinin derivatives from the *Artemisia annua* plant *(36)* changed the paradigm of malaria treatment shifting from quinolone-based to artemisinin-based therapies, which are now the first choice to treat uncomplicated and severe *P. falciparum* malaria given their speed, potency and safety (table 3 summarizes the drug treatment options for treating malaria)

Uncomplicated malaria

The main objectives of the treatment of uncomplicated malaria are to prevent progression to severe disease and death, reduce clinical symptoms and cure the infection as soon as possible (2). In endemic regions, correct and prompt treatment should also help to prevent antimalarial drug resistance and to reduce onward transmission to others. In the past decades, this has relied essentially in the use of inexpensive and widely available antimalarial drugs in combination treatments, rather than as monotherapies.

Current recommendations state that children and adults with uncomplicated *P. falciparum* malaria (except pregnant women in their first trimester) should be treated with one of the following Artemisinin-based combination therapies (ACTs): artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, dihydroartemisinin-piperaquine, artesunate-sulfadoxine–pyrimethamine, and artesunate- pyronaridine. They combine two active drugs with different mechanism of action and different half-lives. ACTs are administered orally in regimens which must in all cases cover a 3-day full course.

Chloroquine is the recommended treatment for uncomplicated malariae, vivax and ovale infections which should be followed by a radical cure with a drug with specific effect

upon the liver hypnozoites. For this purpose, primaquine has been used globally for nearly seven decades, although its toxicity in G6PD deficient individuals (whereby it can cause severe or even life-threatening haemolysis) and compliance issues associated with the duration of recommended treatment (14 days) have made the treatment of *P. vivax* and *P. ovale* suboptimal (37). More recently, tafenoquine, a single dose compound with similar antihypnozoitic potential to the complete primaquine treatment, has been approved by stringent regulatory authorities, although its toxicity liabilities regarding G6PD deficient individuals remain the same, and require its use obligatorily associated with the screening and quantification of G6PD activity (38, 39). In case of resistance to chloroquine, or in countries where both falciparum and vivax coexist frequently, ACTs have become the first line drugs also for the treatment of vivax.

Pregnant women

Pregnant women are a particularly vulnerable group as malaria infection may lead to develop pregnancy loss, severe anaemia, pulmonary edema and hypoglycemia. Mortality in pregnant women with SM may reach 50% and is more likely to occur during 2nd and 3rd trimester, especially in the first pregnancy. Furthermore, pregnant women are at higher risk of treatment failure. In the first trimester of pregnancy it is recommended to use oral quinine and clindamycin, during 7 days (40). The recommended drugs during 2nd and 3rd terms of pregnancy are ACTs which have proven to be safe and effective and it is expected that in a near future they will be also indicated during the first trimester (41). Congenital malaria is rare in endemic countries where mothers have high level of antibodies which they transmit to their offspring, however this complication needs to be taken into consideration when naïve pregnant women with no immunity whatsoever against malaria travel to endemic areas and get infected there. Congenital malaria in newborns behaves as a systemic disease, and needs to be in the differential diagnosis of neonatal sepsis.

Non-immune travelers

Non-immune travelers are individuals from areas without malaria transmission who travel to malaria endemic areas and get exposed to infective bites there. They are generally malaria-naïve and, consequently, a high-risk group that can easily develop SM. They should receive early diagnosis and prompt treatment according to national policies. In non-endemic areas appropriate diagnosis and management can be difficult due to the

lack of familiarity of practitioners, the poor recall of travel as a risk factor, and the limited availability of some antimalarials. Chemoprophylaxis using antimalarial drugs before, during and immediately after the trip is currently considered the best recommendation to prevent malaria in this particular vulnerable group. In case of infection, ACTs or atovaquone-proguanil are the currently recommended regimes for treating uncomplicated malaria in these patients.

Treatment failure

When malaria treatment is not successful, symptoms may recur with an associated positive parasitemia 2-6 weeks after initial regimen. There are some issues which may explain this failure as high parasite burden, the limited availability of pediatric friendly formulations, impaired or reduced drug absorption due to severe disease and vomit, the limited shelf-life of the artemisinin derivatives, the emerging resistance to artemisinin derivatives or existing resistance to the partner drug, and the lack of new drugs/partner drugs. In endemic countries there are additional challenges as difficulties in access to health system; drugs costs, distribution and stock out challenges, and the worrying and under assessed threat provoked by "counterfeit" or substandard drugs (42, 43).

Antimalarial Drug resistance

Emergence of antimalarial drug resistance threatens effective antimalarial drug treatment, malaria control, and elimination. It has been observed for all antimalarials and, more recently, also for the artemisinins. Artemisinin resistance appears to be "partial", is characterized by a slower parasite clearance, and has emerged (like all other resistances documented for antimalarial drugs) in the Greater Mekong Subregion (44). Although sporadic case reports of delayed clearance times for artemisinins have also been reported in SSA, there is no evidence that such a phenotype has reached the most vulnerable continent (45). Polymorphisms in the kelch13 (k13) propeller gene of *P. falciparum* have been associated with artemisinin resistance (46).

Of the five human malaria species, *P. falciparum* and *P. vivax* have developed resistance to antimalarial drugs. There is no conclusive evidence about chloroquine resistance in *P. malariae* (47).

Management of severe and cerebral malaria

Parenteral artesunate is now widely accepted as the standard of care for the treatment of SM, both in adults and children, following the landmark SEAQUAMAT and AQUAMAT trials that demonstrated its superiority over quinine (48, 49). The main advantage of artemisinin derivates is their potency leading to a rapid reduction of the parasite biomass. Although artesunate is more effective than quinine for the treatment of SM, this drug remains highly efficacious in its parenteral form and is still indicated in the treatment of malaria during the first trimester of pregnancy (40). Intramuscular artesunate administration has proven to be non-inferior to intravenous artesunate in reducing parasitemia ≥99% at 24 hours in children with SM (50). Intramuscular artemether seems to be inferior to parenteral artesunate in adults but is more effective that quinine in adults and children, thus being another valuable option when artesunate is not available (51). Parenteral treatment must be shifted to oral when the patient improves and is able to eat and drink. Artesunate is also indicated in its rectal form as a pre-referral option for children under six years of age living in remote areas waiting for immediate transfer to a higher-level (52).

Malaria complications may develop very fast, and may lead to death only a few hours after the first symptoms. Patients with SM may be adequately monitored with frequent measures of vital signs and hematological and biochemical parameters such as glycaemia, hemoglobin or renal and hepatic function. Whenever feasible, monitoring parasite density is desirable until confirming parasite clearance (17) .

Adjunctive therapies for severe malaria

Even with the improved efficacy of artesunate, CFR for SM and CM remain high (48, 49). Therefore, treatment with potent artemisinin-derivatives alone is insufficient to prevent death or neurological disability in all patients with SM. Adjunctive therapies are used in combination with primary antimalarial treatment, with the aim of improving clinical outcomes, reducing mortality, and preventing neurocognitive impairment. Several adjunctive therapies as steroids, immunoglobulin, anti-TNF therapies, antiepileptic drugs, mannitol, nitric oxide or blood transfusions have been evaluated without proving any success (53, 54).

Prevention of malaria

Vector control or bite prevention

Measures to reduce the mosquito population or to limit the contact between humans and mosquitoes are the best preventive measures against malaria (2). Scale up of Insecticide-based, home-centered interventions such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are surely important contributors to the significant decline in the malaria burden witnessed globally from 2000 to 2015 (55). However, such success is threatened by the emergence of mosquitoes resistant to pyrethroids (56), the only insecticide currently use in LLINs, and to other insecticides used as part of other vector control strategies. Additionally, several mosquito behavioural changes are important biological challenges in our fight against malaria, including shifts to outdoor or early biting, quick house exiting right after feeding and even partially feeding upon animals. Such behavioural changes may allow vectors to avoid insecticide and keep transmission high even in the presence of good LLINs or IRS coverage in a phenomenon known as residual transmission (57). Innovative approaches are needed to control residual transmission, some options being considered include the use of drugs (such as for example ivermectin, a potent endectocide (58)) that kill mosquitoes feeding upon treated people or animals, attractive targeted sugar baits and spatial repellents (59).

Chemoprophylaxis

Intermittent preventive treatment

These are innovative preventive schemes for the administration of different antimalarial treatment regimens separated in time to risk groups, benefitting with high coverage encounters of the malaria-endemic populations with the health system. Preventive treatment in pregnant women with sulfadoxine-pyrimethamine (SP) is a recommendation implemented in many African countries with high *P. falciparum* endemicity, and has clear benefits on the health of the pregnant mother and the newborn. WHO currently recommends at least three doses (one month apart) during pregnancy, although the coverage of such a recommendation still remains suboptimal.

.- Seasonal malaria chemoprevention

This is a strategy to protect children under five years of age in the Sahel, where malaria is highly seasonal and transmission occurs only during a few consecutive months

of the year. It consists of administering a monthly dose of an artemisinin-free antimalarial (SP-Amodiaquine) during the malaria-transmission season (usually 3-4 months long). Such a strategy has demonstrated an 80% reduction in malaria episodes and almost 60% reduction in all-cause mortality when implemented appropriately. WHO also recommends intermittent prophylaxis in infants in areas where malaria transmission is moderate-high and *P. falciparum's* resistance to SP is less than 50% (40), but the lack of real-time data on the frequency of this phenotype has hindered this recommendation.

.- Fixed-term prophylaxis

Antimalarial chemoprophylaxis is recommended for everyone, especially children, traveling to malaria-endemic areas. This should be done with the most suitable drug or combination of drugs (atovaquone-proguanil, doxycycline, mefloquine, etc.) for the area to be visited and which best adapts to the idiosyncrasy of the traveller, always following the current recommendations of the WHO or the US Centers for Disease Control and Prevention (CDC) . The drug or combination of drugs should be started before the trip, with the aim of reaching good blood levels upon arrival at the destination, and should generally be continued for an additional 1-4 weeks after returning, to cover the incubation period of a possible infective bite received in the last days of the trip. HIV-infected patients receiving co-trimoxazole as prophylaxis for opportunistic infections are partially protected of malaria infections, but should consider taking additional drugs as this scheme is not sufficient to guarantee full protection.

Vaccines

The great antigenic variability shown by the malaria parasite throughout its life cycle has made the design of effective vaccines a titanic task. The RTS,S/AS01 malaria vaccine, approved by stringent regulatory authorities (European Medicines Agency) in the year 2015 is, currently, the only effective compound, and has shown consistently significant (albeit partial) levels of protection, both against clinical malaria and severe malarial disease (60). A pilot and large-scale implementation program, promoted by the WHO, was launched in 2019 in three African countries, namely Ghana, Kenya and Malawi to evaluate its protective effective, the feasibility of administering 4 doses, the impact on overall infant mortality and the safety of its routine use in endemic countries. It is expected that after three years of implementing such pilot program, a clear recommendation will

emerge on the need to include this vaccine as part of the expanded programme of immunization throughout falciparum endemic settings. The development of a vaccine against *P. vivax* is far behind the development of vaccines against P. falciparum, with no recent clinical trials beyond phase II.

Another approach to the development of an effective vaccine, consists on the attempt to develop an immune response as a result of the inoculation of irradiated and therefore attenuated *P. falciparum* sporozoites. Such an approach, logistically more complex, has however provided initial promising results in human volunteers subject to an experimental malaria challenge, but the efficacy of such a vaccine needs to be confirmed in malaria-endemic areas (61).

Conclusions and Future perspectives

In the third decade of the 21st century, malaria remains a significant challenge to the health of humans living where it is transmitted. The encouraging trends observed in the first years of the millennium are now threatened by a stall in progress reducing malaria cases. The new push for malaria eradication, which monopolized most malaria efforts in the last decade seems to have been tampered by a renewed spirit of enhanced efforts in those countries with higher burdens, recognizing that lack of improvements there will thwart global progress. In this particular moment, innovation and research focussing on new therapeutics, diagnostics and insecticides need to continue at the forefront of global efforts, so as to overcome the biological challenges affecting the tools available, and thus creatively bypass the hurdles that are hampering our fight against this deadly disease.

Competing interests

The authors declare that they have no competing interests

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Table 1: Clinical defining features of severe malaria

Impaired consciousness	A Glasgow Coma Score <11 in adults or a Blantyre coma score <3 in children
Acidosis	A base deficit of >8 meq/l or, if unavailable, a plasma bicarbonate of <15 mM or venous plasma lactate>5 mM. Severe acidosis manifests clinically as respiratory distress – rapid, deep and laboured breathing
Hypoglycaemia:	Hypoglycaemia: Blood or plasma glucose <2.2 mM (<40 mg/dl)
Severe malarial anaemia:	Severe malarial anaemia: A haemoglobin concentration <5 g/dl or a haematocrit of <15% in children <12 years of age (<7 g/dl and <20%, respectively, in adults) together with a parasite count >10 000/µl
Renal impairment (acute kidney injury):	Plasma or serum creatinine >265 µM (3 mg/d) or blood urea >20 mM
Jaundice:	Plasma or serum bilirubin >50 lM (3 mg/dl) together with a parasite count >100 000/µl
Pulmonary oedema	Radiologically confirmed, or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose gums or venipuncture sites; haematemesis or melaena
Shock	Compensated shock is defined as capillary refill ≥3 s or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Hyperparasitaemia:	*P. falciparum* parasitaemia >10%

(**Source**: Severe malaria. Trop Med Int Health. 2014;19 Suppl 1:7-131.)

Table 2: Diagnostic tools for detection of malaria, benefits and limitations

Method	Principle	Benefits	Limitations
Clinical diagnosis	Recognition of symptoms	Rapid and cheap	Very unspecific (over diagnosis and overtreatment)
Microscopy	Observation of malaria parasites in blood smears (thick and thin films)	Species differentiation and quantification	Labor and time consuming
		Follow-up of patients	Requires trained staff and equipment maintenance
Rapid diagnosis test (RDT)	Detection of malaria antigens in blood samples	Fast (5–20 min) and simple to perform and interpret	Possible false positives and negatives
		Affordable and stable in extreme conditions	No species differentiation or quantification
		No need of high qualified staff and equipment	Limited performance for mixed-infections
Polymerase chain reaction (PCR)	Identification of malaria DNA in blood samples	High sensitivity and specificity	High cost
		Species differentiation and quantification	Need of highly technical expertise and equipment maintenance
		Antimalarial resistance detection	

(**Adapted from**: Rubio M, Bassat Q, Estivill X, Mayor A. Tying malaria and microRNAs: from the biology to future diagnostic perspectives. Malar J. 2016; 15: 167)

Table 3: Main principles for the drug treatment of malaria

Severe malaria
Treatment of choice • Artesunate (i.v. or i.m.) 2.4 mg/kg* immediately, then at 12, 24 h and daily until patient is able to drink and eat (*For children <20 kg the parenteral artesunate dose is 3 mg/kg) Alternatives • Artemether (i.m.) 3.2 mg/kg initial dose followed by 1.6 mg/kg daily until oral medication can be taken reliably • Quinine dihydrochloride (20 mg salt/kg) by slow intravenous infusion over 4 h or by i.m. injection split to both anterior thighs, followed by 10 mg salt/kg 8 h until patient is able to swallow.
Uncomplicated malaria
Uncomplicated *P. falciparum* malaria • Artemether 1.4–4 mg/kg body weight + lumefantrine 10–16 mg/kg body weight twice daily for 3 days • Artesunate 4 mg/kg body weight + amodiaquine 10 mg/kg body weight once daily for 3 days • Artesunate 4 mg/kg body weight + mefloquine 8.3 mg/kg body weight once daily for 3 days • Dihydroartemisinin 4 mg/kg body weight + piperaquine 18 mg/kg body weight once daily for 3 days (for children <25 kg the dose of dihydroartemisinin is at least 2.5 mg/kg per day) • Artesunate 4 mg/kg body weight + sulfadoxine–pyrimethamine 25/1.25 mg/kg body weight, once daily for 3 days • Artesunate 4 mg/kg body weight + pyronaridine 7.5-15 mg/kg body weight, once daily for 3 days Uncomplicated C*hloroquine-sensitive*[1] *P. vivax, P. ovale*[2]*, P. malariae, P. Knowlesi* • Chloroquine dose of 10 mg base/kg body weight at days 1 and 2 followed by 5 mg base/kg body weight at day 3.

1. In areas with chloroquine-resistant infections, adults and children with uncomplicated *P. vivax, P. ovale, P. malariae* or *P. knowlesi* malaria (except pregnant women in their first trimester) can be treated with an artemisinin derivative (except artesunate- sulfadoxine–pyrimethamine for *P. vivax*)

2. If there are no contraindications (pregnancy, children <6 months, glucose-6-phosphate-dehydrogenase deficiency) treatment of *P. vivax* and *P. ovale* must be followed by a radical cure with primaquine 0.5-1 mg/kg body weight once daily during 7-14 days. Alternatively, a single dose treatment with tafenoquine has already been FDA approved in patients ≥16 years of age (dose 300 mgs), although not yet widely implemented or recommended by WHO.

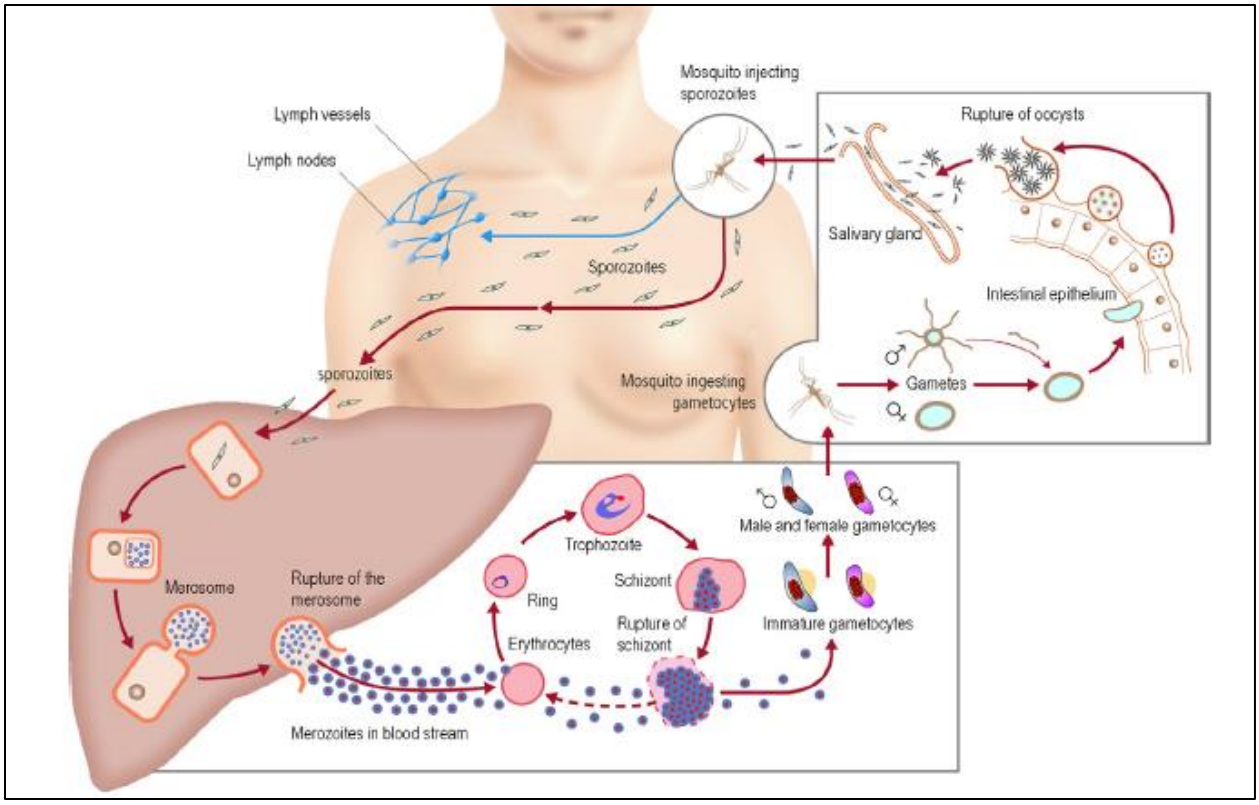

Figure 1: Lifecycle of *P. falciparum* in human body and anopheline mosquito

TITLE: Diagnosis of clinical malaria in endemic settings

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Abstract

Introduction: Malaria continues to be a major global health problem, with over 228 million cases and 405,000 deaths estimated to occur annually. Rapid and accurate diagnosis of malaria is essential to decrease the burden and impact of this disease, particularly in children. We aimed to review the main available techniques for the diagnosis of clinical malaria in endemic settings and explore possible future options to improve its rapid recognition.

Areas covered: literature relevant to malaria diagnosis was identified through electronic searches in Pubmed, with no language or date restrictions and limited to humans.

Expert opinion: Light microscopy is still considered the gold standard method for malaria diagnosis and continues to be at the frontline of malaria diagnosis. However, technologies as rapid diagnostic tests, mainly those who detect histidine-rich protein-2, offer an accurate, rapid and affordable alternative for malaria diagnosis in endemic areas. They are now the technique most extended in endemic areas for parasitological confirmation. In these settings, PCR-based assays are usually restricted to research and they are not currently helpful in the management of clinical malaria. Other technologies, such as isothermal methods could be an interesting and alternative approach to PCR in the future.

Keywords: Malaria, *Plasmodium, parasite,* malaria diagnosis, Microscopy, Rapid Diagnostic tests, PCR, LAMP

1. Introduction

Malaria represents a major global health problem, with over three billion people living in areas where the infection is transmitted, and with a burden currently estimated at over 228 million cases and 405,000 deaths, annually [1]. The majority of cases and deaths are concentrated in young children and circumscribed to Sub-Saharan Africa (SSA), where 9 out of every 10 cases and deaths occur [1]. Malaria can be uncomplicated or even asymptomatic, but it can also progress to severe illness and eventually become fatal. Despite effective antimalarial treatment, the case fatality rate remains high among severe malaria (SM) cases, ranging from 8.5 to 30% [2,3]. The vast majority of those cases are caused by *Plasmodium (P.) falciparum* but there are four additional species which can cause disease in humans: *P. vivax, P. ovale, P. malariae,* and *P. knowlesi.* Charles Louis Alphonse Laveran was the first person to describe the blood stages of the *Plasmodium* parasites in malaria infected patients using a microscope, now 140 years ago. Since Laveran's discoveries, microscopy has remained the gold standard method for malaria diagnosis, but much progress has been achieved in terms of new diagnostic strategies and tools.

Over the last couple of decades, there has been a significant reduction in the burden of malaria around the world as a consequence of different prevention and control strategies. To reinforce these achievements and progress towards global malaria eradication, the World Health Organization (WHO) launched in 2015 the Global Technical Strategy (GTS) for Malaria 2016–2030 [4]. One of the main pillars of this comprehensive framework is to ensure universal access to malaria prevention, diagnosis and treatment. Such a strategy defined the ambitious goal of reducing malaria mortality rates and global incidence by at least 90% by 2030 [4]. Even with recent progress towards achieving these objectives, it is necessary to harness innovation and expand research including, of course, diagnostic tools.

Malaria symptoms are very unspecific. In endemic areas, malaria is usually the most common cause of fever, something that explains why in the past, in areas where transmission was intense, the mere presence of an elevated body temperature was often synonym of the need to establish an empirical treatment with antimalarial drugs. Most patients with uncomplicated malaria only have few abnormal physical findings. SM is a complex multisystem disease that, in children, usually presents with three overlapping syndromes: coma, respiratory distress and severe anemia [5]. Both uncomplicated and SM are difficult to diagnose based solely on clinical assessment. Prompted by the generalized decreasing malaria trends, and by the confirmation that point-of-care

(POC) diagnostic means to confirm malaria had reached sufficient quality standards, the WHO recommended in 2010 that malaria treatment should be restricted to cases where parasitological diagnosis (by any available means) had been confirmed [6,7]. Consequently, efforts were made to scale up diagnostic testing, treatment and surveillance for malaria, like the test, treat and track (T3) initiative [8]. Although the compliance with this new recommendation has steadily improved during the past decade, it still remains suboptimal, since as many as 30% of patients receiving antimalarials lack an appropriate diagnostic test [8]. Today, most commonly used methodologies to confirm the presence of parasites include light microscopy and rapid diagnostic tests (RDTs). Serology based on enzyme-linked immunosorbent assays (ELISAs) to measure antibody response to malaria infection is currently used in the context of vaccine research or epidemiological surveillance [9,10]. However, such techniques have a limited role in the diagnosis and management of clinical malaria so they will not be covered in this review.

Conventional microscopy is an inexpensive and reliable method, allowing for the identification and quantification of malaria parasites. However, it is time-consuming, requires expertise and is hindered by certain logistical limitations. RDTs detect in a few minutes parasite antigens and are easy to perform and interpret with minimal infrastructure requirements. However, they do not allow parasite quantification, have different performance levels regarding species identification, and can present false positive and false negative results. Molecular diagnostic techniques are the most sensitive and specific methods for malaria diagnosis but require specialized and expensive equipment, which precludes their use in resource-constrained settings. This review aims to examine the current available methods for the diagnosis of clinical malaria in endemic countries, discuss their advantages and disadvantages (table 1) and summarize the needs and research gaps regarding the future improvement of malaria diagnosis. Articles were identified through electronic searches of Pubmed, with no particular language or date restrictions, and limited to humans. Pubmed was searched (accessed 29 June 2020) through the use of a broad sensitive filter using following combinations: “malaria AND clinical diagnosis” (7591 results), “malaria AND microscopy” (3166 results), “malaria AND rapid diagnostic test” (1418 results), “malaria AND PCR” (3271 results), “malaria AND molecular diagnosis” (1077 results), “malaria AND LAMP” (127 results). The references of the retrieved papers were used to search for additional studies.

2. Clinical Diagnosis

Malaria signs and symptoms are non-specific and may commonly overlap with other frequent diseases such as viral and bacterial infections, making it difficult to distinguish them based uniquely on a clinical approach. Importantly, malaria may present with similar manifestations to those of other potentially life-threatening diseases, including pneumonia, meningitis or sepsis [11-13]. Fever is the landmark of malaria disease, but other common symptoms include general malaise, fatigue, arthralgias, myalgias, headache, abdominal discomfort, nausea or vomiting [14]. Importantly, the clinical spectrum of malaria is wide, ranging from asymptomatic infections to life-threatening disease. It depends on many factors, including host, parasite and social ones. An important determinant of the clinical phenotype of malaria is the level of immunity acquired to it. Indeed, in high transmission areas, repeated exposition to infective bites leads to the progressive acquisition of a partial immunity against the most severe forms of disease (but not against infection). Young children, theoretically naïve to malaria -and similar to travelers not previously exposed-, are the ones showing more overt clinical symptoms. Conversely, older children and adults, if repeatedly exposed and therefore naturally immunized, will tolerate better infections and show less or no clinical symptomatology [15-17]. This is an important consideration as in certain situations, malaria parasites may be detected in blood, but the attribution of clinical symptoms to such infection will need to be pondered in relation to the immunological background [18,19].

In resource-constrained settings, and particular in most rural areas, health systems are notoriously fragile, and historically there has been a scarcity of supporting diagnostic tools. Even simple laboratory facilities, such as microbiology, hematology or biochemistry tend to be limited, and often unavailable. Additionally, human resources shortages are common, and there is a limited number of well-trained health care providers, clinicians and laboratory technicians. Such a reality was at the basis for the development in the 1990's of the WHO-promoted Integrated Management of Childhood Illness (IMCI) guidelines [20], a set of highly sensitive clinical algorithms whose ultimate objective was to improve the quality of disease management and reduce child mortality [21]. Such clinical algorithms, which have been further developed to include approaches for case management at the community level, have saved hundreds of thousands of lives [22,23], and they have been shown to be highly cost effective [24,25]. Some studies have shown that this algorithm-based approach is highly sensitive [26,27] but, on the other hand, significantly lacks specificity, leading to an important malaria over-diagnosis and over-treatment [28-33]. Overtreating malaria

may also cause inadequate management or treatment delays for other potentially life-threatening diseases with similar symptomatology [34-36].

3. Microscopy

Microscopic visualization and identification of *Plasmodium* parasites in blood of patients remains the gold standard for malaria diagnosis [14,37]. Light microscopy of Giemsa stained slides has been the most widely used tool for examination of thick and thin peripheral blood smears. Ideally, thick films should be used for parasite detection; whereas thin films are more appropriated to identify the Plasmodium species, and both of them can be used to quantify parasitemia [38]. However thin films are often not routinely performed in endemic settings, and in most areas of SSA, where *P. falciparum* clearly predominates, species identification is not proactively conducted An initial negative microscopy evaluation is not necessarily indicative of the absence of malaria parasites, as the identification of malaria parasites is highly dependent on the expertise of the examiner. Moreover, peripheral parasitemias may be different than the overall burden of parasitemia due to cytoadherence and sequestration of parasitized red blood cells in the microvasculature, particularly in *P. falciparum* infections [39]. As a result, and in the event of a high suspicion of malaria but a negative microscopy result, repetition of the test a few hours later is warranted.

The sensitivity and specificity of microscopy varies widely across settings and depends on many factors such as level of peripheral parasitemia, microscopists' expertise, or type of *Plasmodium* species infection. This may debilitate microscopic diagnosis in a context of reduction of malaria prevalence (due to lack of training programs and challenges to replace expert microscopists) and when mixed-species malaria infections are present [31,40]. In a recent study with 551 febrile patients in three different sites in Cameroon [33], light microscopy showed a sensitivity and specificity of 57% and 99%, respectively, when compared against polymerase chain reaction (PCR). In Equatorial Guinea [41] the sensitivity and specificity of microscopy was 55% and 81%, being 74% and 87% in Ethiopia [42], and 50% and 71% in Nigeria [43], all of them using different methods of PCR as reference standard. These studies also showed the common presence of mixed co-infections and differences in accuracy depending on the specific *Plasmodium* species detected. Apart from errors in diagnosis, such inaccuracies may be clinically relevant as misdiagnoses may lead to inadequate or insufficient antimalarial drug treatment,

delayed management and severe complications [44]. Although some countries with coexisting circulating species may adopt common therapeutic approaches for malaria, irrespective of species, it is important to note that *P. vivax* and *P. ovale* infections do uniquely require an 8-aminoquinoline for radical cure and the prevention of relapses, as opposed to *P. falciparum*. It is thought that in routine laboratories microscopy can detect between 50-100 parasites/μl and highly skilled microscopists may detect as little as 10 parasites/μl [45]. However, the accuracy depends on staff proficiency confirming the importance of adequate and continuous training, supervision and quality assessment of staff working with microscopy [41-43,46-48].

In general, microscopy is considered an inexpensive and cost-effective method that, with a small volume of blood and modest laboratory infrastructure [49]. Furthermore, it is currently believed to be the best method for patient follow-up after treatment for malaria.[50]. Disadvantages include the fact that it is time consuming, labor and time intensive, and it requires proper preparation of slides, maintenance of microscopes, skilled staff [51], and quality controls [52,53]. Such limitations become particularly important in regions with the highest burden of malaria like SSA, where only a few of the available laboratories meet international quality standards [54].

Although labor-intensive and time consuming, Giemsa is the most widely used staining method due to its low cost and its good sensitivity and specificity compared with other techniques like Leishman or Field´s stain [55]. Fluorochrome dyes such as acridine orange (AO) have been developed in an effort to improve the performance of microscopic parasite detection [56]. Microscopy using AO has demonstrated good sensitivity and specificity in comparison to Giemsa and, importantly, results may be available quicker (~ 3-10 minutes) [57,58]. The main drawbacks are that sensitivity also decreases with low parasitemia, species identification is more difficult and more specialized equipment is needed (i.e. fluorescent microscopes). The Quantitative Buffy Coat (QBC) technique includes an additional centrifugation step prior to staining with AO to stratify blood components and concentrate infected erythrocytes [59]. QBC has demonstrated high sensitivity compared to light microscopy even with low parasitemia and is simple and fast to perform [60]. However, it may also present difficulties in parasite differentiation and quantification and requires specific equipment and trained staff which may affect its performance [61]. Currently, fluorochrome dyes and QBC technique are not routinely and widely used in endemic areas.

Conventional fluorescent microscopes are expensive and need continuous electricity and darkrooms. Light-emitting diode (LED) technology with low-cost, portable and attached devices has been recently approved for other diseases such as tuberculosis and may surpass the disadvantages associated with fluorescence microscopy. This technology needs less power, can work with batteries and is possible to use it under day-light conditions. Different malaria studies have shown good performance in field conditions and it could represent an affordable, rapid and accurate option for malaria diagnosis [58,62-64]. Partec rapid malaria test (PT) is another fluorescence-based microscope technique that uses a non-specific DNA-binding fluorescent dye [4′-6-diamidino-2-phenylindole (DAPI)]. This method has comparable sensitivity and specificity and seems to be more cost effective in endemic settings than other techniques such as light microscopy, RDTs or QBC and, consequently, may represent a reliable and affordable diagnostic option [65-70]. Like other fluorescent methods, it does not allow species identification and may lead to false positive results due to artefacts. However, an extra advantage is that this microscope has a camera that allows digital visualization and image capturing and storage [71].

Malaria microscopic diagnosis is hampered by the limited number of trained microscopists and the challenges they face in endemic settings. Recently, in order to improve this situation, image analysis and machine learning-based approaches have been developed. These methods require digitized blood smears that are automatically analyzed by computer systems, which have been designed to detect the presence and the quantity of *Plasmodium* parasites [72-78]. These automated techniques may increase the speed and accuracy of the diagnosis, alleviate workloads and reduce costs when compared to other microscopic techniques [55]. However, implementation and performance under field conditions must be further studied to demonstrate their utility [79]. Other innovative trends like deep learning, collaborative intelligence, gamification or mobile smartphones for malaria diagnosis are rapidly evolving and are already offering alternative approaches and perspectives to be considered in the near future [55,80-82].

4. Rapid Diagnostic Tests

The use and expansion of RDTs for malaria diagnosis has sharply increased in the last years. From 2010 to 2017, in SSA alone, the number of laboratory-based malaria tests performed annually increased from 55 million to more than 223 million, and >75% were done using RDTs [1]. This dramatic expansion in RDT use has increased the number of suspected malaria cases

tested in SSA. In 2018 some countries reported more than 80% of cases tested and around 350 million RDTs were sold in the region [1].

RDTs are immunochromatographic lateral flow devices which represent an accurate, rapid and affordable alternative, offering a qualitative diagnosis through the detection of one or more *Plasmodium* proteins. The vast majority of malaria RDTs currently available and used globally detect the *P. falciparum* histidine-rich protein-2 (HRP-2). Lactate dehydrogenase (LDH) and aldolase are glycolytic enzymes present in all *Plasmodium* species which can also be detected by RDTs [83-85]. Of the different antigens detected by commercial malaria RDTs, two are specific for *P. falciparum* [*P. falciparum*-specific HRP-2 (PfHRP-2) and *P. falciparum*-specific lactate dehydrogenase (Pf-pLDH)], two for *P. vivax* [*P. vivax*-specific LDH (Pv-pLDH) and *P. vivax*-specific aldolase (Pv-ALDO)] and two are generic and common to all human *Plasmodium* species (pan-pLDH and pan-aldolase). There are no specific tests available for *P. malariae*, *P. ovale* and *P. knowlesi*. PfHRP2 is a parasite-specific protein produced only by *P. falciparum* throughout its asexual life cycle, and released during schizogony into the peripheral circulation, where it can persist for weeks after the elimination of parasites [86]. [83,87]. On the other hand, LDH and aldolase are cleared faster from blood after initiation of treatment and they indicate active infection [88].

RDTs use dye-labeled monoclonal antibodies to bind the malaria antigen. This antigen-antibody complex migrates across a strip of nitrocellulose membrane often encased in a plastic cassette, where it interacts with a secondary antibody that is bound to the membrane. The interaction of the antigen-antibody complex with the immobilized secondary antibody results in a colorimetric reaction that produces a visible line on the membrane. There is a myriad of commercial tests which can detect one single antigen or different combinations of them, including among many others the following: BinaxNow, CareStart, SD Bioline, ParaHit and OptiMAL-IT [89]

These RDTs may differ not only in the epitopes used for antigen detection, but also in their analytical chemistries, reliability, performance and formats (dipsticks, cards or cassettes) [85]. This heterogeneity led to the development of the WHO RDT evaluation program, WHO-FIND, which seeks to control the quality of the RDTs used, and produces updated summaries on the performance of each of these tests against a series of quality-controlled samples. Since its implementation, this program has evaluated an increasing number of products (now estimated at

332) and established minimal quality standards and requirements to identify the most appropriate tests for each epidemiological and clinical context [85]. A recent study analyzed thirteen RDTs detecting HRP-2 and pLDH among the best-in-class performing products according to WHO-FIND testing program. The study showed that the performance of RDTs detecting *P. vivax* and pan-pLDH varied considerably between products, and that their sensitivity was lower compared to RDTs for PfHRP2 and PfLDH detection of *P. falciparum* [90]. A meta-analysis of fourteen studies also compared the performance of PfHRP2 and pLDH-based detection for *P. falciparum* and showed that although both performed well, overall, PfHRP2 tests are slightly more accurate than pLDH tests. This study also highlighted that, when compared to microscopy and PCR-corrected microscopy, the PfLDH RDT had better specificity (95.9% vs 86.1%) whereas the HRP2-based assays had higher sensitivity (96.3% vs. 82.6%) [91].

In endemic settings where *P. falciparum* and *P. vivax* may coexist, it is necessary to differentiate between species as these parasitic infections require different treatments and discriminating between the two is clinically important. A Cochrane Review estimated that the pooled sensitivity of *vivax*-specific RDTs detecting Pv-pLDH was 95% (95% CI, 86-99), with a specificity of 99% (95% CI, 99-100) when compared to microscopy (the accuracy was lower when compared to PCR) [92]. Another study comparing the performance of LDH and aldolase tests for *P. vivax* malaria with microscopic examination demonstrated that both were highly accurate with sensitivities ranging from 93.5 to 97.4% and specificities from 98 to 100% [93]. The combination of both antigens (rather than the determination of a single one) could enhance the detection capacity and performance of the tests. However, the accuracy of pan-specific RDTs, which distinguish between *P. falciparum* infections (alone or mixed) and solely non-falciparum infections, appear to be much lower. No specific test has been approved for the diagnosis of *P. malariae* or *P. ovale* [89,94-96] and those detecting *P. knowlesi* still do not seem to perform well[97,98].

The density limit of detection (LOD) for a conventional RDT is around 100 parasites/uL [99] while the threshold estimated for light microscopy may range from 10to 500 parasites/μL (and for molecular methods based on PCR is 1-5 parasites/μL [44,45] (figure 1). Sensitivity and specificity for microscopy and RDTs are quite similar [92,100] although the interpretation of RDTs results becomes problematic when comparing discrepant results between both techniques [101]. In any case, the comparison of the performance between both techniques is heavily

dependent on the availability of experienced microscopists, who are rare in endemic settings like Africa [46].

The potential for false positives with HRP2 RDTs needs to be considered, owing to the persistence of HRP2 antigenemia that can remain in peripheral circulation for up to 4-6 weeks post-treatment [102], even after successfully treated infections. Children in particular may remain positive longer than adults [50]. Other causes of false positive results are the presence of autoantibodies, young *P. falciparum* gametocytes, or cross-reactivity between *Plasmodium* species or other infections such as trypanosomiasis, schistosomiasis, leishmaniasis, toxoplasmosis, dengue, hepatitis C, and tuberculosis, although this may be infrequent [89]. False negatives are possible due to low parasite density infections, very high parasite infections (i.e. the prozone effect) [103] or *pfhrp2/3* gene deletions (see below) [104]. Nevertheless, it seems that false-negative results are more likely to be due to poor-quality RDTs brands and batches, poor transport and storage conditions or operator errors [105]. When considering the screening properties of a particular RDT it is important to take into account that they can be affected by the epidemiological context and the infection dynamics of the malaria-endemic population [106]

Recently, there has been interest in developing new RDTs with increased sensitivity to detect low level parasitemias. Ultrasensitive RDTs (uRDTs) have been reported to outperform conventional RDTs in both high and low transmission settings in different use case scenarios, as they have a 10-fold lower LOD [99,107]. uRDTs had a similar workflow as conventional RDTs and were able to detect infections 1.5 times sooner than the latter [99,107]. However, uRDTs fail to identify some infections detected by PCR. Considering these studies, it seems that these new tests could play an important role in the identification of low-density infections in rural and remote areas. However, it is unclear whether uRDTs will have an additional value for the clinical diagnosis and fever management of malaria patients as the currently available RDTs are able to detect the vast proportion of symptomatic malaria infections [108,109].

Lack of *pfhrp2* (and/or *pfhrp3*) in laboratory isolates was soon described after discovery of these proteins, although it was only in 2008 when this deletion was first described among field isolates [86,110]. This deletion renders parasites potentially “invisible” to PfHRP2-based tests, and may therefore result in an inadequate management of malaria cases falsely thought to be negative. There have been recent studies trying to characterize at a global level this problem, showing prevalence of *pfhrp2/3*-negative parasites as high as 20%-100% in Peru or Eritrea, 41%

in Guyana, 36% in Ghana and 23% in Rwanda [104]. On the African continent and in India, there are reports of pooled prevalences of *pfhrp2/3* deletions in false negative RDTs which could affect up to 7% and 69% of the isolates, respectively [111]. Although it is not recommended to use combined HRP2/pLDH RDTs in SSA, some studies have shown that it could mitigate the impact of *pfhrp2/3* deletion in clinical malaria [106]. Currently, WHO recommends switching from HRP2 based RDTs to other diagnostic methods when the prevalence of *pfhrp2/3*-deficient parasites meets the lower 90% confidence interval for a 5% prevalence, which may imply high costs and deployment of resources. To avoid unnecessary losses, there is a need to develop new and effective tools for detection and monitoring of *pfhrp2/3* deletions [112-114].

The benefits of RDTs in comparison to other tests include the fact that they are cheap, easy to perform and interpret, do not require highly trained staff and laboratory support, are stable in extreme conditions and have comparable accuracy compared to other techniques, while producing results much faster. If they are incorporated into clinical diagnosis and the recommendations are adhered to, they can reduce malarial over-diagnosis and over-treatment [115-117]. It has been shown that RDTs, in comparison to microscopy or clinical diagnosis, might be more cost-effective [118] and more feasible to implement [119]. On the other hand, if recommendations are not followed by practitioners, using RDTs can lead to an increase in antibiotic prescriptions and overuse of antimalarials [119,120].

. In endemic settings like SSA it has been demonstrated that RDTs are, in general, used well and their use can increase the availability and feasibility of accurate malaria diagnosis [121]. Nonetheless, RDT performance and adherence to test results may present a high variability between different cadres of clinical staff, and there is a need to build and consolidate robust quality assurance systems [122].

5. Molecular approaches

5.1 PCR

PCR is the most widely used nucleic-acid amplification test (NAAT) used for the diagnosis of malaria. Although there are different types of PCR assays, they are all based on the same principle: repeated amplification cycles to generate numerous copies of specific fragments of genetic material, and the detection and analysis of the products of this amplification process. PCR-based assays are characterized by an excellent analytical sensitivity and they outperform

microscopy and RDTs in terms of detecting a higher percentage of malaria infections [33,123,124]. In a systematic review of endemic population surveys, the prevalence of *P. falciparum* infection measured by microscopy was on average 51% (95% CI: 45-57) of that measured by PCR [125]. Similarly, two systematic reviews reported that an important percentage of *P. vivax* infections detectable by PCR was unnoticed using microscopy [126,127]. PCR assays have typical LOD of ~1-5 parasites/μL (figure 1). However, more sensitive versions have been developed using multi-copy target genes or increasing the blood volumes processed [128,129], and reverse transcription PCR (RT-PCR) assays usually have lower LOD because transcribed RNA sequences are present in higher copy numbers relative to genomic DNA (~0.002-0.02 parasites/μL) [130-133]. Low-density malaria infections constitute a remarkable proportion of all malaria infections [134], but those infections are often subclinical. Their underdiagnosis may delay antimalarial treatment and perpetuate malaria transmission in the community [124]. However, similarly to uRDT, the benefits of using PCR for managing symptomatic fevers remains uncertain [135,136].

PCR has other important strengths apart from high accuracy. PCR-based assays are especially useful in areas where different malaria species are co-endemic as they can differentiate all *Plasmodium* species, even simultaneously in mixed co-infections. . Although microscopy is the gold standard for quantifying parasitemia, its performance with low parasitemias is generally poor, and quantitative PCR (qPCR) is a useful alternative in these situations. A quantitative output can be obtained by using extrapolation against standard curves [137-139]. PCR can also be used to differentiate between asexual and sexual parasite stages [140], distinguish recrudescence from reinfection [47]as well as to detect drug resistant infections [141].

Although PCR assays have advantages over microscopy and RDT for the diagnosis of malaria, their widespread implementation in endemic areas is challenging, and such methods are often considered impractical for use in the field. Malaria PCR assays imply a high cost, results are not immediately available and there are issues regarding standardization for PCR methodology which makes both comparing and interpreting results difficult. Moreover, they require laboratory facilities with stringent cleanliness, a stable source of power, cold chain, technical expertise, as well as regular quality controls and equipment maintenance [142]. Among all PCR assays, real-time PCRs are perhaps best designed in terms of simplicity and speed, but still have many drawbacks, including high costs.

Efforts to improve the field applicability of PCR methodologies include the development of a multiplex malaria sample-ready (MMSR) assay, characterized by reactions tubes with all necessary components for amplification which can be stored at a room temperature. This removes the need for cold-chain although increases the cost [143,144]. Post-amplification detection has also been greatly simplified with the development of PCR-nucleic acid lateral flow immunoassays (PCR-NALFIA), a technology analogous to the one used in RDTs. NALFIA is a sensitive, simple, fast, electricity-free and cheap detection system that can provide results in 10 minutes [145,146]. Progress has also been made in developing methods that circumvent the nucleic acid extraction step, saving time, reagents and simplifying the process [147-149]. Although these and other simplified assays have many strengths and good analytical sensitivity, they are still relatively expensive compared to RDTs and microscopy and have limited applicability in the field due to their dependence on a thermal cycler for amplification.

Considering that WHO recommends microscopy and quality assured RDTs as the primary diagnostic tools for suspected clinical malaria cases in resource-constrained settings, regardless of the malaria epidemiological situation [138], PCR assays are mainly used for research purposes and as a reference standard against which other methods are evaluated.

5.2 LAMP

Isothermal methods are NAATs that achieve amplification of nucleic acids at a constant temperature without the need for thermal cyclers and, thus, a heating block or water bath can be used instead. This, in addition to having all the inherent strengths and versatility of NAATs, makes these methods attractive alternatives to PCR for resource-limited settings. Loop-mediated isothermal amplification (LAMP) and, to a lesser extent, nucleic acid sequence-based amplification (NASBA) are the isothermal amplification assays at the forefront of *Plasmodium* detection [150].

LAMP assays amplify genetic material at a single-temperature in less than an hour, using a polymerase with strand displacement properties. The highly efficient nucleic acid amplification process creates a precipitate that increases the turbidity of the solution, which makes simple visual identification of positive samples possible [151]. LAMP malaria assays have proven to have equivalent accuracy as PCR methods. Very high sensitivities and specificities have been reported for LAMP assays detecting *Plasmodium* parasites, either at the genus or species level [152-154].

A LAMP assay designed to detect *P. falciparum* and used in symptomatic outpatients in a remote Ugandan clinic showed sensitivities of 90-93% when compared to reference nested PCRs [155]. LAMP technology usually shows a LOD of ~1 parasite/μL but may achieve 0.025-0.1 parasites/μL (figure 1) [156]. Apart from its high sensitivity, it has some additional advantages for resource-limited settings: it requires less sophisticated equipment, less technical expertise, shorter turnaround times and has reduced associated costs.

Since the first LAMP assay description for malaria detection in 2006 [157], several modifications have been made in an effort to make LAMP assays more field-deployable. These include simplification of sample preparation, emergence of ready-to-use lyophilized reagents that do not require cold chain, and moving towards instrument- and electricity-free devices [158]. Two examples are the direct blood dry LAMP system, named CZC-LAMP [159], and the non-instrumented nucleic acid (NINA)-LAMP assay that self-generates energy using an exothermic chemical reaction [160,161] In parallel, less subjective and more sensitive alternatives to visual readouts have appeared, but usually these are at the expense of increased cost or risk for contamination: turbidimeters, gel electrophoresis, colorimetric-based [162,163] and fluorescence-based methods [164,165], as well as lateral-flow dipsticks [166].

Currently, to the best of our knowledge, two field-stable LAMP kits are CE-marked and commercially available: the LoopAmp malaria (Pan/Pf/Pv) detection kit (Eiken Chemical Company) and the Alethia™ malaria LAMP assay (Meridian Bioscience). Despite these being highly-sensitive and field-friendly, their protocols could be further simplified [167] and they could be improved to specifically identify all *Plasmodium* species [168]. LAMP technology applied for parasite quantification, gametocyte detection [169] and antimalarial resistance detection [170,171] are in development but not yet available for commercial reagents.

5.3 NASBA

NASBA employs a combination of three enzymes to achieve RNA amplification without the need for thermal cycling [172]. However, unlike LAMP, results cannot be monitored visually by turbidity. NASBA was first applied to the detection of malaria parasites back in 1997 based on amplification of 18S rRNA [173]. Afterwards, different quantitative versions (QT-NASBA) have been developed [174,175]. QT-NASBA assays have been used to detect different *Plasmodium* species and submicroscopic infections in febrile patients [176-178]. Given that gametocytes can

be differentiated according to sexual stage-specific mRNA transcripts, QT-NASBA assays have been designed and extensively used for detection, classification and quantification of gametocytes [179,180].

QT-NASBA assays that detect malaria asexual forms have a LOD of 0.01-0.1 parasites/μL [174-176]. They have been reported to outperform microscopy and RDTs [181,182] and to give equivalent results than PCR assays [174]. In a study conducted in 338 symptomatic children in Kenya and Tanzania, real-time 18S rRNA QT-NASBA could detect a higher percentage of malaria infections than microscopy and RDTs, and it had a sensitivity of 95% (95% CI: 87-98) and a specific of 99% (95% CI: 96-100) with PCR as the reference standard [152,182]. Compared to PCR assays, QT-NASBA technology does not need thermal-cycling equipment, is quicker (~1 hour), uses a simpler sample preparation procedure, can be used with smaller blood volumes and is not prone to interference by DNA [174]. These properties make NASBA an attractive alternative to PCR and for use in malaria POC diagnosis, but further research is needed to reduce costs and make it a field-friendly technology [150].

6. Other innovative approaches

A number of innovative approaches for malaria diagnosis in endemic areas are currently in development, mainly to circumvent the challenges associated with field performance of current available techniques. However, most of them are far from implementation in the field and they still need to surpass logistical and economical concerns. Some of them have been previously cited in the corresponding section. Here, we merely list some additional interesting approaches.

Automated analyzers that detect unusual light scatter patterns generated during routine full blood count may offer accurate, rapid and cost-effective malaria screening, but need microscopy for confirmation of malaria parasites [183]. One of these systems has been used for clinical diagnosis with promising results [184]. Hemozoin, the parasites' waste product, is considered a potential biomarker for malaria diagnosis and represents an alternative indicator of malaria infection. For its detection, apart from flow cytometry, other technologies can be used as well (e.g. spectroscopy, ELISA) [185]. Remarkably, magneto-optical detection of hemozoin is an inexpensive and sensitive method which has been reported to provide results in less than one minute [186]. Additionally, advances in magnetic resonance relaxometry [187] microfluidics [188], biosensing technologies [189] and molecular biology have led to progress in lab-on-chip

diagnostic platforms [190]. These devices adapt common laboratory tests to a self-contained, portable, micro-scale format surpassing the logistical and financial constraints that avoid the introduction of high specialized technology in low-resourced settings [191]**.** Although at a very preliminary stage, they hold promise as novel POC platforms for developing countries.

In parallel, there have also been efforts to develop non-invasive malaria diagnostic tools. This represents an exciting innovation for situations when blood samples are difficult to obtain. Until now, there are some studies with encouraging, but still incipient results, which explore the utility of urine, saliva, breath and transdermal detection tests for malaria diagnosis [192-196].

7. Expert opinion

In the last decade malaria diagnosis has moved away from presumptive treatment -which during years had been the norm- to parasitological confirmation. This approach, encouraged by WHO, tries to surpass the significant limitations of relying on clinical assessment. When the presence of parasites cannot be established, empirical treatment is warranted only in cases with high clinical suspicion, and where withdrawing malarial treatment could imply an unacceptable risk to the life of the patient. Although the compliance with this recommendation has steadily improved during the past decade, improvement is still possible.

Malaria microscopic diagnosis is still considered the gold standard. It is an affordable technique that allows, besides malaria diagnosis, parasite quantification and species identification and gives prognostic and follow-up information of patients. However, it is hampered by the limited number of trained microscopists and the challenges they face in endemic settings (e.g. implementations problems, high burden of work, lack of continuous training and no regular supervision). Some of these limitations are solved by RDTs, which in a way, have "democratized" diagnosis bringing it to those places where it was most needed, and notoriously simplifying the requirements in terms of laboratory infrastructures and trained human resources previously needed. Nowadays, even community health workers have access to affordable, sensitive and specific diagnostic methods, which can guarantee a parasitological confirmation and thus effectively guide malaria management. Such POC methods are sufficiently robust for the detection of symptomatic patients and have essentially replaced microscopy as the diagnostic tool of choice, even though microscopy still offers many advantages and should remain in use as a complementary diagnostic methodology. RDTs do have disadvantages, and contrary to microscopy, show

limitations in terms of identifying species, cannot quantify parasitemia and cannot confirm parasitological response to treatment. In addition, they can provide false positives and false negative results due to multiple reasons, which can lead to an incorrect or suboptimal management of malaria and other febrile illnesses.

Both advantages and disadvantages have to be taken into account when considering the use of light microscopy and RDTs. The choice of test needs to be based in the malarial epidemiological context and the logistical and staff capabilities of each particular setting. Having said that, it is important to consider both techniques as complementary rather than mutually exclusive. For example, wherever possible, microscopy should help to confirm RDTs result in order to improve the individual management of malaria patients. The undisputable benefits of RDTs and their massive deployment in endemic areas must not put in question the need to continue strengthening local laboratory capacities, staff training and supervision.

Molecular assays are usually too expensive and have limited applicability in the field. At the moment, they are mainly used in research activities and as reference methods of evaluation. Despite the wide availability and variety of assays, they currently show little additional value to cheaper alternatives for the clinical assessment and guiding of management decisions for patients. Their high intrinsic sensitivity, an initially positive trait, can make interpretation of positive results difficult in some situations, as these could arise from parasite DNA persistence after treatment. Similarly, the use of molecular assays that do not distinguish between gametocytes and asexual forms, can lead to challenges in the interpretation of results, as these can arise from the detection of gametocytes (which are not responsible for clinical symptoms). Nonetheless, they also have interesting features and there are current efforts to make NAATs more affordable and field-deployable. LAMP and NASBA technologies are specially promising fields and ongoing work can make POC implementation of molecular diagnostics a reality for malaria in the near future.

In the coming years, the use of malaria diagnostic techniques will be influenced by the changing epidemiology of malaria in a global scenario with some countries running towards malaria elimination, and others struggling with persistent high burden. In areas with continuous transmission, RDTs will continue to grow in popularity and, in some settings, will replace definitively traditional techniques as light microscopy. Microscopy availability will also be hampered by the reduction in malaria prevalence and the lack of expert microscopists. Consequently, main priorities will be focused on addressing the intrinsic limitations of RDTs and

facing new emerging biological and epidemiological challenges. The parasite's capacity to evade RDTs by not expressing the main protein they detect, and the transition from moderate-to-high to low transmission areas are some of those difficulties. Future efforts will be directed to improve accuracy and performance of existing tests for *P. falciparum* and *P. vivax*, to develop specific RDTs for the different *Plasmodium s*pecies and for mixed co-infections, to find alternative targets for cases of *pfhrp2/3* gene deletion and to develop tests capable of reliably detecting infections of low parasite biomass. These tests will also be the model for PCR-based assays and isothermal technologies to find POC alternatives to be implemented in field conditions. **Conclusions**

Significant gaps still remain in access to malaria diagnosis, even if good tools are available, and efforts need to be directed at improving the availability of such diagnostic methods where they remain most needed. In parallel, further work needs to be conducted so as to guarantee that technological innovation is at the core of the design and conceptualization of new diagnostic tools, always keeping in mind that the natural end-users are the populations living in malaria-endemic settings.

Key issues

- Light microscopy of Giemsa stained blood slides is still considered the gold standard method for malaria diagnosis.
- Rapid diagnostic tests, particularly those who detect histidine-rich protein-2, are an accurate, rapid and affordable technology for malaria diagnosis in endemic areas. In fact, they are currently the most widely used method for parasitological confirmation.
- In resource poor areas the use of PCR-based assays is restricted to research and needs further optimization to become a helpful approach in the management of clinical malaria.
- Isothermal methods, specially LAMP and NASBA technology, are attractive alternatives to PCR for resource-limited settings.

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Table 1. Advantages and disadvantages of current malaria diagnostic methods
Abbreviations: RDT=rapid diagnostic test, PCR=polymerase chain reaction

METHOD	ADVANTAGES	DISADVANTAGES
Clinical diagnosis	• Fast • Inexpensive • No specialized equipment required	• Non-specific due to symptoms overlapping with other infectious diseases • Malaria misdiagnosis and over-treatment
Microscopy	• Low cost • Allows parasite quantification • Allows species identification • Provides prognostic information • Allows patient follow-up	• Technical expertise needed • Labor and time-consuming • Continuous training and equipment maintenance needed • Logistical limitations • No antimalarial resistance detection
RDTs	• Affordable • User-friendly • Fast (5-20min) • Easy to interpret • Field deployable • No need of highly trained staff or laboratory support • Stable in extreme conditions	• Possible false positives and false negatives • Limited species identification • No parasite quantification • No antimalarial resistance detection
PCRs assays	• High sensitivity and specificity • Some allow a quantitative approach • Some allow species identification • Some allow antimalarial resistance detection	• High cost • High turnaround times (>1h) • Technical expertise needed • Continuous training and equipment maintenance needed • Little methodology standardization • No currently available field-deployable options
Isothermal assays	• High sensitivity and specificity • Potential to be field-deployable • Relatively short turnaround time (~1h) • Some allow a quantitative approach • Potential for species identification • Potential for antimalarial resistance detection	• Only two commercial assays available, still under development and improvement • Technical expertise needed • Continuous training and equipment maintenance needed • Higher costs than other diagnostic tools, but cheaper than PCRs

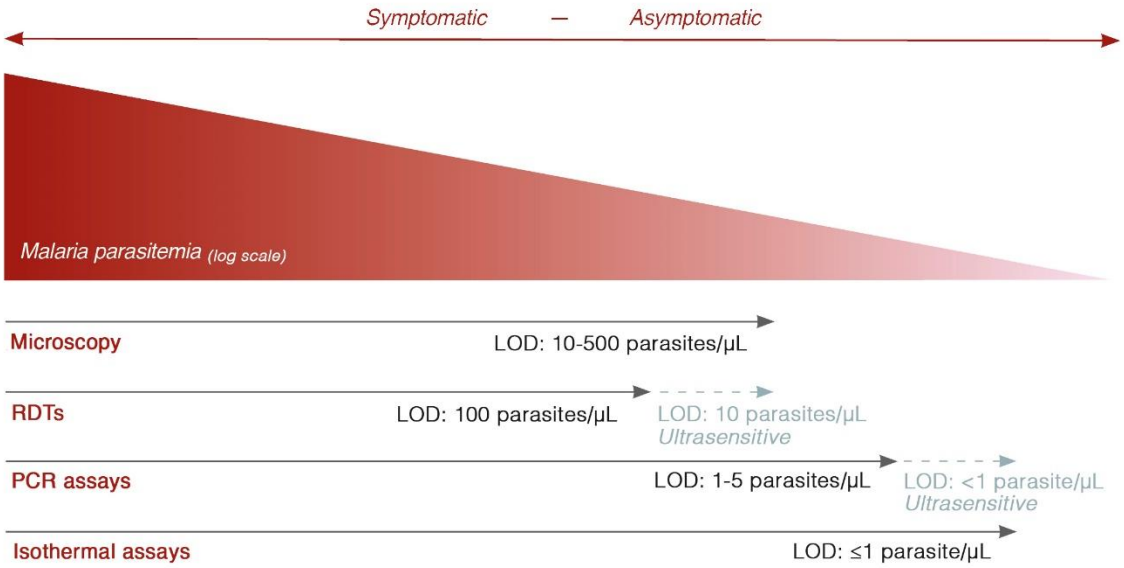

Figure 1. Limit of detection (LOD) of different malaria diagnostic methods. Microscopy techniques have a LOD of ~10-500 parasites/µL, with variation depending on many factors like microscopists' expertise or type of Plasmodium species infection. Conventional RDTs have a LOD of ~100 parasites/µL, but a newly developed ultrasensitive RDT has been reported to detect ≤10 parasites/µL. PCR assays have a typical LOD of ~1-5 parasites/µL, although there are more sensitive versions able to detect <1 parasite/µL. Isothermal assays are also sensitive enough to detect infections of ≤1 parasite/µL. The transition between a symptomatic and an asymptomatic infection depends on different factors (such as parasite virulence, host malaria immunity, host genetic factors or co-morbidities),but asymptomatic carriers usually have lower parasitemias.

Abbreviations: LOD= Limit of detection, RDT=rapid diagnostic test, PCR=polymerase chain reaction

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Case Report

OXFORD

CASE REPORT

Leukoerythroblastosis in a Young Child with Severe Malaria and Superimposed Gram Negative Infection

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ABSTRACT

Background: Leukoerythroblastosis, a non-specific and often short-lasting response of the bone marrow to different diseases such as malignancies or infections, is characterized by the presence in the peripheral blood of immature red and white cells.
Methods: We present a case of leukoerythoblastosis occurring in a 24 months old Mozambican girl, in the context of a severe malaria episode and an associated urinary tract infection. Peripheral blood smear was used for diagnosis of malaria and leukoerythroblastosis. *Enterobacter cloacae* isolation and antibiotic susceptibility testing were performed by conventional microbiology.
Results: Peripheral blood smear was positive for *Plasmodium falciparum* and showed a leukoerythroblastosis with red cell anisopoikilocytosis and left shifted neutrophils. Urine culture confirmed the presence of a multi-resistant *E. cloacae*. Treatment of underlying conditions resolved the leukoerythroblastic reaction.
Conclusions: Leukoerythroblastosis may be related to different infectious diseases and may also appear in the context of severe malaria. Bacterial superinfection needs to be investigated.

KEYWORDS: leukoerythroblastosis, malaria, gram negative infection, urinary tract infection

INTRODUCTION

Malaria patients can present with different haematological alterations affecting all cell lines. White blood cell count (WBCC) is often affected, and changes in every white blood cell line have been described. If WBCC left shift is accompanied by red blood cells (RBCs), leukoerythroblastosis should be contemplated. Leukoerythroblastosis is defined as the presence of immature cells of the myeloid series and nucleated red blood cells (nRBCs) in the circulating blood, with or

without anaemia [1]. Leukoerythroblastosis can be associated with different pathologies, including infectious diseases [2]. Leukoerythroblastic reactions have been mainly found to affect adults [3] and only a few cases have been reported in children [4–7]. Some of those cases were attributed to infections. We present a leukoerythroblastic reaction in a Mozambican child, occurring in the context of a severe malaria episode with an associated bacterial sepsis from urinary origin.

CASE REPORT

The patient, a 24 months old Mozambican girl, was admitted with a 3 day history of fever and general malaise after starting treatment for uncomplicated malaria on a local facility 2 days before admission. On the day of admission, she developed four episodes of generalized tonic-clonic seizures, and was transferred to Manhiça District Hospital with suspicion of severe malaria. The patient had no relevant past clinical history. On examination, the child was fully conscious but in a general poor condition, presenting fever, tachypnoea, tachycardia, prostration and pallor. There were no other relevant findings on examination. Peripheral blood smear was positive for *Plasmodium falciparum* with a parasitaemia of 47 065 parasites/μl. Human immunodeficiency virus test on admission was negative. Initial laboratory tests showed a haemoglobin of 5.5 g/dl, RBCs of 2.03 × 10^3/μl, haematocrit of 16.6% and a WBCC of 33.73 × 10^3 leukocytes/μl with a differential count of neutrophils 37.4%, lymphocytes 54%, monocytes 8.3%, eosinophils 0.1% and basophils 0.1%. No others important laboratory results were reported. Treatment including parenteral artesunate, empirical ceftriaxone and blood transfusion was initiated. The patient showed a good clinical evolution, and fever disappeared after 32 h. Parasite clearance was demonstrated at 42 h. After three doses of artesunate, a complementary full course of oral artemether-lumefantrine was administered, according to Mozambican National guidelines for treatment of malaria. Forty-eight hours after admission, the patient's clinical status worsened, developing fever again and showing an escalating WBCC peaking at 138.5 × 10^3 leukocytes/μl at Day 5 after admission. A peripheral blood smear obtained on Day 6 confirmed an elevated WBCC (manually calculated at around 28 × 10^3 leukocytes/μl), although of a much lesser magnitude than the 78.13 × 10^3 leukocytes/μl reported at the same time by the automated coulter haemogram counter. The haematopathologist report described a leukoerythroblastosis with red cell anisopoikilocytosis and left shifted neutrophils (Figure 1). Although bone narrow examination is essential for making a definitive diagnosis, no bone marrow sample could be obtained because of lack of site resources. Malignancies and erythrocyte abnormalities were ruled out considering the information provided by the blood smear and the clinical evolution. New samples of blood and urine were taken for cultures, and antibiotic was switched empirically from ceftriaxone to ciprofloxacin with temperature normalization 24 h later. Subsequently, the urine culture obtained on Day 6 grew a multi-resistant *Enterobacter cloacae* resistant to ceftriaxone and sensible to ciprofloxacin. No microorganism was isolated in the first and second blood cultures. The patient also presented a drop of haemoglobin at 98 and 174 h to 4.8 and 4.3 g/dl, respectively, requiring two additional blood transfusions. After those interventions, the patient improved swiftly, with temperature and WBCC normalization, and was discharged on Day 10 with a haemoglobin of 7.9 g/dl and WBCC of 17.27 × 10^3/μl. On Day 14 after admission, an outpatient clinical and laboratory control was made. The patient remained asymptomatic and her haemogram showed a haemoglobin of 7.7 g/dl, RBCs of 2.03 × 10^3/μl, haematocrit of

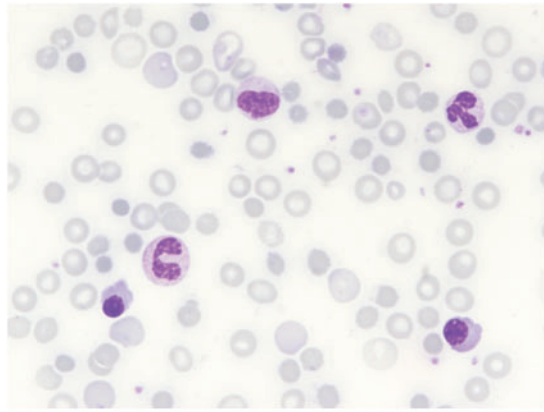

Fig. 1. Peripheral blood smear (May-Grunwald Giemsa staining) showing a leukoerythroblastic reaction, with some neutrophils, one metamyelocyte and two erythroblasts, together with marked anisopoikilocytosis of the erythrocytes.

26% and WBCC of 12.17 × 10^3 leukocytes/μl with a normal WBCC.

DISCUSSION

Different leukocyte alteration patterns have been reported in relation to malaria infections, including normal WBCC, leukopenia and leukocytosis [8–13]. Differential diagnosis of WBCC alterations is challenging, particularly in poor settings where diagnostic resources and specialized staff are scarce. Those alterations are normally detected by automated haematology analysers, and in certain circumstances, it is recommended to obtain a peripheral blood smear, and whenever possible, conduct a bone marrow examination [14]. The microscopic observation of the peripheral blood smear may not only quantify the real magnitude of the WBBC elevation but can also detect qualitative abnormalities in all haematological series, including leukocytes, erythrocytes and platelets [14]. If WBCC left shift is accompanied by RBCs, leukoerythroblastosis should be considered. Leukoerythroblastosis is characterized by the appearance of immature cells of the myeloid series and nRBCs in the circulating blood, with or without anaemia [1]. Leukoerythroblastosis has been better defined in adults [3] and can appear as a result of a wide range of diseases including leukaemia or malignancies, infections, haemorrhages or drug reactions [14, 15]. In children, only a handful of cases have been reported, associated with malignancy [4], parvovirus B19 infections [5, 6] or inguinal abscess [7]. When the underlying condition is treated, WBCC normalizes rapidly, leading to the resolution of the leukoerythroblastic reaction, as occurred in this case. Although the relative weight of malaria infection and its relationship with leukoerythroblastosis cannot be ruled out, the timeline of events suggests that in this case it was mainly caused by a bacterial sepsis of urinary origin. The coexistence of malaria and superimposed bacterial infections is relatively frequent and entails a poorer prognosis [16]. This case report is illustrative of such an association, with the initial severe *P. falciparum* infection being complicated by a urinary tract infection because of a multi-resistant *E. cloacae*, with the previously unreported particularity of being expressed as a leukoerythoblastosis.

CONCLUSIONS

Leukoerythroblastosis in childhood may be related to different diseases including infections of a transient and acute nature. Differential diagnosis is challenging in poor settings with lack of laboratory facilities and specialized staff. In the context of a severe malaria episode, it may reflect a coexisting infection that needs to be further investigated.

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Malaria, immunity and mental disorders: A plausible relationship?

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Malaria is the most common and dangerous parasitic disease, being responsible every year for nearly half a million deaths, and an estimated 219 million clinical cases, globally [1]. The devastating short-term effects that an acute malarial infection can have on any given individual have been historically well characterized, and there are also abundant data on the subacute and chronic sequelae derived from severe malarial episodes, which are understandable in the context of the sudden and profound insult that such an aggressive infection may have in the central nervous system and other key organs [2]. However, much scarcer information exists regarding other more subtle or prolonged deleterious effects of malarial infection and disease on well-being, beyond its acute phase, particularly in regard to common mental disorders (CMD) and neuro-psychiatric health.

In this recent article of *EBioMedicine* [3] Rachel Jenkins and colleagues explore the three-way relationship between malaria, mental disorders and immunity in a representative sample of adults randomly selected from the Kombewa Health and Demographic Surveillance system running in Kisumu, a highly malaria-endemic county located in Western Kenya [4]. To do so, authors applied a myriad of validated structured questionnaires designed to assess mental health and collected blood samples of the studied individuals to confirm malaria parasitaemia and test for common markers of immune function (CD4/CD3 ratio, CD4 counts, IL-1β, IL-6, IL-8, and IL-10, TNF-β, TNF-α). Although no associations were found between malaria and psychotic symptoms, their results confirm a statistically significant association between malaria and CMD (OR 1.7, $p = 0.014$), a heterogeneous group of distress states which typically manifest with anxiety, depressive and unexplained somatic symptoms. Their results also hint at the potential mediating role that the cytokine TNF-α may play in this association.

The association between mental health and non-communicable diseases has been widely studied [5], with evidence of inflammation significantly contributing to the pathogenesis of major psychiatric disorders (including depression [6] or schizophrenia [7], among others), and the inflammasome -a multiprotein oligomer responsible for the activation of inflammatory responses- proposed as a central mediator [8]. Conversely, the association between mental health and communicable diseases has been historically neglected and thus largely unexplored, perhaps with the exception of some chronic infections such as HIV [9]. Findings reported in this study are of public health relevance, as they propose for the first time a solid association between malaria infection and CMD, while venturing a hypothetical cytokine pathway for such an association. The association between TNF-α and depression has been previously described and may be explained by its influence in the hypothalamo-pituitary-adrenocortical axis, neuronal serotonin transporters and in the indoleamine 2,3-dioxygenase pathway [10]. Additionally, TNF-α may play a dual role in both the pathogenesis of and protection against malaria, although the actual implication of this cytokine in the course of disease has not been fully understood [11]. In fact, anti-TNF therapies have been tested as adjuvant therapies in severe malaria but have shown no effect in its prognosis [12].

In spite of its large sample size ($n = 1158$) and high consent rates (97.2% for the interviews, 91.4% for the blood samples), factors for which authors are to be praised, this study has important limitations that need to be highlighted. First, it is possible that some of the conclusions derive from chance findings, due to the multiple comparisons conducted. Second, concomitant conditions such as anemia or HIV, both highly prevalent in the area, could have acted as confounders of the association, and would need to be determined in future studies. Third, a more comprehensive approach to understanding the immune response is needed, including both the cellular and humoral components, and with a longitudinal design. Finally, malaria infections may lead to a wide spectrum of clinical symptomatology, ranging from pure asymptomatic carriage of parasites, to life-threatening disease [13]. Thus, one should avoid talking of malaria as a single clinical entity. It is probable that adults in this highly-endemic malarious setting have acquired a significant amount of immunity and tolerance to the infection, allowing them to carry malaria parasites without clinical expression, something that undermines some of the author's hypotheses regarding the causal relationship. Understanding the role of such asymptomatic infections, as opposed to those with overt clinical symptoms (and its different clinical phenotypes), and how they may differentially expose the individual to the risk of developing mental illness is necessary and would require more precisely designed studies.

This study raises many questions and opens highly relevant gateways that need to be further substantiated with additional research. The first critical issue is that for these findings to unequivocally support the author's conclusions, they need to be replicated in other malaria-

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endemic areas, possibly with distinct transmission and epidemiological conditions (*i.e.* involving other malaria species and other intensity of transmission scenarios). A wider use of the mental health screening tools used here should therefore be encouraged, not only for the purposes of replication, but also to more widely ascertain mental health problems at the population level of low-income settings, an area unfortunately so far neglected [14]. Second, due to its cross-sectional nature, and although authors venture some hypotheses on how malaria could lead to depression or *vice versa*, inference on directional causality is impossible. To further understand causal relations, prospective epidemiological studies following cohorts of population at risk of acquiring malaria should be established, and repeatedly evaluated to record both their malaria infection status and the incidence of newly acquired mental health problems. Additionally, by selectively inducing some of the conditions under consideration, or activating some of the inflammatory pathways proposed, research with animal models could experimentally confirm some of these findings. Other areas warranting further research include the impact of malaria elimination campaigns on the mental health of both adults and children in endemic areas; and possible target areas for preventive or therapeutic interventions to avoid incident mental health problems in malaria endemic areas.

Authors' contribution

RV wrote the first draft of the manuscript, together with QB. RV and QB critically revised the manuscript. Both authors approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interest.

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RESEARCH ARTICLE Open Access

A randomised, double-blind clinical phase II trial of the efficacy, safety, tolerability and pharmacokinetics of a single dose combination treatment with artefenomel and piperaquine in adults and children with uncomplicated *Plasmodium falciparum* malaria

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Abstract

Background: The clinical development of a single encounter treatment for uncomplicated malaria has the potential to significantly improve the effectiveness of antimalarials. Exploratory data suggested that the combination of artefenomel and piperaquine phosphate (PQP) has the potential to achieve satisfactory cure rates as a single dose therapy. The primary objective of the study was to determine whether a single dose of artefenomel (800 mg) plus PQP in ascending doses is an efficacious treatment for uncomplicated *Plasmodium falciparum* malaria in the 'target' population of children ≤ 5 years of age in Africa as well as Asian patients of all ages.

Methods: Patients in six African countries and in Vietnam were randomised to treatment with follow-up for 42–63 days. Efficacy, tolerability, safety and pharmacokinetics were assessed. Additional key objectives were to characterise the exposure–response relationship for polymerase chain reaction (PCR)-adjusted adequate clinical and parasitological response at day 28 post-dose (ACPR28) and to further investigate Kelch13 mutations. Patients in Africa ($n = 355$) and Vietnam ($n = 82$) were included, with 85% of the total population being children < 5 years of age.

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Results: ACPR28 in the per protocol population (95% confidence interval) was 70.8% (61.13–79.19), 68.4% (59.13–76.66) and 78.6% (70.09–85.67) for doses of 800 mg artefenomel with 640 mg, 960 mg and 1440 mg of PQP respectively. ACPR28 was lower in Vietnamese than in African patients (66.2%; 54.55–76.62 and 74.5%; 68.81–79.68) respectively. Within the African population, efficacy was lowest in the youngest age group of ≥ 0.5 to ≤ 2 years, 52.7% (38.80–66.35). Initial parasite clearance was twice as long in Vietnam than in Africa. Within Vietnam, the frequency of the Kelch13 mutation was 70.1% and was clearly associated with parasite clearance half-life (PCt1/2). The most significant tolerability finding was vomiting (28.8%).

Conclusions: In this first clinical trial evaluating a single encounter antimalarial therapy, none of the treatment arms reached the target efficacy of > 95% PCR-adjusted ACPR at day 28. Achieving very high efficacy following single dose treatment is challenging, since > 95% of the population must have sufficient concentrations to achieve cure across a range of parasite sensitivities and baseline parasitaemia levels. While challenging, the development of tools suitable for deployment as single encounter curative treatments for adults and children in Africa and to support elimination strategies remains a key development goal.

Trial registration: ClinicalTrials.gov, NCT02083380. Registered on 7 March 2014.

Keywords: Artefenomel, OZ439, Piperaquine, Single dose combination treatment, Pharmacokinetics, Dose–response, modelling and simulation, Phase II B, Uncomplicated *Plasmodium falciparum* malaria, Children,

Background

Since 2000 the incidence of malaria has fallen by 41% and mortality rates have declined by 62% globally, due to increased deployment of new interventions including artemisinin-based combination treatments and insecticide treated bed nets (WHO 2016) [1]. However, despite these gains, in 2015 there were 212 million new cases and an estimated 429,000 malaria-related deaths, with Africa continuing to bear the heaviest burden, accounting for approximately 9 in 10 malaria cases and deaths, the vast majority of which were in young children [1]. The high death rate in young children is believed to be linked to low acquired immunity, coupled with greater vulnerability to the infection. Since lower immunity may mean that young children require higher drug exposures than older patients to achieve cure, it is important to tailor dose selection to this population. Patients in Asia may also have lower immunity due to low endemicity.

Numerous studies have suggested that in 'real-life' community settings, poor to moderate adherence to current standard 3-day treatment regimens is common, and this could impact morbidity and mortality as well as drive the development of resistance, although definitive data are difficult to obtain [2–4]. Availability of a highly efficacious 'single encounter treatment' would be expected to improve effectiveness of malaria treatment and delay selection of resistant parasites. An effective cure that can be administered as a single treatment, directly observed if required, would also provide an important tool to support malaria elimination efforts [5, 6]. Medicines for Malaria Venture (MMV) and its partners have undertaken to develop single dose treatments for malaria [7].

Exploratory clinical data suggested that artefenomel (OZ439) plus piperaquine phosphate (PQP) in combination could be efficacious as a single encounter cure. Artefenomel, a novel synthetic trioxolane, contains a similar peroxidic pharmacophore to artemisinins and has demonstrated rapid parasite clearance in patients, with a median parasite reduction ratio at 24 h (log10) post-treatment (PRR24) for *Plasmodium falciparum* ranging from 0.9 to 1.88 [8]. PQP is a long-acting antimalarial currently marketed in a fixed dose combination with dihydroartemisinin (DHA), administered once daily for 3 days.

We report the results of the first clinical efficacy study of the combination of artefenomel and PQP in a design which allowed rapid progression from adult African patient to children ≤ 5 years of age and Asian patients of all ages to ensure that dose finding for phase III is carried out in populations most likely to require the highest exposures to achieve cure. The study also employed interim futility analyses in order to drop doses with a low probability of success early.

Methods

Study objectives

The primary objective of the study was to determine whether a single dose combination of artefenomel plus PQP is an efficacious treatment for uncomplicated *P. falciparum* malaria.

Secondary and exploratory objectives included determination of the incidence of recurrence, recrudescence and new infection, estimation of parasite clearance kinetics and exploration of the relationship between Kelch13 genotype and parasite clearance half-life (PCt1/2) in Asian patients.

An additional key exploratory objective was to characterise the dose/exposure–response relationship for the combination for the primary efficacy endpoint across the patient population and to identify significant covariates influencing efficacy. Safety, tolerability and pharmacokinetics (PK) were also assessed. Details of the study objectives, design and endpoints are summarised in Additional file 1: S1 Study protocol, Section 1 Study synopsis and in more detail in Sections 4, 5.1 and 5.10 respectively.

The study was conducted at nine study sites across six African countries, Benin (Cotonou), Burkina Faso (Nanoro, Banfora and Niangoloko) [9, 10], Democratic Republic of Congo (Kinshasa) [11], Gabon (Libreville, Lambaréné) [12], Mozambique (Manhiça) [13] and Uganda (Tororo) [14], and four sites in Vietnam (Quang tri, Gia Lai, Khanh Hoa, Binh Phuoc) [15]. Malaria prevalence is hyperendemic to holoendemic, and transmission is perennial in all sites with seasonal variation. Drug resistance of *P. falciparum* against chloroquine and sulfadoxine-pyrimethamine is widespread at all African sites, and evidence of artemisinin resistance was confirmed at the Vietnamese sites [16].

Study design, participants and interventions

This was a randomised, double-blind, single dose study to investigate the efficacy, safety, tolerability and PK of artefenomel 800 mg in loose combination with three doses of PQP (640, 960, 1440 mg) in male and female patients aged ≥ 6 months to < 70 years (body weight ≥ 5 kg to ≤ 90 kg) with uncomplicated *P. falciparum* malaria. The artefenomel dose of 800 mg was expected to deliver close to the maximum well-tolerated exposure, and PQP doses were selected to span a range of adequate clinical and parasitological response at day 28 (ACPR28) values, with the highest dose estimated to give a mean maximum placebo corrected change from baseline QTcF of 18 ms [17].

Patients presenting with microscopically confirmed *P. falciparum* mono-infection in the range 1000 to 100,000 asexual parasites/μL of blood, and with fever (axillary temperature ≥ 37.5 °C) or history of fever in the preceding 24 h, were included following their submittal of written informed consent, and all eligible patients were randomised via an Interactive Web Response System (IWRS) in a ratio of 1:1:1 to one of the three treatment arms (see supplementary material). Important exclusion criteria were the presence of severe malaria (according to the WHO definition [18]), haemoglobin below 8 g/dL, exclusions relating to cardiac and hepatic safety and prior antimalarial treatment within specified time frames. Full inclusion/exclusion criteria are given in Additional file 1: S1 Study protocol, Sections 1 Study synopsis and 6 Selection of patients.

The study was initiated in patients aged > 15 years, and following review of safety data by an Independent Safety Monitoring Board (ISMB), sequentially younger patients were recruited in a step-down procedure described in Additional file 1: S1 Study protocol, Section 6.5.2 and illustrated in Figure 1 (S1 Study protocol), Step-down procedure. The aim was to recruit a population predominantly of African children ≤ 5 years of age and also to include Asian patients (the most important target populations). Patient recruitment and follow-up were conducted between July 2014 and August 2015.

Fasted patients ≥ 35 kg received artefenomel 800 mg in loose combination with PQP doses of 640, 960 or 1440 mg at day 0. Patients who weighed < 35 kg received body weight-adjusted doses [19] within weight bands predicted to achieve similar exposure ranges to patients ≥ 35 kg. The dose for a given weight band was adjusted by scaling clearance allometrically, using the relationship $CL = (\text{body weight}/70)^{0.75}$. Artefenomel was administered as a suspension formulation containing α-tocopherol polyethylene glycol (TPGS). PQP was included in the suspension (for patients < 24 kg) or was administered as separate tablets (patients ≥ 24 kg), with blinding maintained by administering matching placebo tablets. For the lowest weight band the dosing volume was 75 mL (plus 2× 15 mL rinses). Patients who vomited within 5 min of start of dosing were re-dosed once. Details of study drug treatments and administration are given in Additional file 1: S1 Study protocol, Section 10 Treatment.

Following drug administration, patients were followed for 42 days or 63 days at some centres (patients were consented separately for days > 42 to 63). Patients remained in the clinical unit for a minimum of 48 (African patients > 5 years old) or 72 h (African patients ≤ 5 years old and all Asian patients) and were discharged provided parasite and fever clearance had been achieved. Patients returned for assessment on days 3, 5, 7, 10, 14, 21, 28, 42 and 63 at selected centres. Blood films (thick and thin) were prepared and axillary temperatures were measured at screening/pre-dose, 6, 12, 18, 24, 30, 36, 48, 72 h and days 5, 7, 10, 14, 21, 28, 42 and 63. Assessments for safety included haematology, clinical chemistry, urinalysis and a triplicate 12-lead electrocardiogram (ECG). Clinical assessments were taken according to the schedule presented in detail in Additional file 1: S1 Study protocol, Section 2 Schedule of assessments.

For screening, thick blood films were stained with 10% Giemsa for 10 min, and thick and thin films for baseline to follow-up were stained with 2% Giemsa for 30 min. Expert microscopists determined parasite densities and examined thick blood films for parasites and thin blood films for non-*falciparum* infections. A second

microscopist, blinded to initial readings, re-read all slides, and a third resolved discrepant readings. A slide was considered negative in the absence of asexual parasites per 1000 counted leukocytes using a 100× magnification oil immersion objective. Parasite density was calculated as follows: (number of counted parasites/counted leukocytes) × most recent absolute leukocyte count per microliter. Details of additional methods are given in the supplementary material.

Analysis populations

The intention to treat (ITT) and safety analysis sets were identical and included all patients who provided informed consent, received the study drug (entire or partial dose) and had a confirmed positive blood film for *P. falciparum* asexual parasitaemia at inclusion. The ITT subset defined for the Kaplan–Meier (KM) estimates of recurrence, recrudescence and new infection rate included only those who consumed the entire dose.

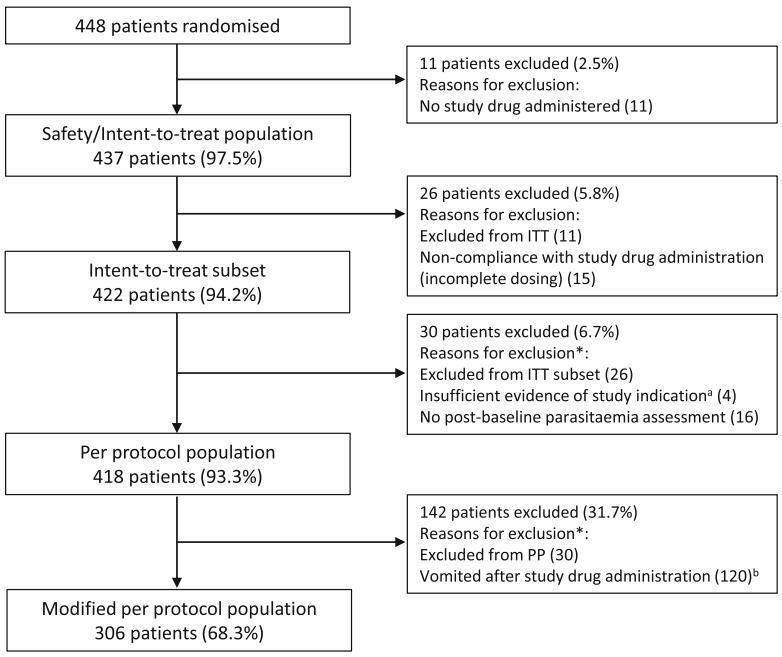
The per protocol (PP) set was the primary analysis set and included all patients comprising the ITT set who consumed the entire dose and were without major protocol deviations. The modified PP analysis set in addition excluded patients who vomited between > 5 min and ≤ 4 h after start of drug administration. Patients could be excluded from a population for more than one reason (see Fig. 1). The PK population included all patients in the ITT set with at least one evaluable PK sample after treatment administration.

Ethical considerations

The study (MMV_OZ439_13_003) conformed to the Declaration of Helsinki and Standard Operating Procedures that meet current regulatory requirements and guidelines established by the International Conference on Harmonization for Good Clinical Practice in Clinical Studies. It was approved by the relevant Independent Ethics Committees (IECs), national Institutional Review Boards and, where relevant, local regulatory authorities at each of the participating sites (for more details see the supplementary material). The study protocol was registered and the study results are reported on ClinicalTrials.gov (NCT02083380).

Endpoints

The primary efficacy endpoint was polymerase chain reaction (PCR)-adjusted ACPR28 in the PP analysis set. We also report the following secondary and exploratory endpoints: PCR-adjusted ACPR at day 42 (ACPR42) and day 63 (ACPR63) and crude ACPR at days 28, 42 and 63 for the ITT and PP analysis sets and Kaplan–Meier incidence rate of recrudescence over 63 days (ITT subset); in the PP analysis set, percentage of patients achieving parasite clearance at 72 h post-



* Reasons for exclusion. Patients may be excluded for more than 1 reason;
[a] An asexual parasite count of < 500 parasites/μL (3 patients), or without any indication of fever at screening/baseline, or with a mixed infection at screening (1 patient). In addition, an asexual parasite count > 200,000 parasites/μL at baseline (1 patient);
[b] Vomited within ≤ 5 minutes and not successfully re-dosed or vomited between > 5 minutes and ≤ 4 hours post-study drug administration.

Fig. 1 Analysis sets and exclusions

dose, PCt1/2, Kelch13 genotype and the relationship between Kelch13 genotype and PCt1/2 are also reported.

ACPR was defined according to the WHO [20] as absence of parasitaemia on day X, irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure (ETF), late clinical failure (LCF) or late parasitological failure (LPF). The definition of ETF was a slight modification of the WHO definition [20] (see Additional file 2: S2 Statistical analysis plan, Section 15 Efficacy outcomes). The derivation of crude (unadjusted) and PCR-adjusted ACPR for both the ITT and PP populations (for details see Additional file 2: S2 Statistical analysis plan, Section 15 Efficacy outcomes) and of re-emergence, recrudescence and new infection was also done according to the principles set down by the WHO and MMV [20, 21]. The PCR methodology was in accordance with the procedures to identify parasite populations recommended by the WHO and MMV [22]. Three polymorphic genetic markers, MSP1, MSP2 and GluRP, were used to distinguish recrudescence from new infections, according to WHO-recommended procedures and as previously described by Snounou et al. [21, 23]. Recrudescence was defined as at least one identical allele for each of the three markers in the pre-treatment and post-treatment samples. New infections were diagnosed when all alleles for at least one of the markers differed between the two samples. Kelch13 genotyping (baseline) was determined by the method of the Pasteur Institute [24], PCt1/2 was calculated using the WWARN calculator [25] and the relationship between Kelch13 genotype and PCt1/2 was explored by site and by mutation graphically, and summary statistics reported. Details of the derivation and definitions of other endpoints are provided in Additional file 2: S2 Statistical analysis plan, Section 15 Efficacy outcomes).

Safety and tolerability endpoints included incidence of adverse events (AEs) and serious AEs (SAEs), vital signs, physical measurements, laboratory safety measurements, liver function tests (LFT) increase, cases fulfilling the Hy's law definition and ECG abnormalities including absolute QTc value categorisation and change from baseline QTc. Further details are given in Additional file 2: S2 Statistical analysis plan, Section 16 Safety outcome.

Treatment emergent adverse events of special interest (TEAESIs), requiring rapid reporting, were also defined in the protocol to ensure careful monitoring:

1) Hepatic: Hy's law definition cases; any alanine transaminase (ALT) or aspartate transaminase (AST) ≥ 5 × the upper limit of the normal range (ULN); any elevation in total bilirubin ≥ 2.5 × ULN (> 35% direct); any AST or ALT ≥ 3 × ULN with the appearance of fatigue, nausea, vomiting, right upper quadrant pain or tenderness, fever, rash and/or eosinophilia (eosinophil percent or count above the ULN); or ALT ≥ 3 × ULN that persisted for > 4 weeks
2) Cardiac: QTcF prolongation from baseline of > 60 ms; QTcF at any time; QTcF > 450 ms; T-wave liability or T-wave morphologic changes during therapy; bundle branch block; and any arrhythmia
3) Haematological: Haemoglobin (Hb) drop > 2 g/dL from baseline; Hb drop < 5 g/dL; absolute neutrophil count (ANC) < 1000/μL
4) Pregnancy.

Statistical considerations

The aim of the study was to determine whether any of the treatment arms reached a target PCR-adjusted ACPR28 of ≥ 95% (PP analysis set). The study was not powered for comparison between dosing arms. ACPR28 was categorised as Cure and Failure according to the WHO method [20]. Descriptive statistics and a 95% Clopper–Pearson two-sided confidence interval (CI) were constructed around the single binomial proportion per treatment arm and for all treatment arms combined. Similar analyses were performed for the ITT and modified per protocol population (mPP) analysis sets across all days, and for crude ACPR. Descriptive summary statistics were also produced for all secondary and exploratory endpoints. Further details can be found in Additional file 2: S2 Statistical analysis plan, Section 15 Efficacy outcomes and Section 6.6 Statistical tests.

Trial simulations suggested that a treatment arm size of 106 should be required if the dose combination is effective [26]. Recruitment was to be capped at a maximum of 150 patients from the target population (patients ≤ 5 years old in Africa or patients of any age in Asia) per treatment arm. The study design was adaptive, allowing interim assessment of response for the purpose of concluding futility after recruitment of 50 evaluable patients per arm (target population). Futility was to be concluded if the probability that ACPR28 was ≤ 90% was greater than or equal to 0.3 [27]. Additionally, African patients > 5 years old were recruited during the age step-down, and these patients were included in the final analysis but not the interim futility analysis. Further details are given in Additional file 1: S1 Study protocol, Section 11 Statistical methods and data management.

Pharmacokinetic analysis

In adult patients (weighing > 35 kg) blood samples for pharmacological analysis were collected at 15 to 16 time points: pre-dose, 2, 4, 6, 12, 24, 48, 72 h and days 5, 7, 10, 14, 21, 28, 42, 63. In paediatric patients the number

was between 3 and 10 samples. Artefenomel and piperaquine PK data were analysed separately using non-linear mixed effect modelling (population PK analysis) in Monolix (version 4.3.3) or NONMEM (version 7.3 or later) respectively. For artefenomel, additional data from two mono-therapy clinical phase II studies in adult Asian patients were included in order to extend the dose range (100–1200 mg); see Additional file 3: S3 Pharmacokinetic analysis details.

Subsequently, exposures of artefenomel and piperaquine for each patient were derived from the individual PK parameters estimated in the population PK analysis. Maximum plasma concentration (Cmax), time to reach maximum concentration (Tmax) and concentration on day 7 (Cday7) were obtained from the simulated profiles, and the area under the curve (AUC) extrapolated to infinity (AUCinf) was calculated directly from the estimated PK parameters. More details are provided in Additional file 3: S3 Pharmacokinetic analysis details.

Exposure–response analysis

The relationship of the binary outcome of ACPR28 response to the estimated artefenomel and piperaquine Cday7 and other covariates was evaluated in a logistic regression model using the statistical software R (version 3.2.2). Within the single dose setting of the study, Cday7 is highly correlated with other exposure variables, such as AUCinf or Cday14. However, Cday7 was preferred to allow future extrapolation to multi-dose regimens as well for its scientific rationale (concentrations of any given drug may be required to exceed the minimum parasiticidal concentration for at least 7 days to achieve full parasite clearance). Additional covariates evaluated were presumed immunity status (low for African patients ≤ 5 years and Asian patients of all ages), region, baseline parasitaemia, age and Kelch13 genotype. All patients in the ITT set with ACPR28 values and exposure for both drugs were included in the analysis. More details are provided in Additional file 4: S4 Exposure–response analysis details.

Dose–response simulations

The objective of the simulations was to evaluate the dose–response relationship for single dose combination treatment with artefenomel and PQP based on the developed population PK and exposure–response models. The simulations were performed for a range of single dose combination doses (for a TPGS formulation) for the African population ≤ 5 years of age. Actual doses assumed the same body weight bands and dose adjustments applied to this study. For further details see Additional file 4: S4 Exposure–response analysis details.

Results

Interim analysis

An interim futility analysis was carried out after recruitment of approximately 50 evaluable patients from the target population per treatment arm as planned. All doses were concluded to have reached the futility criteria (probability of ACPR28 < 90% was 0.9999); hence, the study was stopped. Recruitment to the study continued during the futility analysis process.

Final analysis

Analysis populations

The analysis populations are shown in Fig. 1.

Patient disposition and demographics

A total of 448 patients were randomised equally to the three treatment groups (randomised set, Table 2), and 437, $n = 355$ in Africa and $n = 82$ in Asia (Vietnam) received the study drug (ITT/safety set, Table 1). Demographics and patient characteristics by region are given in Table 1.

Demographic characteristics were similar across treatment arms and analysis sets. Age (and hence weight) differed by region due to the enrolment structure. For the ITT/safety set, in Africa, 81.1% of patients were ≤ 5 years old, whereas in Asia 96.3% were > 15 years old. No patients ≤ 5 years old were recruited in Asia. There was also a difference in sex; 48.3% of patients in Africa and 93.9% in Asia were male.

Median baseline asexual parasitaemia across all treatment arms was 12,913/μL (range 187/μL to 220,240/μL), was similar in Asian and African patients ≤ 5 years, 13,140/μL (range 1065/μL to 123,080/μL) and 14,029/μL (range 187/μL to 229,240/μL) respectively and was slightly lower in African patients > 5 years, 9714/μL (range 835/μL to 160,040 μL).

Of those randomised, 178 (39.7%) completed the study up to day 42 (or 63) (Table 2). Of the 270 patients (60.3%) prematurely discontinued from the study, the majority (47.1%) were discontinued due to meeting the multiple criteria to receive antimalarial rescue treatment, either from failure to clear baseline parasitaemia (1.6%) or from parasite recurrence (i.e. treatment failure; 45.5%). Thus, premature study discontinuation prior to day 28 (and prior to day 42 or 63) is linked with the efficacy endpoint ACPR.

Compliance

Protocol defined compliance; consumption of the total volume of study drug without vomiting (or successful re-dosing in the event of vomiting within 5 min of dosing) was 65% in the African population and 91.5% in the Asian population. Non-compliance was predominantly due to vomiting, with an overall incidence of

Table 1 Demographics and patient characteristics (safety set)

All patients		800:640 (*N* = 143)	800:960 (*N* = 148)	800:1440 (*N* = 146)	Total (*N* = 437)
Africa	Number (*n*)	116	121	118	355
Age (years) (derived)	Median	3.30	3.20	2.90	3.10
	(Min., max.)	(0.5, 54.3)	(0.8, 44.6)	(0.5, 37.7)	(0.5, 54.3)
> 15.0 years	*n* (%)	15 (12.9)	16 (13.2)	14 (11.9)	45 (12.7)
> 5.0 to ≤ 15.0 years	*n* (%)	7 (6.0)	7 (5.8)	8 (6.8)	22 (6.2)
> 2.0 to ≤ 5.0 years	*n* (%)	69 (59.5)	72 (59.5)	70 (59.3)	211 (59.4)
≥ 0.5 to ≤ 2.0 years	*n* (%)	25 (21.6)	26 (21.5)	26 (22.0)	77 (21.7)
Male	*n* (%)	56 (48.3)	63 (52.1)	70 (59.3)	189 (53.2)
Vietnam	*n*	27	27	28	82
Age (years) (derived)	Median	27.30	27.30	28.60	27.45
	(Min., max.)	(12.5, 48.5)	(9.7, 60.0)	(13.3, 57.6)	(9.7, 60.0)
> 15.0 years	*n* (%)	26 (96.3)	26 (96.3)	27 (96.4)	79 (96.3)
> 5.0 to ≤ 15.0 years	*n* (%)	1 (3.7)	1 (3.7)	1 (3.6)	3 (3.7)
> 2.0 to ≤ 5.0 years	*n* (%)	0	0	0	0
≥ 0.5 to ≤ 2.0 years	*n* (%)	0	0	0	0
Male	*n* (%)	27 (100.0)	22 (81.5)	28 (100.0)	77 (93.9)

n number of patients in each category/%)

vomiting of 28.8% (35% in Africa and 7% in Asia). Compliance and vomiting incidence were similar in the African population across age. There were anecdotal reports that, in some centres, young children were unable to ingest the full study dose, despite a reported success rate of administration of study drug (with or without subsequent vomiting) of > 95% in all populations.

Efficacy: ACPR

Crude and PCR-adjusted ACPR results are reported in Table 3 for the ITT analysis set. Re-emergence, crude and PCR-adjusted ACPR results for the PP analysis set are reported in Table 4.

For the primary analysis set (PP) and endpoint (ACPR28), none of the treatment arms reached the target efficacy of ≥ 95% and there was no clear dose–response relationship, although efficacy was highest for PQP 1440 mg (78.6%; 95% CI 70.09–85.67) across the populations (Table 4; Fig. 2b).

Efficacy in the mPP population (excluding patients who vomited) was similar to that in the PP population; e.g. for a PQP dose of 1440 mg, ACPR28 was (80.0%; 95% CI 69.92–87.90) across the populations.

ACPR28 appeared lower in Asian (Vietnamese) than in African patients; thus, for all treatment arms combined, ACPR28 was 66.2% (95% CI 54.6–76.6) and 74.5%

Table 2 Patient disposition (randomised set)

All patients		800:640 (*N* = 148)	800:960 (*N* = 151)	800:1440 (*N* = 149)	Total (*N* = 448)
Treated	*n* (%)	143 (96.6)	148 (98.0)	146 (98.0)	437 (97.5)
Completed	*n* (%)	57 (38.5)	56 (37.1)	65 (43.6)	178 (39.7)
Premature study discontinuation	*n* (%)	91 (61.5)	95 (62.9)	84 (56.4)	270 (60.3)
Primary reason for premature study discontinuation					
Criteria met for established anti-malarial treatment	*n* (%)	68 (45.9)	79 (52.3)	64 (43.0)	211 (47.1)
Study drug discontinued	*n* (%)	0	4 (2.6)	3 (2.0)	7 (1.6)
Withdrawal of consent	*n* (%)	9 (6.1)	3 (2.0)	7 (4.7)	19 (4.2)
Investigator's opinion	*n* (%)	1 (0.7)	1 (0.7)	0	2 (0.4)
Patient non-compliant	*n* (%)	0	1 (0.7)	0	1 (0.2)
Adverse event	*n* (%)	0	0	1 (0.7)	1 (0.2)
Lost to follow-up	*n* (%)	5 (3.4)	3 (2.0)	3 (2.0)	11 (2.5)
Other	*n* (%)	8 (5.4)	4 (2.6)	6 (4.0)	18 (4.0)

n number of patients in each category/%)

Table 3 Crude and PCR-adjusted ACPR by day: ITT analysis set

		800:640 (*N* = 143)	800:960 (*N* = 148)	800:1440 (*N* = 146)	Total (*N* = 437)
Day 28					
Crude ACPR	*n/r* (%)	76/143 (53.1)	79/148 (53.4)	92/146 (63.0)	247/437 (56.5)
	95% CI[a]	[44.63; 61.53]	[45.01; 61.61]	[54.64; 70.85]	[51.73; 61.23]
PCR-adjusted ACPR	*n/r* (%)	77/143 (53.8)	82/148 (55.4)	95/146 (65.1)	254/437 (58.1)
	95% CI[a]	[45.32; 62.21]	[47.02; 63.57]	[56.75; 72.76]	[53.34; 62.79]
Day 42					
Crude ACPR	*n/r* (%)	63/143 (44.1)	66/148 (44.6)	68/146 (46.6)	197/437 (45.1)
	95% CI[a]	[35.77; 52.59]	[36.43; 52.98]	[38.29; 55.01]	[40.35; 49.88]
PCR-adjusted ACPR	*n/r* (%)[a]	67/143 (46.9)	72/148 (48.6)	73/146 (50.0)	212/437 (48.5)
	95% CI[a]	[38.47; 55.37]	[40.36; 56.99]	[41.62; 58.38]	[43.74; 53.31]
Day 63[b]					
Crude ACPR	*n/r* (%)[a]	50/136 (36.8)	49/140 (35.0)	58/135 (43.0)	157/411 (38.2)
	95% CI[a]	[28.67; 45.45]	[27.14; 43.51]	[34.48; 51.76]	[33.48; 43.09]
PCR-adjusted ACPR	*n/r* (%)[a]	50/136 (36.8)	54/140 (38.6)	59/135 (43.7)	163/411 (39.7)
	95% CI[a]	[28.67; 45.45]	[30.47; 47.16]	[35.19; 52.50]	[34.90; 44.57]

n number of patients in each category achieving ACPR, *r* total number of patients in the relevant analysis set with a defined response of Cure or Failure, *N* total number of patients in relevant analysis set

[a]Clopper–Pearson

[b]Patients followed up to day 63 consented separately from the patients followed up to day 42; hence, total patient population is lower for day 63

(95% CI 7.81–79.7) respectively (Fig. 2a). In the African population, efficacy was lowest in the youngest age group (≥ 0.5 to ≤ 2 years), 52.7% (95% CI 38.8–66.4) for all treatment arms combined (Fig. 2b).

In the PP population, recurrence in African and Vietnamese patients was 37.0% and 31.6% respectively at day 28, with all but one determined by PCR to be recrudescence in the Vietnamese population, whereas in the African population, approximately one third of recurring parasites was determined to be a new infection at day 28.

In Asia, in the PP population, the number and percentage of recrudescences and new infections, with 95% CIs, was 11/80 (13.8%; CI 7.07–23.27) and 0/80 at day 14, 18/79 (22.8%; CI 14.10–33.60) and 0/79 at day 21, 23/79 (29.1%; CI 19.43–40.42) and 1/79 (1.3%; CI 0.03–6.85) at day 28, 26/78 (33.3%; CI 23.06–44.92) and 1/78 (1.3%; CI 0.03–6.94) at day 42, and 27/75 (36.0%; CI 25.23–47.91) and 2/75 (2.7%; CI 0.32–9.30) at day 63.

In Africa, in the PP population, the number and percentage of recrudescences and new infections, with 95% CIs, was 34/325 (10.5%; CI 7.35–14.31) and 1/325 (0.3%) at day 14, 57/321 (17.8%; CI 13.74–22.39) and 19/321 (5.9%; CI 3.60–9.09) at day 21, 61/319 (19.1%; CI 14.95–23.87) and 32/319 (10.0%; CI 6.96–13.87) at day 28, 65/313 (20.8%; CI 16.41–25.69) and 50/313 (16.0%; CI 12.09–20.51) at day 42, and 62/277 (22.4%; CI 17.61–27.75) and 49/277 (17.7%; 13.38–22.70) at day 63.

Kaplan–Meier estimates of the fraction of patients with recrudescence and new infection over time by region and age group are presented in Figs. 3 and 4 respectively.

Parasite clearance and association with Kelch13 mutation

A regional difference in the percentage of patients who cleared parasites by 72 h post-dose was evident, with 92.9% (95% CI 89.6–95.4) of African patients achieving parasite clearance by 72 h post-dose compared with 35.0% (95% CI 24.7–46.5) in Vietnamese patients. There was no clear difference in the percentage of patients who cleared parasites across the different piperaquine doses or in the African population > 5 years compared with those ≤ 5 years.

The median time to 50%, 90% and 99% parasite clearance was approximately twice as long in Asia compared with Africa (e.g. time to 90% parasite clearance was 23.6 (interquartile range, IQR 16.20–29.10) h in Asian patients and 14.30 (IQR 10.90–17.60) h in African patients.

Initial clearance of parasites was rapid in the African population. Median parasite clearance half-life (PCt1/2) was calculated using the WWARN Parasite Clearance Estimator (PCE), details of which are published [25]. PCt1/2 is the estimated time for parasitaemia to decrease by half, derived from the clearance rate constant 1/h. Parasite clearance values were reported only for results with $R^2 > 0.75$. PCt1/2 was longer in Vietnam versus Africa (6.1 h [minimum 1.1, maximum 12.7] versus 3.5 h [minimum 1.2, maximum 7.7]). Within Vietnam, PCt1/2 was similar across the four study centres.

A total of 20 known Kelch13 genotypes were tested for (in Africa four new genotypes were identified at low frequency, 0.3–1.7%: A578S, A626V, M562T, Y541F, none associated with artemisinin resistance) [28]. In

Table 4 Re-emergence, crude and PCR-adjusted ACPR by day: PP analysis set

		800:640 (*N* = 139)	800:960 (*N* = 140)	800:1440 (*N* = 139)	Total (*N* = 418)
Day 28					
Re-emergence	*n/r* (%)	48/129 (37.2)	56/136 (41.2)	39/133 (29.3)	143/398 (35.9)
Recrudescence	*n/r* (%)	25/129 (19.4)	37/136 (27.2)	22/133 (16.5)	84/398 (21.1)
	95% CI[a]	[12.95; 27.26]	[19.93; 35.50]	[10.67; 23.97]	[17.20; 25.45]
New infection	*n/r* (%)	11/129 (8.5)	10/136 (7.4)	12/133 (9.0)	33/398 (8.3)
	95% CI[a]	[4.33; 14.75]	[3.58; 13.11]	[4.75; 15.23]	[5.78; 11.45]
Indeterminate	*n/r* (%)	3/129 (2.3)	0	0	3/398 (0.8)
Negative	*n/r* (%)	2/129 (1.6)	1/136 (0.7)	0	3/398 (0.8)
Missing	*n/r* (%)	7/129 (5.4)	8/136 (5.9)	5/133 (3.8)	20/398 (5.0)
Crude ACPR	*n/r* (%)	74/129 (57.4)	77/136 (56.6)	89/133 (66.9)]	240/398 (60.3)
	95% CI[a]	[48.36; 66.03]	[47.85; 65.09]	[58.23; 74.83	[55.31; 65.14]
PCR-adjusted ACPR	*n/r* (%)	75/106 (70.8)	80/117 (68.4)	92/117 (78.6)	247/340 (72.6)
	95% CI[a]	[61.13; 79.19]	[59.13; 76.66]	[70.09; 85.67]	[67.58; 77.32]
Day 42					
Re-emergence	*n/r* (%)	59/127 (46.5)	64/134 (47.8)	56/130 (43.1)	179/391 (45.8)
Recrudescence	*n/r* (%)	29/127 (22.8)	37/134 (27.6)	25/130 (19.2)	91/391 (23.3)
	95% CI[a]	[15.86; 31.12]	[20.24; 36.00]	[12.85; 27.07]	[19.17; 27.78]
New infection	*n/r* (%)	16/127 (12.6)	17/134 (12.7)	18/130 (13.8)	51/391 (13.0)
	95% CI[a]	[7.38; 19.65]	[7.57; 19.53]	[8.42; 21.00]	[9.87; 16.79]
Indeterminate	*n/r* (%)	3/127 (2.4)	1/134 (0.7)	3/130 (2.3)	7/391 (1.8)
Negative	*n/r* (%)	3/127 (2.4)	1/134 (0.7)	0	4/391 (1.0)
Missing	*n/r* (%)	8/127 (6.3)	8/134 (6.0)	10/130 (7.7)	26/391 (6.6)
Crude ACPR	*n/r* (%)	61/127 (48.0)	65/134 (48.5)	67/130 (51.5)	193/391 (49.4)
	95% CI[a]	[39.09; 57.07]	[39.79; 57.29]	[42.62; 60.39]	[44.30; 54.43]
PCR-adjusted ACPR	*n/r* (%)[a]	65/100 (65.0)	71/108 (65.7)	72/100 (72.0)	208/308 (67.5)
	95% CI[a]	[54.82; 74.27]	[55.99; 74.60]	[62.13; 80.52]	[61.99; 72.73]
Day 63[b]					
Re-emergence	*n/r* (%)	59/114 (51.8)	70/122 (57.4)	53/116 (45.7)	182/352 (51.7)
Recrudescence	*n/r* (%)	29/114 (25.4)	37/122 (30.3)	23/116 (19.8)	89/352 (25.3)
	95% CI[a]	[17.75; 34.45]	[22.33; 39.30]	[13.00; 28.25]	[20.83; 30.16]
New infection	*n/r* (%)	15/114 (13.2)	20/122 (16.4)	16/116 (13.8)	51/352 (14.5)
	95% CI[a]	[7.56; 20.77]	[10.31; 24.18]	[8.09; 21.43]	[10.98; 18.61]
Indeterminate	*n/r* (%)	4/114 (3.5)	3/122 (2.5)	4/116 (3.4)	11/352 (3.1)
Negative	*n/r* (%)	3/114 (2.6)	1/122 (0.8)	0	4/352 (1.1)
Missing	*n/r* (%)	8/114 (7.0)	9/122 (7.4)	10/116 (8.6)	27/352 (7.7)
Crude ACPR	*n/r* (%)[a]	48/114 (42.1)	48/122 (39.3)	57/116 (49.1)	153/352 (43.5)
	95% CI[a]	[32.92; 51.71]	[30.62; 48.59]	[39.74; 58.58]	[38.22; 48.82]
PCR-adjusted ACPR	*n/r* (%)[a]	48/83 (57.8)	53/90 (58.9)	58/84 (69.0)	159/257 (61.9)
	95% CI[a]	[46.49; 68.60]	[48.02; 69.16]	[58.02; 78.69]	[55.63; 67.83]

n number of patients in each category achieving ACPR, *r* total number of patients in the relevant analysis set with a defined response of Cure or Failure, *N* total number of patients in relevant analysis set

[a]Clopper–Pearson

[b]Patients followed up to day 63 consented separately from the patients followed up to day 42; hence, total patient population is lower for day 63

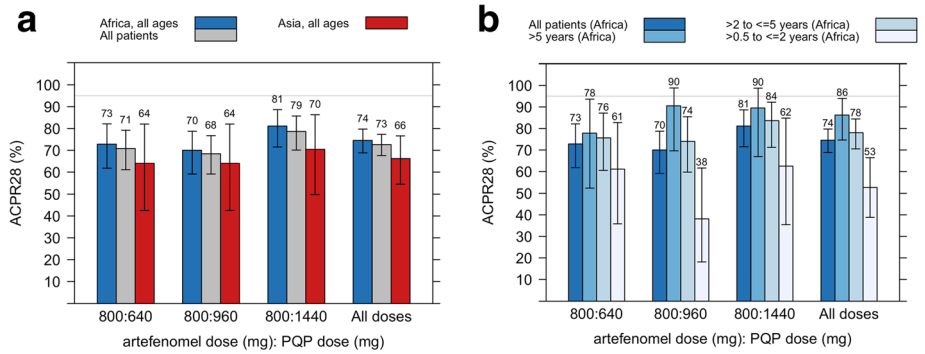


Fig. 2 Efficacy: PCR-adjusted ACPR28 in the PP population, **a** by region (all ages), **b** by age in African patients, percentage in each category with 95% confidence intervals. The numbers presented above the bars are the percent ACPR28. The majority of treatment failures were late parasitological failures (32.9% across the populations and treatment arms), with one early treatment failure in an African patient > 5 years (PQP 640 mg arm)

Vietnam, a high frequency of Kelch13 mutation was observed (70.1%). Five mutations were detected, four of which, C580Y, I543T, P553L and V568G, are defined according to the WHO [28] as validated or candidate markers for partial artemisinin resistance. The exception is C469P, which is not known to be associated with artemisinin resistance [28].

PCt1/2 appeared to be associated with Kelch13 genotype; median PCt1/2 values for C580Y and P553L, the two most frequently occurring mutations, were 7.9 ($N = 24$; minimum 2.4, maximum 12.3 h) and 8.1 h ($N = 19$; minimum 5.5, maximum 12.7) respectively, versus 2.6 h ($N = 19$; minimum 1.4, maximum 5.4) for wild type (WT) (Fig. 5). Mutations that were present at a lower frequency (1/67, 1.5%) also had greater PCt1/2 values; C469P (8.3 h), I543T (5.5 h) and V568G (7.4 h). PCt1/2 in patients with Kelch13 WT in Vietnam was similar to that in the African population (Fig. 5).

Safety and tolerability

No deaths were reported. One TEAE of vomiting occurring 19 min post-dose (treatment-related) was reported to have led to study treatment discontinuation.

Six treatment emergent serious adverse events (TESAEs) were reported in four patients. One patient had severe anaemia (2 days post-dose), one patient had a reversible haemoglobin drop < 5 g/dL (26 days post-dose) and one presented with febrile convulsions (7 h post-dose). One patient had three TESAEs, two of reversible grade 3 transaminase elevations and one of neutropenia (28 days post-dose). None of these TESAEs led to premature study discontinuation. Five of the six TESAEs were considered to be potentially related to the study treatment, the exception being that of febrile convulsion which was considered not related. No AEs due to drug-induced liver toxicity (Hy's law or increase of ALT/AST with clinical symptoms for more than 4 weeks) were reported.

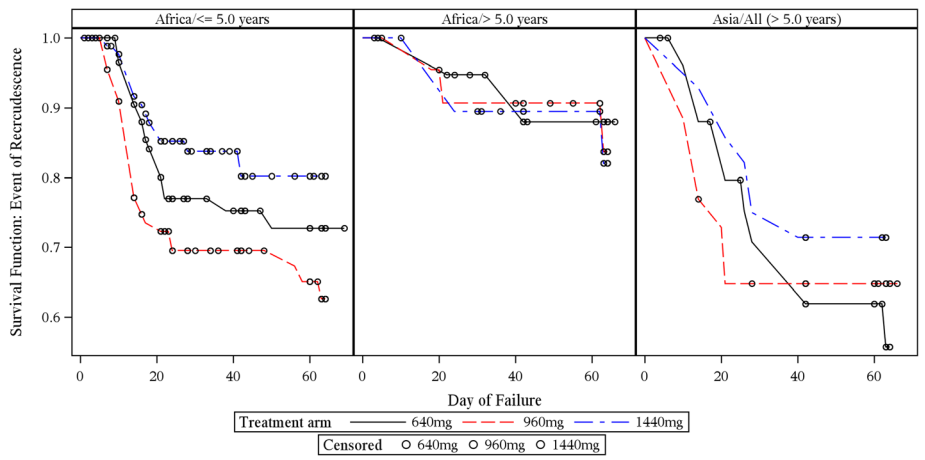


Fig. 3 Kaplan–Meier population at risk of recrudescence by region and age group over time (ITT subset). Note that the *y*-axis is expanded (survival range 0.5–1.0) to clearly visualise the failure rates

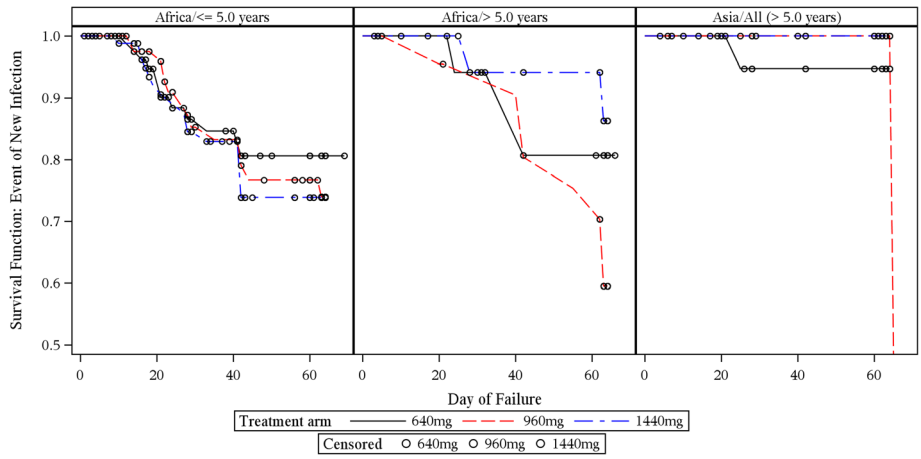


Fig. 4 Kaplan–Meier population at risk of new infection by region and age group over time (ITT subset). Note that the *y*-axis is expanded (survival range 0.5–1.0) to clearly visualise the failure rates

Table 5 gives the incidence of TEAEs occurring in ≥ 5% of the study population up to 28 days post-dose, by System Organ Class. All events of transaminase elevations were reversible, except in one subject who withdrew consent, interrupting the liver function test follow-up.

The most frequently reported TEAESI was QT prolongation in ECG: 24 (16.8%), 37 (25.0%) and 38 (26.0%) of patients in the PQP 640 mg, 960 mg and 1440 mg treatment arms respectively. A first degree atrioventricular (AV) block reported as grade 1 at 48 h post-dose (PR interval 226.33 ms, heart rate 55 beats per min and QTcB and QTcF within the normal range) was reported in one patient (PQP 640 mg). The event resolved at day 7 post-dose (PR interval 185 ms and heart rate 78 beats per min). One patient (PQP 1440 mg) had a mild reversible sinus bradycardia which resolved in 4 days. QTcF increase from baseline of 30–60 ms occurred in 55 (38.7%), 59 (40.4%) and 71 (49.7%) and of > 60 ms in 8 (5.6%), 11 (7.5%) and 27 (18 .9%) of patients respectively in the 640, 960 and 1440 mg PQP dosing arms. All but one QTcF value was < 480 ms (QTc value = 501 ms).

One patient (PQP 1440 mg) experienced a reversible TEAESI of hyperbilirubinaemia (total bilirubin > 2.5 × ULN) in the System Organ Class hepatobiliary disorders. This event was associated with a TESAE of anaemia (Hb drop > 2 g/dL from baseline). Other frequent TEAESIs were neutrophil count decreased < 1000/μL (41 patients; 9.4%) and Hb decreased (drop > 2 g/dL from baseline or Hb < 5 g/dL: 40 patients; 9.2%). The most significant tolerability finding was vomiting (28.8%) according to the compliance data. The high rate of vomiting is thought to be partly related to the 'high volume' TPGS formulation used in the study (although the reason for the regional difference in vomiting rate is unclear).

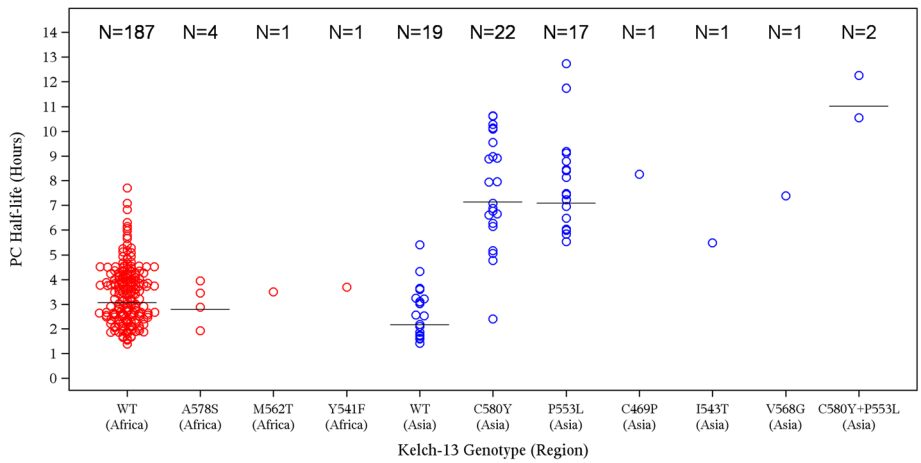


Fig. 5 Association of parasite clearance half-life and Kelch13 Status (PP analysis set). Vietnam (*blue circles*), Africa (*red circles*), median (*black line*), C580Y + P553L polyclonal infection. Note that the plot includes only patients with both genotyping and PCt1/2 results

Table 5 Incidence of treatment-emergent adverse events in ≥ 5% of study population up to day 28 post-dose (safety analysis set)

	Artefenomel mg: PQP mg			
System Organ Class Preferred term	800:640 (*N* = 143)	800:960 (*N* = 148)	800:1440 (*N* = 146)	Total (*N* = 437)
At least 1 TEAE (*n* (%) *E*)	115 (80.4) 266	127 (85.8) 324	122 (83.6) 308	364 (83.3) 898
Infections and infestations (*n* (%) *E*)	74 (51.7) 102	76 (51.4) 106	63 (43.2) 82	213 (48.7) 290
Malaria	43 (30.1) 44	45 (30.4) 48	34 (23.3) 34	122 (27.9) 126
Bronchitis	13 (9.1) 16	13 (8.8) 19	10 (6.8) 11	36 (8.2) 46
Rhinitis	11 (7.7) 11	9 (6.1) 10	10 (6.8) 10	30 (6.9) 31
Plasmodium falciparum infection	9 (6.3) 9	10 (6.8) 10	7 (4.8) 7	26 (5.9) 26
Investigations (*n* (%) *E*)	58 (40.6) 75	68 (45.9) 100	70 (47.9) 95	196 (44.9) 270
Electrocardiogram QT prolonged	27 (18.9) 29	41 (27.7) 48	44 (30.1) 56	112 (25.6) 133
Neutrophil count decreased	18 (12.6) 18	11 (7.4) 11	12 (8.2) 13	41 (9.4) 42
Haemoglobin decreased	9 (6.3) 9	20 (13.5) 22	11 (7.5) 11	40 (9.2) 42
Gastrointestinal disorders (*n* (%) *E*)	31 (21.7) 39	47 (31.8) 60	44 (30.1) 54	122 (27.9) 153
Diarrhoea	11 (7.7) 12	21 (14.2) 21	20 (13.7) 20	52 (11.9) 53
Vomiting	14 (9.8) 14	20 (13.5) 20	16 (11.0) 16	50 (11.4) 50
Abdominal pain	5 (3.5) 5	8 (5.4) 9	12 (8.2) 13	25 (5.7) 27
General disorders and administration site conditions (*n* (%) *E*)	11 (7.7) 11	14 (9.5) 16	17 (11.6) 21	42 (9.6) 48
Pyrexia	5 (3.5) 5	12 (8.1) 13	9 (6.2) 11	26 (5.9) 29

N number of subjects affected (%), *E* number of events

Pharmacokinetic results

The final artefenomel and piperaquine population PK models, including details of the analysis and model diagnostics, are provided in Additional file 3: S3 Pharmacokinetic analysis details.

The PK of both artefenomel and piperaquine in adult and paediatric patients could be described by three compartment disposition models. All (apparent) clearance and volume parameters were related to body weight allometrically. Additional covariates identified for the PK of artefenomel were vomiting, artefenomel dose and age. In particular, relative bioavailability was a function of age; it was 40% lower for a patient of 1 year versus 20 years of age. For the PK of piperaquine, the only additional covariate identified was vomiting. No covariate effects of region, age (for piperaquine), sex, protocol-defined noncompliance or actual or adult equivalent PQP dose were identified.

Individual artefenomel and piperaquine exposures were estimated for 427 and 426 patients respectively (including patients who vomited and were not successfully re-dosed). Summaries of the individual estimated exposures by region and age group are provided in Additional file 3: S3 Pharmacokinetic analysis details). Cday7 is summarised across region and age group in Fig. 6.

Geometric mean artefenomel exposure was lowest in the African population 0.5 to ≤ 2 years of age (which includes body weight bands 5–14.9 kg). Thus, AUCinf and Cday7 were 7.6 μg*h/mL (coefficient of variation, CV 105%) and 2.0 ng/mL (CV 147%) respectively compared with 10.0 μg*h/mL (CV 111%) and 3.3 ng/mL (CV 141%) for African patients > 5 years. Asian patients > 5 years (all but one over 35 kg) had higher mean exposures than the African patients; AUCinf of 16.9 μg*h/mL (CV 66%) and Cday7 of 5.1 ng/mL (CV 95%) (Fig. 6a). Artefenomel exposures and between-patient variability were similar across the three PQP treatment arms.

Piperaquine exposures increased approximately proportionally with dose, although there was considerable overlap between the treatment arms. Exposures tended to be lower in the African population ≤ 2 years of age (including body weight band 5–9.9 kg) and in the Asian population (Fig. 6b). Exposures of artefenomel and piperaquine were about 50% and 70% lower respectively in patients who vomited relative to those who did not. See Additional file 3: S3 Pharmacokinetic analysis details for further details and Additional file 4: S4 Exposure–response analysis details for two patient exposure examples.

Exposure–response relationship

When exploring the observed ACPR28 by artefenomel exposure bins (categorical Cday7 ranges) rather than dose, the relationship between exposure and ACPR28 is clearly visible (Fig. 7). The relationship was different between the two regions but similar for the two African age groups. Thus, in the Asian population, a lower ACPR28 was achieved for the same artefenomel Cday7 compared with the African population. Both the region effect and lack of age effect were confirmed in the subsequent statistical analysis (logistic regression).

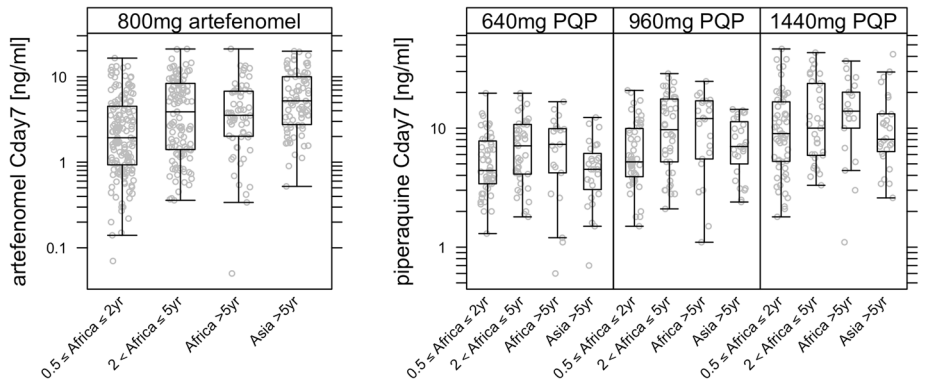


Fig. 6 Estimated Cday7 for artefenomel (**a**) and PQP (**b**) by region/age group. Patients who vomited are included. Median (horizontal line), box (interquartile range) and whiskers represent the most extreme individual point which is not more than 1.5× the length of the box

The relation between artefenomel and piperaquine exposure and ACPR28 was described with a logistic regression model. Details of the model, analysis and model diagnostics are provided in Additional file 4: S4 Exposure–response analysis details. The analysis data set comprised 348 patients.

The probability of achieving ACPR28 (pACPR) was found to be a function of artefenomel and piperaquine exposures, the baseline parasitaemia ($p \le 0.0001$) and region (interaction between region and artefenomel Cday7) ($p = 0.002$):

$$\log\left(\frac{p}{1-p}\right) = 3.23 + 0.22 * Cday7_{PQ} + (0.73 - 0.59[if\ Asia]) * Cday7_{OZ} - 1.27 * log10(BasePar) + 0.46\,[if\ Asia]$$

where p is the probability of ACPR28 and *BasePar* is the baseline parasitaemia (parasites/μL).

There was no statistically significant influence of either Kelch13 genotype or age on pACPR, and (once region was in the model) there was no additional influence of presumed immunity status. In addition, no interaction between the exposures of the two drugs was identified.

Three-dimensional (3D) graphical representations of the exposure–ACPR28 model are shown in Fig. 8 for African and Vietnamese patients, assuming a baseline parasitaemia of 10,000 parasites/μL. These plots show that both artefenomel and piperaquine exposure (Cday7) contribute to efficacy (ACPR28) in a concentration-dependent manner, and that this relationship is different for African versus Vietnamese patients; that is, higher artefenomel exposure (but not higher piperaquine exposure) is required to achieve the same ACPR28 in Vietnamese (Asian) versus African patients (see the equation).

This difference in sensitivity to artefenomel (about five times lower in Asian versus African patients) is illustrated by the difference in the isoboles plotted in Fig. 9 (red versus blue isoboles).

For example, if each drug were administered by itself (Cday7 = 0 for the partner drug), assuming a baseline parasitaemia of 100,000 parasites/μL in African patients, an artefenomel Cday7 of 8 ng/mL would be required for a 0.95 probability of achieving ACPR28, compared to 40 ng/mL in Asian patients. For each presented scenario, any exposure combination of artefenomel and piperaquine to the right of the isobole lines is associated with > 0.95 probability of achieving ACPR28.

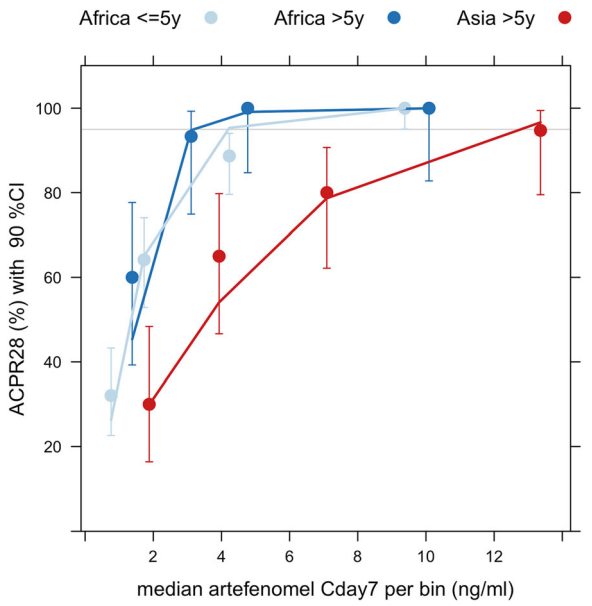


Fig. 7 Summary of the observed ACPR28 by estimated artefenomel concentration at day 7 together with the logistic regression model predictions. The *dots* and *error bars* represent the observed ACPR28 with 90% CI for five artefenomel concentration bins (categories). The *lines* represent the final logistic regression model predictions taking into account the median artefenomel Cday7, as well as the median piperaquine Cday7 and median baseline parasitaemia, for each bin. Number of patients per bin: Africa ≤ 5 years $n = 53$, Africa > 5 years $n = 15$, Asia $n = 20$

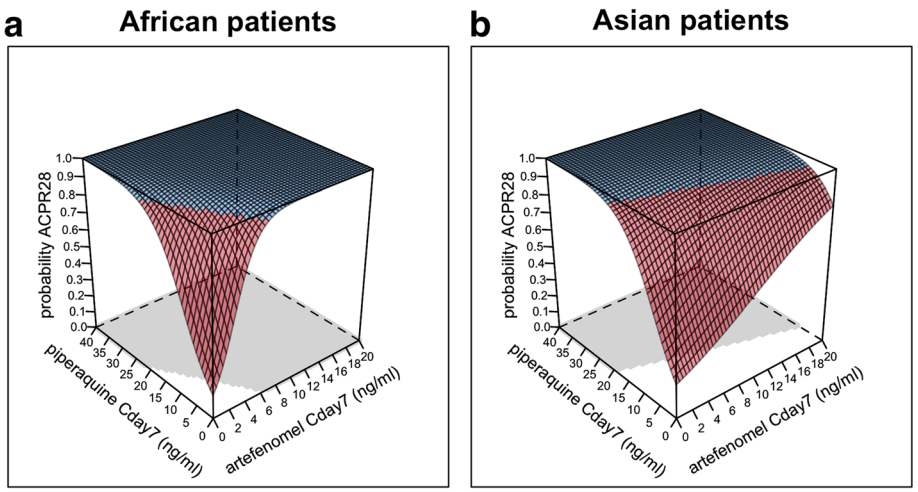


Fig. 8 Estimated exposure–ACPR28 relationship for the combination of artefenomel with piperaquine for a baseline parasitaemia of 10,000 parasites/μL. **a** African patients, **b** Vietnamese patients, 3D representation; *blue* highlights the ACPR28 > 0.95. The *shaded area* shows the concentrations required to achieve a probability of ACPR28 > 0.95

Figure 9 also shows the observed individual exposure combinations flagged as Cure (open symbols) or Failure (closed symbols). These exposures are associated with a wide range of baseline parasitaemia levels. The data points are the actual data used to estimate the model, which is represented by the isoboles, and thus it is clear which part of the isobole is supported directly by the data and which is an extrapolation. For example, the inference that in African patients for a baseline parasitaemia of 100,000 parasites/μL an artefenomel Cday7 of 8 ng/ml or higher (administered alone) is predicted to have pACPR > 0.95 is an extrapolation, since there are no patient data supporting this scenario.

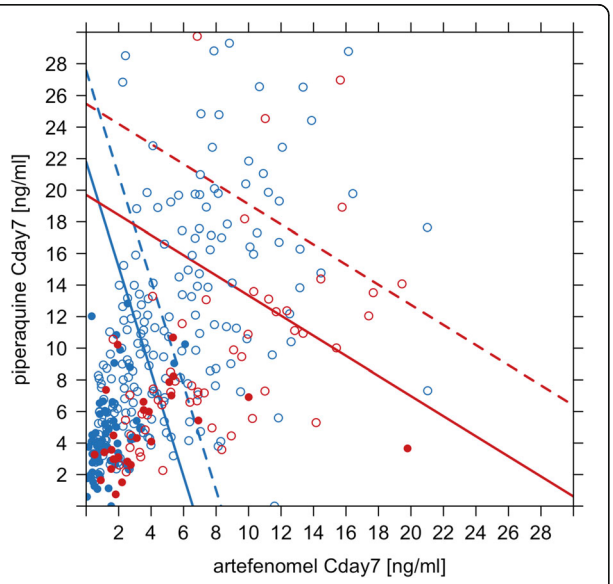


Fig. 9 Concentrations associated with a probability of ACPR28 of 0.95: model-predicted isobolograms by region and baseline parasitaemia. Asian patients = *red*, African patients = *blue*. Baseline parasitaemia 10,000 parasites/μL, *solid isobole*; baseline parasitaemia 100,000 parasites/μL, *dotted isobole*. Includes actual estimated individual exposures associated with Cure (ACPR28), *open symbol* or Failure (recrudescence), *closed symbol*

Dose–response relationship

The projected ACPR28 simulations for various dose combinations, taking into account between-subject variability as well as model parameter uncertainty in both PK and exposure response, are shown in Table 6. The identified age effect on the relative bioavailability of artefenomel was not included in the simulations. Thus, hypothetically, if similar exposures to African patients > 5 years were achieved in patients ≤ 5 years (e.g. alternative formulation or alternative dose adjustment), the doses highlighted in Table 6 may achieve an ACPR28 > 95%. Note that the projections for several dose combinations in the table are extrapolations from the model, both the mono-therapy (exposure–response component) and the higher dose levels (PK component).

Discussion

We report the results of the first phase II dose-ranging study to assess the potential of a single encounter curative treatment (artefenomel plus PQP) for uncomplicated *P. falciparum* malaria in adults and children in Africa and Asia (Vietnam).

None of the treatment arms reached the pre-specified target efficacy of ≥ 95% PCR-adjusted ACPR28 in the overall population or in any of the subpopulations, including African patients > 5 years of age, demonstrating that a single encounter treatment with artefenomel

Table 6 Simulated % ACPR28 with 90% confidence intervals for single dose combinations of artefenomel and PQP in non-vomiting African children ≤ 5 years old

PQP adult-equivalent dose, mg	Artefenomel adult-equivalent dose, mg					
	0	200	400	800	1200	1600
0	NR (–)	22 (14–32)	39 (29–49)	70 (59–79)	86 (78–92)	94 (88–97)
320	27 (18–37)	35 (28–44)	52 (45–59)	79 (73–84)	91 (86–94)	**96 (93–98)**
640	40 (29–52)	48 (39–58)	64 (58–70)	85 (81–88)	94 (91–96)	**97 (95–99)**
960	51 (38–64)	59 (48–69)	72 (65–78)	89 (85–92)	**95 (93–97)**	**98 (97–99)**
1440	64 (49–76)	70 (59–80)	80 (73–86)	92 (89–95)	**97 (95–98)**	**99 (98–99)**
2000	74 (59–85)	79 (67–88)	86 (80–91)	**95 (93–97)**	**98 (97–99)**	**99 (98–100)**
2800	83 (70–91)	86 (76–93)	91 (85–95)	**97 (95–98)**	**99 (98–100)**	**> 99 (99–100)**
3600	88 (78–95)	91 (83–96)	94 (90–97)	**98 (96–99)**	**99 (99–100)**	**> 99 (99–100)**

For the stimulations, the identified age effect on artefenomel PK was not included; it was assumed, therefore, that exposures in the youngest patients were similar to those in the older age groups. *NR* not reported
Predicted ACPR28 > 95%; outcome predicted with a lower bound > 95%

800 mg plus up to 1440 mg PQP does not provide sufficient exposure for a sufficient duration to achieve the required efficacy. Efficacy appeared lower in Vietnamese than in African patients overall; however, the lowest efficacy was observed among the youngest African age group (> 0.5 to ≤ 2 years old).

The study was not powered to compare outcomes between treatment arms, and no clear PQP dose trend was identified in the primary analysis for ACPR28. This was due to large exposure variability and the limited dose range studied, resulting in overlapping exposures between treatment arms, coupled with the binary nature of the clinical endpoint. However, this large exposure range allowed establishment of an exposure–response relationship for both drugs in combination, which in turn resulted in identification of factors influencing efficacy, thereby providing a fuller understanding of the study results.

Thus, the exposure–response analysis demonstrated that both drugs contribute to efficacy (ACPR28) in a concentration-dependent manner, and as might be expected, higher baseline parasitaemia requires higher exposures to provide the same ACPR28.

Within Africa, the concentration–response relationship for artefenomel and piperaquine did not appear to differ with age (or presumed immunity). While age was not identified as a significant covariate for ACPR28, the numbers of African patients > 5 years old was relatively small and may have been insufficient to identify a difference. Instead, the lower efficacy in the youngest African patients appeared due to lower exposure to artefenomel (and to a lesser extent piperaquine). There was insufficient information to be able to identify the reason for the lower exposure. This may have been a consequence of incorrect dose adjustment to account for clearance/bioavailability differences across the age range; however, failure of the youngest children to consume the entire dose (there were anecdotal reports of this despite high reported compliance) or vomiting may have contributed.

The exposure–response analysis indicated that the lower efficacy (ACPR28) in the Vietnamese relative to the African population was due to lower sensitivity to artefenomel (but not to piperaquine), that is, a regional difference in the concentration–response relationship. The mechanism of this lower sensitivity is not known.

Kelch13 genotyping indicated a high frequency of patients infected with artemisinin resistant parasites within the Vietnamese sites. The most common Kelch13 genotypes were C580Y, the predominant validated marker of artemisinin resistance across the Greater Mekong Subregion, and P553L, a candidate marker of artemisinin resistance found in the Western Greater Mekong Subregion [28].

Artemisinin resistance is characterised by a decrease in the rate of parasite clearance following artemisinin mono-therapy or artemisinin-containing combination treatments in patients infected with parasites with mutations in the Kelch13 gene [29]. In vitro this is manifested by a decreased sensitivity to artemisinin of the early rings stage of the parasite lifecycle [30], and as such is considered partial resistance. Artemisinin partial resistance has not been shown to reduce the cure rate unless partner drug resistance is also present.

The current study is the first to fully evaluate the efficacy of a combination containing a synthetic endoperoxide (artefenomel) in patients infected with artemisinin resistant parasites, and the association between PCt1/2 following artefenomel/PQP treatment and Kelch13 mutation suggests that these mutations may drive a similar decrease in the rate of parasite clearance for artefenomel-containing combinations as with artemisinin-containing combinations [29]. In vitro data suggest that, similar to DHA, there is reduced sensitivity of early mutant rings to artefenomel [30]. However, Kech13 mutation

was not identified as a significant covariate for ACPR28 in the model. Caution should be exercised here, as the sample size may well not have been sufficient to identify any association between Kelch13 and ACPR28 in the exposure–response analysis.

High rates of DHA–piperaquine treatment failures are now reported in the Greater Mekong Subregion, suggesting co-segregation, or at least coexistence of artemisinin and piperaquine resistance. Three of the four sites in Vietnam involved in the study (Gia Lai, Binh Phauc and Khanh Hoa) are located in provinces bordering Cambodia, and so conceivably artemisinin and piperaquine resistant genes could coexist. It is noteworthy that no difference was detected in the sensitivity (exposure–response relationship) for piperaquine between Vietnam and Africa. However, the sensitivity to piperaquine of *P. falciparum* parasites collected in this study is not currently known. Recent work to identify genetic markers of piperaquine resistance has confirmed that increased copy numbers of plasmepsin 2 and plasmepsin 3 genes, along with Pfmdr1 gene de-amplification, are independently associated with resistance to piperaquine, and that these markers of piperaquine resistance are prevalent in Cambodia and coexist with Kelch13 [31, 32]. We intend to investigate the frequency of genetic markers of piperaquine resistance in samples collected during this study and to investigate the relationship between Kelch13 and genetic markers of resistance, and PCt1/2 and ACPR.

We are also currently investigating the efficacy of artefenomel in combination with ferroquine. Both parent drug and circulating active metabolite have significantly longer half-life values than piperaquine, and hence this combination has a greater probability of achieving the target efficacy. Ex vivo studies performed with Cambodian clinical isolates suggest negligible impact of piperaquine resistance on ferroquine potency [33].

The significant rate of vomiting and the high dosing volume, particularly for young children, were problematic and may have contributed to the lower drug exposures in the youngest children. The development of age-appropriate formulations is key to the success of individual studies and to development programmes as a whole. Asymptomatic QTc increases from baseline were frequently reported as expected for PQP.

The study results also illustrate the challenges in developing a single encounter combination treatments. Firstly, the administered dose needs to be higher to achieve the required duration of exposure compared to multiple day treatments; therefore, the ratio of Cmax to overall exposure will be greater. Secondly, high between-subject variability in drug exposures, due in part to a limited number of body weight bands for dosing (and which in this study may have been compounded by challenges in administering large dosing volumes to sick children), in addition to a large between-subject variability in baseline parasitaemia and potentially parasite sensitivity, means that the majority of patients will be required to be 'overdosed' if a very high cure rate is to be achieved with a single (adult equivalent) dose level. Both factors mean that a wide therapeutic window is required.

Limitation of the study

A significant limitation of the study was that, although the formulation used had been tested in adult healthy subjects, it had not been tested in adult and, more importantly, paediatric malaria patients. It is possible that the palatability of the formulation and/or volume of administration contributed to the higher than expected rate of vomiting in the study. In addition, although compliance data on drug consumption were collected, insufficient detail was recorded to truly capture compliance, since despite a high reported success rate of drug administration, there were anecdotal reports that young children were unable to ingest the full dose. The effect of this was that the study was unable to conclude on the best weight-based dose adjustment for patients weighing < 35 kg; i.e. drug exposure in young children was lower than in adults. However, it could not be concluded whether this was due to the weight-based dose adjustments or because the intended dosage was not successfully administered.

However, the dose–response simulations suggest that, even if the young children had similar exposures to the adults as well as no vomiting, the target efficacy would not have been achieved.

Conclusions

None of the treatment arms reached the target efficacy of > 95% PCR-adjusted ACPR at day 28. Achieving very high efficacy following single dose treatment is challenging since > 95% of the population must have sufficient concentrations to achieve cure across a range of parasite sensitivities and baseline parasitaemia levels, meaning that a significant number of patients are 'overdosed'. Drugs with a substantial therapeutic window are therefore required. Projected single dose combination doses of artefenomel plus PQP that may achieve the target efficacy in the African population were not markedly higher than those tested in this study, but are most likely not clinically viable due to tolerability and practical dose size.

A high frequency of patients in Vietnam were infected with artemisinin resistant parasites, and an association between PCt1/2 following artefenomel/PQP treatment and Kelch13 mutation suggests that these mutations may drive a similar decrease in the rate of parasite clearance for artefenomel-containing combinations as observed with artemisinin-containing combinations [28].

While challenging, the development of tools suitable for deployment as single encounter curative treatments for adults, and particularly children in Africa, and to support elimination strategies remains a key development goal. There is currently no evidence that full artemisinin resistance has emerged in Southeast Asia; i.e. there is no evidence that reduced sensitivity of the early ring stage has progressed to full resistance [28]. The results of the efficacy study of artefenomel and ferroquine will inform us of the potential of artefenomel-containing combinations to treat malaria in both Africa and Southeast Asia where partial resistance to artemisinin and resistance to piperaquine is widespread.

Additional files

Additional file 1: S1. Study Protocol. (PDF 1822 kb)
Additional file 2: S2. Statistical analysis plan. (PDF 1515 kb)
Additional file 3: S3. Pharmacokinetic analysis details. (DOCX 689 kb)
Additional file 4: S4. Exposure–response analysis details. (DOCX 269 kb)

Abbreviations

ACPR: Adequate clinical and parasitological response; ACPR28: Adequate clinical and parasitological response on day 28; AE: Adverse event; ALT: Alanine transaminase; ANC: Absolute neutrophil count; AST: Aspartate transaminase; AUCinf: Area under the plasma concentration curve extrapolated to infinity; Cday7: Plasma concentration on day 7; CI: Confidence interval; Cmax: Maximum plasma concentration; CV: Coefficient of variation; DHA: Dihydroartemisinin; E: Number of events; ECG: Electrocardiogram; ETF: Early treatment failure; Hb: Haemoglobin; IEC: Independent Ethics Committee; IQR: Interquartile range; ISMB: Independent Safety Monitoring Board; ITT: Intention to treat; IWRS: Interactive Web Response System; KM: Kaplan–Meier; LCF: Late clinical failure; LPF: Late parasitological failure; MMV: Medicines for Malaria Venture; mPP: Modified per protocol population; n: Number of patients; OZ439: artefenomel; P.: Plasmodium; pACPR: probability of achieving adequate clinical and parasitological response; PCR: Polymerase chain reaction; PCt1/2: Parasite clearance half-life; PK: Pharmacokinetics; PP: Per protocol; PQP: Piperaquine phosphate; PRR24: Parasite reduction ratio at 24 hours (log10) post-treatment; SAE: Serious adverse event; TEAE: Treatment emergent adverse event; TEAESI: Treatment emergent adverse event of special interest; Tmax: Time to reach maximum plasma concentration; TPGS: α-tocopherol polyethylene glycol; ULN: Upper limit of the normal range; WHO: World Health Organization; WT: Wild type

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Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FM, SD, DL, MS, CF, IL, MR, MEG, NG, EHC, JTM and BEL substantially contributed to analysis and interpretation of data. YA, ABT, TTD, GMN, MBA, HT, QB, SI, MA, NVH, COM, JLK, JAL, RZM, MG, ANK, JM, AB, SMO, SC, KM, OS, JDB, AS, RV, AM, GM and HD substantially contributed to acquisition of data. FM, SD, AK, AKT, PGK, BQP, AO and MR substantially contributed to conception and design. FM, BEL, SD, DL, MS, CF, IL and SB were involved in drafting the manuscript. YA, ABT, TTD, GMN, MBA, HT, QB, SI, BEL, FM, AK, AKT, PGK, BQP, AO and MR critically revised the manuscript for important intellectual content. All authors contributed to drafting and critically reviewing of the manuscript and have approved its final version. All authors read and approved the final manuscript. The authors agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

The study was approved by the relevant IEC, national Institutional Review Boards and, where relevant, local regulatory authorities at each of the participating sites. Participants provided written informed consent prior to inclusion. The ethics committees that approved the study are the following:
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The Comité d'éthique pour la recherche en Santé du Burkina Faso (National ethical committee)
Comité d'Ethique pour la recherche de la santé, Ouagadougou, Burkina Faso

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Ethics Committee of the Kinshasa School of Public Health's, Kinshasa, Democratic Republic of Congo
Comité National d'Ethique pour la Recherche (CNER), Libreville, Gabon
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Competing interests
The authors declare that they have no competing interests.

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Treatment of Uncomplicated Malaria

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Synonyms

Management; Therapeutics; Treatment

Introduction

Malaria is a medical emergency which requires prompt diagnosis and expedited treatment, so as to minimize the risk of progression to a life-threatening condition. Of the nearly 220 million malaria cases occurring annually, only 1–2% will progress to severe disease (WHO 2018). The greatest burden of severe and fatal disease is borne by children under 5 years of age (particularly in sub-Saharan Africa) which may account for over 60% of the nearly half a million annual malaria deaths (WHO 2018). Such a significant burden places malaria as one of the global biggest killers and highlights the need of establishing optimal management strategies for this deadly infection, either based on the implementation of currently existing drugs or on the development of new effective antimalarial interventions (Murray et al. 2012; Varo et al. 2018; WHO 2018).

An uncomplicated malaria case may be defined as a patient with a parasitological confirmed *Plasmodium* infection, with clinical symptoms, and not fulfilling the criteria for severe malaria. The huge percentage of infections by malaria parasites are considered to be benign or mild, and only a small proportion of *Plasmodium falciparum* infections (and to a lesser extent of other plasmodia species) will develop severe manifestations. The first symptoms of the disease are nonspecific including general malaise, fatigue, arthromyalgias, headache, abdominal discomfort, nausea, or vomiting which are normally followed by fever, the landmark of the disease. Indeed, in malaria-endemic areas, malaria remains the most common cause of fever. In children, respiratory symptoms are also frequent, and the infection can be misdiagnosed with other common conditions of childhood. In areas of stable transmission, young children with recurrent infections can present with an enlarged spleen and chronic anemia. Most patients with uncomplicated malaria only have few abnormal physical findings, and a

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variety of laboratory alterations have also been well characterized.

The main objectives of the treatment of uncomplicated malaria are to prevent progression to severe disease, reduce clinical symptoms, and cure the infection as soon as possible. The World Health Organization (WHO) defines "cure" as elimination of all parasites from the body (WHO 2018). From a public health perspective, correct and prompt treatment should help to prevent antimalarial drugs resistance and to stop further transmission of the infection to other individuals. Effective case management remains therefore the cornerstone of malaria treatment, and it is based on early and accurate diagnosis and treatment. In the past decades, this has relied essentially in the use of inexpensive and widely available antimalarial drugs, and similarly to what occurs with other infections, in combination with treatments, rather than as monotherapies. In the last decade, artemisinin-based combination therapies (ACTs) have become the mainstay of the treatment of uncomplicated malaria. Table 1 summarizes the main principles in the management of uncomplicated malaria.

Treatment of Uncomplicated Malaria, Table 1 Main principles for the treatment of uncomplicated malaria

1. The treatment of uncomplicated malaria is important to prevent progression to severe disease, to reduce clinical symptoms, and to cure infection
2. All malaria cases must be confirmed by laboratory procedures. An isolated negative thick blood smear or rapid diagnostic test does not exclude a malaria diagnosis
3. In case of high clinical suspicion, treatment must be started even in the absence of parasitological confirmation
4. The treatment must be guided by the type of *Plasmodium* species and the resistance profile of the area
5. Artemisinin-combination therapies (3-day regimen) are the treatment of choice for uncomplicated malaria
6. In case of inability to tolerate oral treatment, parenteral treatment must be initiated
7. In case of developing severe manifestations, prompt parenteral treatment must be started
8. Although clinical symptoms may disappear quickly, treatment needs to be completed according to manufacturer's recommendations
9. Treatment of uncomplicated malaria in cases of *Plasmodium vivax, Plasmodium ovale* spp., *Plasmodium malariae*, and *Plasmodium knowlesi* must follow these same principles
10. In cases of *Plasmodium vivax* or *Plasmodium ovale* infection, radical cure treatment with primaquine or tafenoquine is recommended, after exclusion of G6PD deficiency

WHO recommends that all malaria cases should be treated after parasitological confirmation using light microscopy or rapid diagnostic tests (RDTs) and restricts treatment based on presumptive diagnosis only to cases where a parasitological diagnosis is not possible. The number of malaria cases treated after testing positive for malaria has progressively increased during the last years, but it has been estimated that as many as 30% of patients receiving antimalarials are still treated in the absence of an adequate malaria testing (WHO 2015).

Current Difficulties Regarding the Treatment of Uncomplicated Malaria

There are many potential challenges for a successful treatment of malaria, which can be divided in two major areas: (1) factors related to the currently available drugs and (2) wider issues related to access to treatment in those settings where malaria is endemic. Regarding the former, most important issues relate to the limited availability of new drugs/partner drugs, the limited availability of pediatric-friendly formulations (important limitation, considering that the bulk of malaria cases occur in the youngest populations), the limited shelf life of the artemisinin derivatives, and the emerging resistance to artemisinin derivatives. Regarding the latter, it is important to highlight that in many malaria-endemic areas, access to the health system is not granted, and many malaria cases remain untreated at the community. Additionally, costs, distribution and stock out challenges, and the increasing problem posed by "counterfeit" or substandard drugs further hinder a correct access to a lifesaving treatment.

Antimalarial Drugs

Artemisinin-Based Combination Therapies

Artemisinin-based combinations (ACTs) are the most effective antimalarial drugs currently available and as such remain the recommended choice for the treatment of uncomplicated malaria caused by *Plasmodium falciparum*. ACTs are designed to be administered orally for the treatment of uncomplicated malaria. For children who cannot swallow pills, dispersible formulations of some ACTs have been designed, so as to guarantee that they can be safely taken by the youngest. ACTs combine two active drugs with different mechanisms of action and different half-lives. This combination is essential to counteract the potential emergence of resistance which has been observed for all the rest of existing antimalarial drugs. Treatment with an artemisinin-based combination should therefore rapidly decrease the parasite biomass through the fast-acting potent artemisinin component and expose the few remaining parasites to a partner drug with a much slower mechanism of action and elimination half-life. However, the high rates of artemisinin tolerance detected among circulating parasites in some areas of the Greater Mekong Subregion (GMS), in Southeast Asia, are a matter of concern, and such mutant parasites will need to be contained prior to spreading to other parts of the world.

ACTs are currently considered the most potent and *fast-acting antimalarials* due to their rapid absorption and strong activity against different stages of the malaria life cycle: from young asexual forms (rings) to most sexual forms (early-stage gametocytes). They are able to reduce parasite burden by a factor of around 10,000 in each asexual cycle (Eastman and Fidock 2009; Nosten and White 2007). Current recommendations from WHO state that children and adults with uncomplicated *Plasmodium falciparum* malaria (except pregnant women in their first trimester) should be treated with one of the following ACTs:

1. Artemether + lumefantrine (AL)
2. Artesunate + amodiaquine (ASAQ)
3. Artesunate + mefloquine (ASMQ)
4. Dihydroartemisinin + piperaquine (DHA-PQP)
5. Artesunate + sulfadoxine–pyrimethamine (AS-SP)

A sixth ACT (artesunate + pyronaridine), already approved by stringent regulatory authorities, should soon be included in the recommendation list from WHO. ACT regimens must in all cases cover a 3-day full course of treatment which might ensure adequate efficacy, promote good adherence, and avoid incomplete treatments in order to minimize the risk of drug resistance. Courses of less than 3 days are not recommended because they have less effect on parasite clearance (including gametocytes), and they provide a shorter protection for the long-acting partner drug. On the other hand, a 3-day course of ACTs which covers two asexual cycles ensures that the artemisinin component will leave only a small proportion of parasites for clearance by the partner drug and consequently will reduce the risk of resistance against it. An important factor to consider in endemic areas where the incidence of malaria is high is the protective capacity against new incident infections conferred by drug levels attained in blood. This is called the posttreatment prophylactic effect and generally depends on the partner drug's half-life, understanding that the artemisinin derivatives quickly disappear from blood. Thus, theoretically, the longer the half-life of the partner drug, the longer the protection conferred against new infections.

Artemether + Lumefantrine

This is the combination more widely available and used as first-line therapy in most malaria-endemic countries (White et al. 2014). It was the first fixed-dose combination of an artemisinin derivative with an unrelated long-acting drug. Lumefantrine is an antimalarial agent classified as an amino alcohol in the same group as halofantrine. Lumefantrine is not available as monotherapy, and, theoretically, it has been proposed that it could show cross-resistance with other drugs such as halofantrine and mefloquine. This combination remains effective against human malaria parasites in most regions of the world, with some reports showing delayed clearance in the GMS.

Artemether is biotransformed to the metabolite dihydroartemisinin, the active component responsible for the clearance of most of the parasite biomass during the first two drug-exposed cycles. However, the artemether and dihydroartemisinin components are eliminated very rapidly from the body, and lumefantrine accounts for the elimination of the remaining parasites during subsequent cycles. In fact, it has been demonstrated that for this drug combination, the principal factor of cure in acute falciparum malaria is the plasma lumefantrine concentration time curve (AUC), or its surrogate, the day 7 lumefantrine levels. The absorption of lumefantrine is dose-dependent and varies markedly between individuals, with a theoretical risk of decreased exposure and treatment failure among some specific groups: children <3 years, pregnant women, large adults, smokers, patients with high parasitemias, patients in very low transmission areas with emerging parasite resistance, and patients taking mefloquine, rifampicin, or efavirenz (WHO 2015; WWARN 2015). Patients in those groups must be monitored for ensuring full adherence, and, in some of them, a prolonged regimen of 5 days has been proposed to ensure effectiveness of treatment, although this is not yet an official recommendation (Kloprogge et al. 2013; Tun et al. 2018; WWARN 2015). Levels of lumefantrine depend also on co-administration with fats, and the manufacturers recommend to take AL simultaneously with food or drink (especially on the second and third day of treatment). Although there is a higher risk of increased exposure in patients receiving lopinavir–lopinavir/ritonavir-based antiretroviral agents, this is not translated in a higher toxicity, and there is no need for treatment adjustment (WHO 2015).

Current formulations of AL are dispensed in fixed-dosed combinations containing 40 or 80 milligram (mg) of artemether and 120 or 240 mg of lumefantrine. The dose is weight-based and should ensure a total dose of 5–24 mg/kilogram (kg) body weight of artemether and 29–144 mg/kg body weight of lumefantrine (WHO 2015). This combination was first introduced as a four-dose regimen, but it was shown that plasma concentrations of lumefantrine were insufficient to completely eliminate the parasite. To increase the AUC and the cure rate, a six-dose regime was then evaluated, proving to have excellent cure rates and being well tolerated. Thus, the current recommended dosage regimen is to give AL twice daily during 3 days with the first two doses preferably given 8 hours (h) apart (WHO 2015).

As mentioned before, this combination is the most widely ACT utilized due to its excellent safety profile (it may provoke mild side effects as nausea, dizziness, and headache), its affordable costs (around $1per treatment), and an available child-friendly dispersible formulation (Bassat et al. 2015).

Artesunate + Amodiaquine

This ACT combination is the second more widely used globally, after AL. Amodiaquine is a 4-aminoquinoline as chloroquine and piperaquine (Nosten and White 2007). Although there may be some cross-resistance, amodiaquine is usually effective against chloroquine-resistant strains. However, in recent years there has been an increase in the reports of resistance in some areas of Africa and Asia.

Artesunate, similarly to artemether, is rapidly converted in dihydroartemisinin which is active against all erythrocytic malaria stages. On the other hand, amodiaquine is converted to desethylamodiaquine which has most of its antimalarial activity. ASAQ is effective against all asexual forms of human malaria parasites. Amodiaquine does not need dosage adjustment in relation to age or pregnancy, but children underweight should be monitored due to a theoretical higher risk of treatment failure. In patients receiving zidovudine, efavirenz, or co-trimoxazole, ASAQ should only be used if this is the only ACT available because there is an increased risk of neutropenia. Others severe side effects as agranulocytosis or liver toxicity, which have been described when this ACT is used as prophylaxis, are rare when used for the treatment of acute malaria. ASAQ is well tolerated but can present with adverse events such as gastrointestinal disturbances or general side effects as insomnia, weakness, fatigue, and insomnia (WHO 2015).

The current recommendation is to administer this fixed drug combination with the following dosages: artesunate (4 (2–10) mg/kg body weight) and amodiaquine (10 (7.5–15) mg/kg body weight) daily for 3 days. The drug is dispensed in tablets containing a fixed-dosed combination of artesunate and amodiaquine: 25 + 67.5, 50 + 135, or 100 + 270 mg, respectively (WHO 2015).

Artesunate + Mefloquine

Mefloquine is a 4-methanolquinoline agent structurally related to quinine. Mefloquine resistance was soon documented in Asia after its introduction as monotherapy treatment, probably associated to the previous widespread use of quinine in the area. Mefloquine has an elimination half-life of around 3 weeks, conferring a posttreatment prophylactic effect which adds benefit in high-endemic settings. ASMQ is widely used in Southeast Asia. However, data on resistance and reports on side effects have hindered the introduction of this ACT in areas like sub-Saharan Africa, where the drug, manufactured according to recommended standards, is not yet fully available. Although mefloquine may present a higher risk of neurological/neuropsychiatric and gastrointestinal adverse events when used as monotherapy, this combination is generally well tolerated. The most frequent side effect when it is used as treatment is vomiting, which may affect adherence, but the differences with other ACTs like AL are not significant (Sirima et al. 2016). Mefloquine has been associated with neuropsychiatric symptoms as dysphoria, psychosis, convulsions, or dizziness, mainly in patients with past cerebral malaria, with history of psychiatric illness or those people on long-term prophylaxis (WHO 2015). In those situations, administration of mefloquine should be contraindicated. Those effects are minimized when the overall dose of mefloquine (25 mg/kg) is given in 3 days.

The current recommendation is to administer 4 (2–10) mg/kg body weight per day of artesunate and 8.3 (5–11) mg/kg body weight per day of mefloquine, given daily for 3 days. As other ACTs, ASMQ is available in a fixed-dose combination for children (25 mg artesunate +55 mg mefloquine hydrochloride) and adults (100 mg artesunate +220 mg mefloquine hydrochloride).

Dihydroartemisinin + Piperaquine

DHA-PQP is effective against all asexual forms of human malaria parasites. Dihydroartemisinin is an active metabolite of artesunate and artemether and, like them, belongs to the family of sesquiterpene peroxide agents (WHO 2015). Similarly to mefloquine, piperaquine is a 4-methanolquinoline related to chloroquine and effective against chloroquine-resistant parasites. Piperaquine was first introduced in China where its extensive use as monotherapy, in mass treatment and mass prophylactic campaigns, triggered the development of resistance to the drug. It was afterward combined with dihydroartemisinin, and the combination has been shown to be similarly safe, well tolerated, and effective as other ACTs (WANECAM 2018). In addition, DHA-PQP has a longer posttreatment prophylactic effect, as a result of the relatively longer half-life terminal elimination of piperaquine (around 4 weeks) (WANECAM 2018). Oral bioavailability can be increased with high-fat meals, and for this reason, and to avoid potential side effects secondary to higher than expected plasma concentrations, it is recommended to fast when the drug is administered. Young children, malnourished children, and pregnant women may present lower drug concentrations, although the need to adjust the dosage in these groups is debatable. Current recommendations, based on modelling, suggest that the dosage of DHA-PQP needs to be increased in children under 25 kg of body weight, so as to achieve similar plasmatic concentrations than those seen in heavier children or adults (WHO 2015). There has been a traditional concern about the arrhythmic potential of piperaquine which, as other related antimalarials currently in use, can prolong QT interval and affect ventricular repolarization. In spite of these concerns, DHA-PQP has not been associated with a higher risk of sudden cardiac death, so the unjustified cardiotoxic reputation of this ACT should not be a barrier for its use (Chan et al. 2018; Millat-Martinez and Bassat 2018).

The target dose of dihydroartemisinin is similar in children and adults (4 (2–10) mg/kg body

weight per day) but must be increased in those patients weighing < 25 kg (24 (20–32) mg/kg body weight per day) in comparison to those weighing ≥25 kg (18 (16–27) mg/kg body weight per day). Fixed-dose combination tablets are available, either containing 20 mg dihydroartemisinin and 160 mg piperaquine or 40 mg dihydroartemisinin and 320 mg piperaquine for patients ≥20 kg. A pediatric-friendly dispersible formulation is also under development.

Artesunate + Sulfadoxine–Pyrimethamine

This ACT combination is perhaps the less utilized one, globally. Sulfadoxine is a long-acting sulfonamide combined in a fixed presentation with the antifolate pyrimethamine. Both are active against *Plasmodium falciparum*, and they have a synergistic activity (Sinclair et al. 2009). This combination is usually well tolerated, and side effects are minor and uncommon. Increasing resistance to SP has been well documented and is likely to worsen because of the widespread use of SP for intermittent preventive treatment (IPT) and seasonal malaria chemoprevention (SMC) purposes and other similar drugs such as trimethoprim–sulfamethoxazole (co-trimoxazole) and sulfalene–pyrimethamine. AS-SP should not be used in areas with SP-established resistance and is contraindicated in those HIV patients receiving co-trimoxazole (WHO 2015). Currently, no fixed-dose combination of AS-SP is available. Artesunate is given daily for 3 days in tablets containing 50 mg (4 mg/kg body weight per day), and SP is given as a single dose on day 1 as fixed-dose combination tablets containing 500 mg sulfadoxine +25 mg pyrimethamine (25/1.25 mg/kg body weight sulfadoxine–pyrimethamine).

Pyronaridine + Artesunate

This ACT, registered under the stringent European regulatory agency EMA, has yet to be included in the WHO guidelines for malaria treatment. It has however already been included in WHO's Essential Medicines list and will be a potential alternative for ACT diversification (WANECAM 2018). Pyronaridine is a benzonaphthyridine closely related to amodiaquine which was first synthesized and used as monotherapy in China. Pyronaridine is highly effective against *Plasmodium falciparum*, including chloroquine-resistant strains and *Plasmodium vivax* species (Croft et al. 2012). Resistance against pyronaridine has emerged but seems delayed when used in combination with artesunate (Croft et al. 2012). Pyronaridine is well distributed in the body and has a slow elimination of around 9–13 days. This ACT has demonstrated to be well tolerated and to have a similar efficacy to other first-line ACTs (WANECAM 2018). It can be given with or without food. Pyronaridine is less toxic than chloroquine, but the initial studies showed potential liver toxicity that has not been confirmed subsequently. Pyronaridine-artesunate has been developed in a fixed-dose combination containing 180 mg of pyronaridine and 60 mg of artesunate. It is available in tablets for adults and in granules for children weighing 5–20 kg and is given as a once-daily, 3-day treatment regimen.

Other Antimalarial Agents

Quinine (QNN), an alkaloid originally obtained from the bark of the cinchona tree, was the first effective treatment against malaria in Western medicine and for centuries was the only available treatment for the disease. Its synthetic version, developed in 1820, is still indicated in the treatment of uncomplicated malaria during the first trimester of pregnancy, on account of the few alternative drugs that are considered safe and efficacious in this particular vulnerable window of the pregnancy. Although it has been well established that artesunate is more effective than QNN for the treatment of severe malaria, this drug remains highly efficacious in its parenteral form. The main problem with quinine is the common occurrence of adverse effects such as "cinchonism," which may present with different manifestations: headache, nausea, vomiting, dizziness, vertigo, tinnitus, diarrhea, abdominal pain, and auditory and visual symptoms. Especially in young children, elderly, and pregnant women, quinine can cause hypoglycemia. Quinine may also have cardiotoxic and cardiovascular side effects (WHO 2015).

Chloroquine (CQ) is an antimalarial of the 4-aminoquinoline family which was first synthesized in 1934 and designated as the drug of choice of uncomplicated malaria after the Second World War. During the next four decades, CQ was the main pillar of the treatment of malaria globally, providing a safe, cheap, and effective alternative. By the end of the 1970s, resistance to CQ was documented in South America and Southeast Asia, spreading shortly after to East Africa and then to the rest of the continent. This situation forced the WHO to change treatment recommendations for the treatment of uncomplicated *Plasmodium falciparum* malaria from chloroquine to ACTs, particularly in countries where *Plasmodium falciparum* had become resistant to CQ (WHO 2015). Since then, the use of CQ has been progressively abandoned, and the only place in the world where this drug remains as first line for the treatment of *Plasmodium falciparum* is the Hispaniola Island (Haiti and Dominican Republic). The documentation that sensitivity to CQ can be rapidly regained after only a few years of discontinuation has put renewed interest of the potential uses of this drug as a complementary tool to other drugs for malaria control in the future, considering that this drug remains cheap, and safe, even throughout pregnancy (Galatas et al. 2017).

Atovaquone–proguanil is a fixed-dose combination of two antimalarial drugs belonging to the napthoquinones and biguanides families, respectively. Their actions are synergistic, and they are both active against all parasites stages. This combination is usually used as prophylaxis of malaria, but it is also indicated for treating uncomplicated malaria episodes in nonimmune travelers. In children, it has been shown to be effective, but there is no pediatric formulation usable in children under 12 kg of weight (Tahar et al. 2014). Atovaquone-proguanil is well tolerated and safe with a good side effect profile. The main barrier of this combination is its current high cost which jeopardizes its wide availability and utilization in malaria-endemic countries.

Treatment of Uncomplicated Malaria in Special Situations

Pregnant Women

Pregnant women are a susceptible group because they are more likely to develop anemia, severe malaria, pregnancy loss, and death. Malaria in pregnancy is also associated with an increased risk of low birth weight, premature labor, and stillbirth. Thus, malaria treatment during pregnancy should also be considered a medical emergency and a public health priority, but unfortunately data on the tolerability, safety, efficacy, or pharmacokinetics of antimalarial drugs during pregnancy are scarce, particularly during the first trimester.

Recent reports confirm that during the second and third terms of pregnancy, ACTs do not seem to have an increased risk of deleterious effects on pregnancy outcomes, in comparison to quinine, and are highly efficacious (Dellicour et al. 2017). Indeed, the benefits of ACTs treatment during the second and third terms of pregnancy are likely to outweigh the adverse outcomes of quinine treatment, and thus ACTs are the recommended drugs for uncomplicated malaria during the later stages of pregnancy. However, evidence of ACTs' safety during the first term is limited, and concerns have been raised because of a demonstrated embryotoxic potential in animal studies. As a result, the current recommendation precludes the usage of ACTs in the early stages of pregnancy, at least until sufficient evidence of their safety is generated, and proposes the use of oral quinine and clindamycin, during 7 days (WHO 2015) for the treatment of malaria infections in the first trimester of pregnancy. This combination presents some problems, including the common side effects of quinine which may result in poor compliance and recrudescence, and the frequent unavailability of clindamycin in many places, leading to a factual use of quinine as monotherapy.

Children

ACTs are safe and well tolerated in children, and they are the treatment of choice for uncomplicated malaria. All ACTs previously presented can be

used in children, but SP should be avoided in the first weeks of life (WHO 2015). The main problem of the antimalarial agents in children is the reduced tolerability due to vomiting or regurgitation (WHO 2015). This situation can hamper the correct intake of the drug, jeopardizing the efficacy of the treatment in the group with the highest risk of developing severe episodes (Eastman and Fidock 2009). There are scarce pharmacokinetic data in children and some concerns about the adequate dose regimen in younger or malnourished patients. Furthermore, there are few specific pediatric formulations which could improve the drug's tolerability and intake (Gargano et al. 2018). This situation should however not delay the initiation of treatment and can justify a lower comparative threshold for the use of parenteral options, due to the higher risk of children to deteriorate quickly.

Nonimmune Travelers

Nonimmune travelers are individuals from areas without malaria transmission who travel to malaria-endemic areas. They are generally malaria-naïve as a result of not having been previously exposed to malaria infections and therefore are completely unprotected against malaria, as opposed to people having been previously exposed routinely to infective bites. As a result, they are a high-risk population and can easily develop severe malaria. They should receive early diagnosis and prompt treatment according to national policies. In non-endemic areas appropriate management can be difficult because the diagnosis may be delayed due to the lack of familiarity of practitioners and because some antimalarial drugs are not easily available. Chemoprophylaxis before, during, and immediately after the trip is currently considered the best recommendation to prevent malaria in this particular vulnerable group. If chemoprophylaxis has not been received, or has only been partially used, and a malaria infection is diagnosed, then the patient should receive a different drug for treatment. ACTs or atovaquone–proguanil are the currently recommended regimens for treating uncomplicated malaria in these patients.

Conclusions

Treatment of uncomplicated malaria seeks to cure infection and prevent progression to severe disease. Correct treatment may also help to stop transmission and dissemination of resistance. Whenever possible it must be based on parasitological confirmation of malaria infection. Currently, ACTs are the treatment of choice as they have demonstrated to be the safest, most effective, and well-tolerated option.

Cross-References

- ▶ Adjunct Therapies for Malaria
- ▶ Antifolate-Drugs
- ▶ Case Management Diagnosis of Malaria, Overview
- ▶ Clinically Relevant Drug Interactions for Malaria
- ▶ Control of Malaria During Pregnancy: Safety of Antimalarial Drugs
- ▶ Investigational Drugs, Quality and Drug Formulations for Malaria: Pharmacology of Antimalarial Drugs, Current Anti-malarials
- ▶ Treatment of Malaria in Special Patient Populations
- ▶ Treatment of Non-falciparum, Mixed Species and Non-human Malaria
- ▶ Treatment of Severe Malaria

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CLINICAL THERAPEUTICS

Efficacy and Tolerability Outcomes of a Phase II, Randomized, Open-Label, Multicenter Study of a New Water-Dispersible Pediatric Formulation of Dihydroartemisinin-Piperaquine for the Treatment of Uncomplicated *Plasmodium falciparum* Malaria in African Infants

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ABSTRACT Artemisinin combination therapies are considered the mainstay of malaria treatment, but pediatric-friendly formulations for the treatment of infants are scarce. We sought to evaluate the efficacy and safety of a new dispersible-tablet formulation of dihydroartemisinin/piperaquine phosphate (DHA/PQP) in comparison to the marketed tablet (Eurartesim) in the treatment of infants with uncomplicated *Plasmodium falciparum* malaria. Reported here are the results of a large phase II, randomized, open-label, multicenter trial conducted in African infants (6 to 12 months of age) from Mozambique, Burkina Faso, The Gambia, the Democratic Republic of the Congo, and Tanzania. Primary efficacy endpoint was the PCR-corrected adequate clinical and parasitological response (ACPR) at day 28. Analysis was performed for the intention-to-treat (ITT) and per-protocol (PP) populations. A total of 201 patients received the dispersible-tablet formulation, and 99 received the conventional one administered as crushed tablets. At day 28, the PCR-corrected ACPRs were 86.9% (ITT) and 98.3% (PP) in the dispersible-tablet group and 84.9% (ITT) and 100% (PP) in the crushed-tablet group. At day 42, these values were 85.9% (ITT) and 96.5% (PP) in the dispersible-tablet group and 82.8% (ITT) and 96.4% (PP) in the crushed-tablet group. The comparison between survival curves for time to new infections showed no statistically significant differences ($P = 0.409$). The safety and tolerability profile for the two groups was similar in terms of type and frequency of adverse events and was consistent with that expected in African infants with malaria. A standard 3-day treatment with the new dispersible DHA/PQP formulation is as efficacious as the currently used tablet in African infants and has a comparable safety profile. (This trial was registered at ClinicalTrials.gov under registration no. NCT01992900.)

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The last 15 years have witnessed impressive advances in the global fight against malaria, including in sub-Saharan Africa, where the highest burden of morbidity and mortality is still concentrated (1, 2). However, an estimated 429,000 malaria-attributable deaths still occurred in 2016, the vast majority of which were among African children, making it clear that major efforts are still required in the fight against this disease (3). One of the major threats in the global strategy against malaria is posed by the emergence and potential spread of drug-resistant parasites. In response to this, the World Health Organization (WHO) recommended in 2006 the use of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria (4). The artemisinin derivatives are currently the most rapidly acting and potent antimalarials (5). The pharmacodynamic effects of ACTs are due to the rapid absorption of artemisinins and their strong activity against several stages of the malaria life cycle from young asexual forms (rings) to early sexual forms (gametocytes) (6).

Eurartesim is a fixed-dose combination composed of dihydroartemisinin (DHA) and piperaquine phosphate (PQP) (7). This partner compound has a different mechanism of action from that of artemisinins and a much longer half-life (several weeks), facilitating the initial clearing of parasites, and a long posttreatment prophylactic effect (8, 9).

The efficacy and safety of Eurartesim for the treatment of uncomplicated *Plasmodium falciparum* malaria has been demonstrated in two phase III trials, respectively conducted in 1,553 African children and 1150 Asian adults and children (10, 11). Both studies demonstrated an equivalent efficacy of DHA/PQP versus the comparative ACT. In addition, they evidenced a superiority of DHA/PQP versus the comparative drugs in a longer posttreatment prophylaxis period that suppress new infections from emerging during follow-up.

In 2011, Eurartesim obtained a marketing approval by the European Medicine Agency for all countries of the European Union, manufactured as film-coated tablets (12). The ACT was marketed for its use in children and adults and has been adopted by several countries where malaria is endemic as the first-line treatment of uncomplicated malaria (3). Finally, Eurartesim obtained by the WHO the prequalification status on 9 October 2015 (13).

One of the greatest challenges of malaria treatment is ensuring adequate intake and compliance among young children, the population group most at risk of developing life-threatening episodes (14). As of today, very few antimalarials are available in good laboratory practice (GLP)-produced pediatric friendly formulations (15). Since infants and very young children are generally unable to swallow oral tablets, Eurartesim was administered in previous trials as an oral suspension of crushed tablets mixed with water. However, the bitter taste of the crushed tablet could easily compromise tolerability in this age group, and crushing tablets is a suboptimal preparation procedure, which could result in loss of drug and a reduced dose ingested. To overcome these problems, a new water-dispersible tablet formulation of DHA/PQP was developed for oral administration in infants and young children.

We present here the results of a multicenter trial in sub-Saharan Africa aiming to assess the efficacy, tolerability and safety of the new dispersible-tablet formulation of DHA/PQP with respect to the marketed formulation, administered as a crushed tablet, to infant patients (from 6 to 12 months of age) with uncomplicated *P. falciparum* malaria. These results are part of a large phase II trial, which was designed to also collect pharmacokinetic data for dispersible and crushed formulations of DHA/PQP in African infants with malaria.

RESULTS

Patient disposition as presented in the case report forms is summarized in Fig. 1. Overall, 443 patients were screened, and 300 were randomized: 201 to receive the

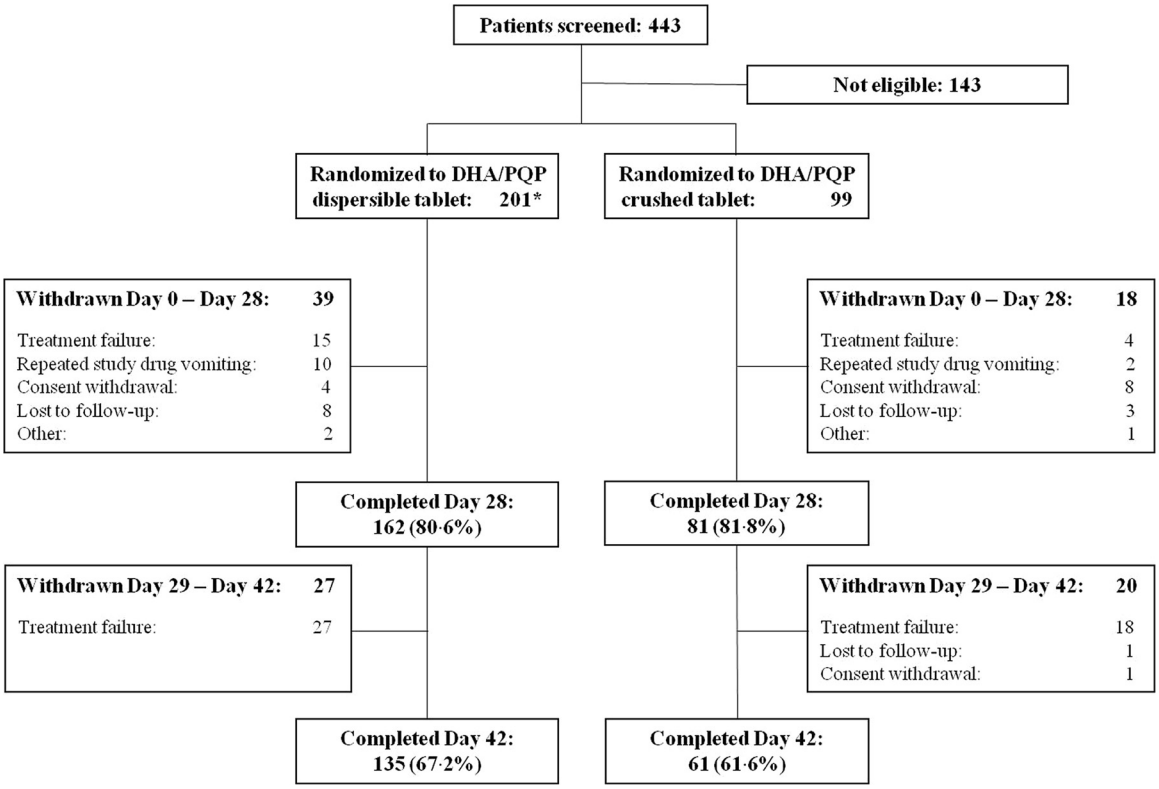

FIG 1 Flow chart of study participants with uncomplicated *P. falciparum* malaria treated with a 3-day course of either dispersible or crushed tablets of dihydroartemisinin-piperaquine (DHA/PQP). *, Two patients randomized in the dispersible treatment group were excluded from all analysis due to unconfirmed malaria diagnosis at day 0 (classified as "Other") and informed consent withdrawal before first study drug administration.

dispersible-tablet formulation and 99 to receive the crushed-tablet formulation. However, two patients randomized in the dispersible-tablet group were excluded by all analysis because one of them did not have parasites at day 0 (as determined by a confirmatory blood smear reading) and the other withdrew informed consent before the first drug administration (these patients, respectively, are reported as "other" and "consent withdrawal" by day 28 in Fig. 1).

The most common reasons for study withdrawal were malaria recurrence (classified as treatment failure) and informed consent withdrawal, occurring with a similar frequency in both treatment groups. Repeated vomiting after drug administration was more frequent in the dispersible arm compared to the crushed arm (5% versus 2%), although such a difference was not statistically significant ($P = 0.349$ by the Fisher exact test). Among patients reported as "other" in Fig. 1, one more patient in the dispersible-tablet group dropped out due to family movement to a another area, while one patient in the crushed-tablet group left the hospital after the first study drug intake.

The number of patients enrolled in each country and their attribution to ITT and PP populations are shown in Table 1. The patients excluded from the PP population due to major protocol violations were 28 in the dispersible-tablet group (including the two patients above described, who were also excluded from the ITT population) and 15 in the crushed-tablet group (Table 2). All of them fulfilled the *a priori* declared major violations or did not complete the study due to a reason different from malaria recurrence or any other AE causing withdrawal from the study (i.e., consent withdrawal or lost to follow-up). Two more patients were excluded from the PP population (one per each treatment group) since it was not possible to assess their outcome on day 28 not having the patients attended this visit and presenting malaria recurrence at day 42. There were also seven randomized patients presenting baseline parasitemia outside the range indicated in inclusion criteria, but all of these violations were judged as "minor"

TABLE 1 Patient accountability by country in ITT and PP populations[a]

Study population	DHA/PQP dispersible-tablet group				DHA/PQP crushed-tablet group			
	ITT		PP		ITT		PP	
	n	%	*n*	%	*n*	%	*n*	%
Total	199	100	173	100	99	100	84	100
Mozambique	90	45.2	83	48.0	51	51.5	40	47.6
Gambia	11	5.5	6	3.5	3	3.0	3	3.6
DR-Congo	26	13.1	25	14.5	7	7.1	7	8.3
Burkina Faso	67	33.7	57	33.0	37	37.4	33	39.3
Tanzania	5	2.51	2	1.2	1	1.0	1	1.2

[a]*n*, number of patients. DR-Congo, Democratic Republic of the Congo.

by the Data Safety Monitoring Board (DSMB), and according to the study protocol these patients were included in the PP population. Overall, there was a good balance for demographic and baseline characteristics between treatment arms (Table 3).

Efficacy outcomes. Both dispersible and crushed formulations were efficacious. Descriptive and inferential statistics for the cure rates are shown in Table 4 and efficacy results are summarized in Fig. 2. In the PP population, the PCR-corrected adequate clinical parasitological responses (ACPRs) at day 28 were very high for both treatment arms (98.3 and 100% for the dispersible and crushed groups, respectively), showing homogeneity among cure rates with overlapping two-sided asymptotic confidence intervals (CI). Similarly, the PCR-uncorrected ACPR was comparable between treatment arms and still above 90% for both groups. At day 42, the PCR-corrected ACPRs were slightly lower than that observed at day 28 but remained very similar among treatment arms (96.5 and 96.4% for the dispersible and crushed groups, respectively), whereas the uncorrected ACPRs for the dispersible arm were to some extent higher with respect to the crushed arm (76.3% versus 72.6%).

In the ITT population, the PCR-corrected ACPRs at day 28 were indeed similar between treatment arms (86.9 and 84.9% for the dispersible and crushed groups, respectively), while the uncorrected ACPRs were nearly identical (80.9 and 80.8%). At day 42, the PCR-corrected ACPRs were to some extent lower than those observed at day 28 for both arms (Fig. 2). Similar to the findings observed in the PP population, the uncorrected ACPRs in the ITT population for the dispersible-tablet group were slightly higher with respect to the cure rate for the crushed-tablet group. Overall, for both PCR-corrected and uncorrected ACPRs, the asymptotic 95% CI values showed a good homogeneity with overlapping values and including the zero value for the computed differences (Table 4). Considering both ITT and PP populations overall, the comparison

TABLE 2 Summary of major protocol violations

Category	DHA/PQP treatment group	
	Dispersible tablet (*n* = 199)	Crushed tablet (*n* = 99)
Total no. of patients with major violations	26	15
% of patients with major violations	13.1	15.2
Protocol violation, no. (%) of patients		
Repeated study drug vomiting	10 (5.0)	2 (2.0)
Study drug noncompliance	2 (1.0)	0
Presence of jaundice at screening	1 (0.5)	0
Consent withdrawal	3 (1.5)	8 (8.0)
Visit at day 28 not performed and malaria recurrence at day 42	1 (0.5)	1 (1.0)
Lost to follow-up at or before day 42	8 (4.0)	3 (3.0)
Moved away from the study site	1 (0.5)	0
Left the hospital after first drug administration	0	1 (1.0)

TABLE 3 Demographic and baseline characteristics (ITT population)

Characteristic	DHA/PQP treatment group	
	Dispersible tablet (n = 199)	Crushed tablet (n = 99)
No. (%) of patients		
Male	89 (44.7)	47 (47.5)
Female	110 (55.3)	52 (52.5)
Mean age and wt ± SD		
Age (mo)	9.1 ± 1.8	9.2 ± 2.0
Wt (kg)	7.9 ± 1.2	8.0 ± 1.1
Clinical data		
Fever, no. (%) of patients	119 (59.8)	61 (61.6)
Mean temp in °C (range)	37.8 (35.8–40.3)	37.8 (35.8–40.2)
Mean parasite density (range)	53,282 (771–226,707)	56,480 (108–322,194)
Presence of gametocytes, no. (%) of patients	10 (5.0)	5 (5.1)
Mean hemoglobin in g/dl (range)	8.96 (5.40–12.00)	8.98 (6.90–12.40)

between the dispersible and crushed groups for the corrected and uncorrected ACPRs, assessed at day 28 or 42, showed no statistically significant differences.

The occurrence of malaria recurrences by time points, as well as other reasons for treatment failure, are shown in Table 5. At day 28, the numbers of new infections were similar between the treatment arms (6 and 4% for the dispersible and crushed groups, respectively), whereas only two recrudescences occurred in the dispersible-tablet group. By day 42, the proportion of patients experiencing new clinical malaria episodes increased similarly in both dispersible and crushed groups (ITT population), reaching, respectively, 18.1 and 21.2% of the new infections (P = 0.519 [chi-square test]) and 2.5 and 1.0% of recrudescences (P = 0.667 [Fisher exact test]). When estimated by Kaplan-Meier survival analysis, the cumulative risks of new infection at day 42 were 19.8 and 23.6% for the dispersible and crushed groups, respectively (Fig. 3), whereas those for recrudescence were 3.1 and 1.3% (ITT population). Similar results were obtained for the PP population (data not shown). As for the cure rates, comparison by log-rank tests between treatment groups on time to new infection and time to recrudescence was not statistically significant in both ITT and PP populations (i.e., new infections, P = 0.409 and P = 0.568, respectively; recrudescences, P = 0.404 and P = 0.162, respectively).

One ETF occurred during the study due to the evolution of uncomplicated into severe malaria in a patient enrolled 2 days after initial malaria symptoms. The complication occurred 20 h after the first study drug intake and was probably due to the late starting of antimalarial treatment. At day 42, the overall proportions of treatment

TABLE 4 PCR-corrected and uncorrected adequate clinical and parasitological response by time points in ITT and PP populations

Study population[b]	Cure rate (ACPR)	No. (%) of patients in DHA/PQP treatment group		Treatment difference (DHA/PQP dispersible–DHA/PQP crushed)		
					Two-sided 95% CI[a]	
		Dispersible table	Crushed tablet	Δ (%)	Without CC	With CC
Day 28						
ITT (n = 199, 99)	PCR corrected	173 (86.9)	84 (84.9)	2.08	−0.064–0.106	−0.071–0.113
	Uncorrected	161 (80.9)	80 (80.8)	0.09	−0.094–0.096	−0.101–0.103
PP (n = 173, 84)	PCR corrected	170 (98.3)	84 (100)	−1.73	−0.037–0.002	−0.046–0.011
	Uncorrected	158 (91.3)	80 (95.2)	−3.91	−0.101–0.023	−0.110–0.032
Day 42						
ITT (n = 199, 99)	PCR corrected	171 (85.9)	82 (82.8)	3.10	−0.058–0.120	−0.065–0.127
	Uncorrected	135 (67.8)	61 (61.6)	6.22	−0.053–0.178	−0.061–0.186
PP (n = 173, 84)	PCR corrected	167 (96.5)	81 (96.4)	0.10	−0.047–0.049	−0.056–0.058
	Uncorrected	132 (76.3)	61 (72.6)	3.68	−0.078–0.151	−0.087–0.160

[a]CI, confidence interval; CC, continuity correction.

[b]Numbers in parentheses correspond to the numbers of individuals treated with the dispersible tablet and the crushed tablet, respectively.

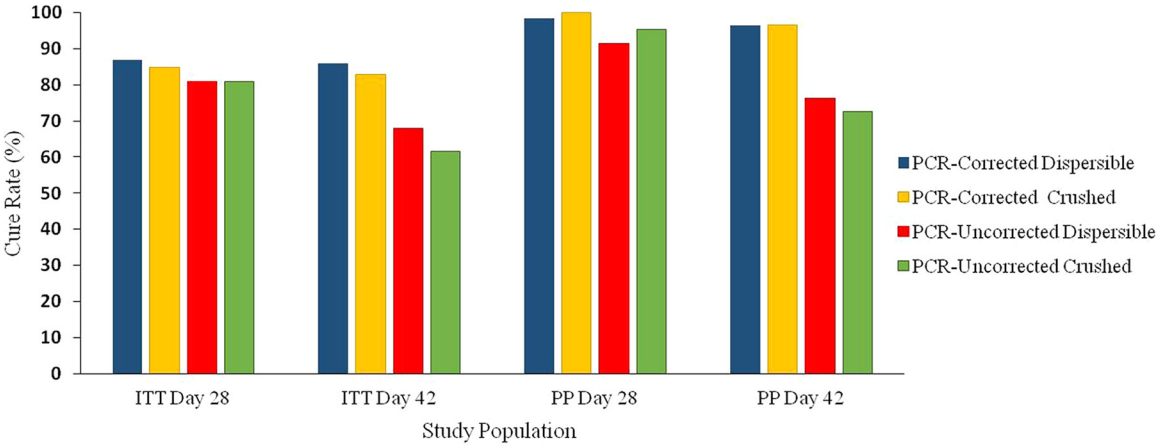

FIG 2 ACPR at days 28 and 42 of treatment with dispersible and crushed DHA/PQP formulations administered to infant patients with uncomplicated *P. falciparum* malaria.

failures (ETFs plus LTFs) were similar between the treatment arms, corresponding to 42 cases (21.1%) in the dispersible-tablet group and 22 cases (22.2%) in the crushed-tablet group ($P = 0.825$ [chi-square test]; ITT population).

Parasite clearance was rapid in both treatment arms. In the ITT population, fewer than half of the patients were parasitemic at day 1 (46.2 and 48.5% in the dispersible and crushed groups, respectively), with only few of them remaining parasitemic at day 2 (3.0% versus 2.0%). With the exception of one patient who was still parasitemic up to day 7 (dispersible-tablet group), all other patients were aparasitemic by day 3. No statistically significant differences in parasitemia clearance between treatment groups were found using the log-rank test ($P = 0.653$).

At baseline, about 60% of the patients had fever. At day 2, more than 96% of the patients were afebrile in both groups. Upon enrollment, only 5% of the patients in both groups presented gametocytes, which quickly disappeared in the first few days. Ga-

TABLE 5 Treatment failure by time point (ITT population)

	No. and % of patients in each treatment group							
	Day 28				Day 42			
	Dispersible tablet ($n = 199$)		Crushed tablet ($n = 99$)		Dispersible tablet ($n = 199$)		Crushed tablet ($n = 99$)	
Reason for failure	*n*	%	*n*	%	*n*	%	*n*	%
Recrudescence[a]	2	1.0	0	0.0	5	2.5	1	1.0
New *P. falciparum* infection	12	6.0	4	4.0	36	18.1	21	21.2
Early treatment failure (ETF)	1	0.5	0	0.0	1	0.6	0	0.0
Late treatment failure (LTF)	14	7.0	4	4.0	41	20.6	22	22.2
Late clinical failure (LCF)	3	1.5	2	2.0	18	9.1	12	12.1
Late parasitological failure (LPF)	11	5.5	2	2.0	23	11.6	10	10.1
Lost to follow-up	8	4.0	3	3.0	8	0.0	4	1.0
Repeated vomiting	10	5.0	2	2.0	10	5.0	2	2.0
Withdrawal before the time of analysis (day 28): any reason except lost to follow-up	4	2.0	9	9.1	4	2.0	9	9.1
Day 28 visit missing and parasitemia at day 42	1	0.5	1	1.0	-	-	-	-
Adverse event	0	0.0	0	0.0	0	0.0	1	1.0
Total no. of failures	38	19.0	19	19.2	64	32.2	38	38.4

[a]For two patients (one for each group) with malaria recurrence, PCR analysis was not performed. Therefore, DSMB categorized these patients as treatment failures in the ITT population and missing in the PP population.

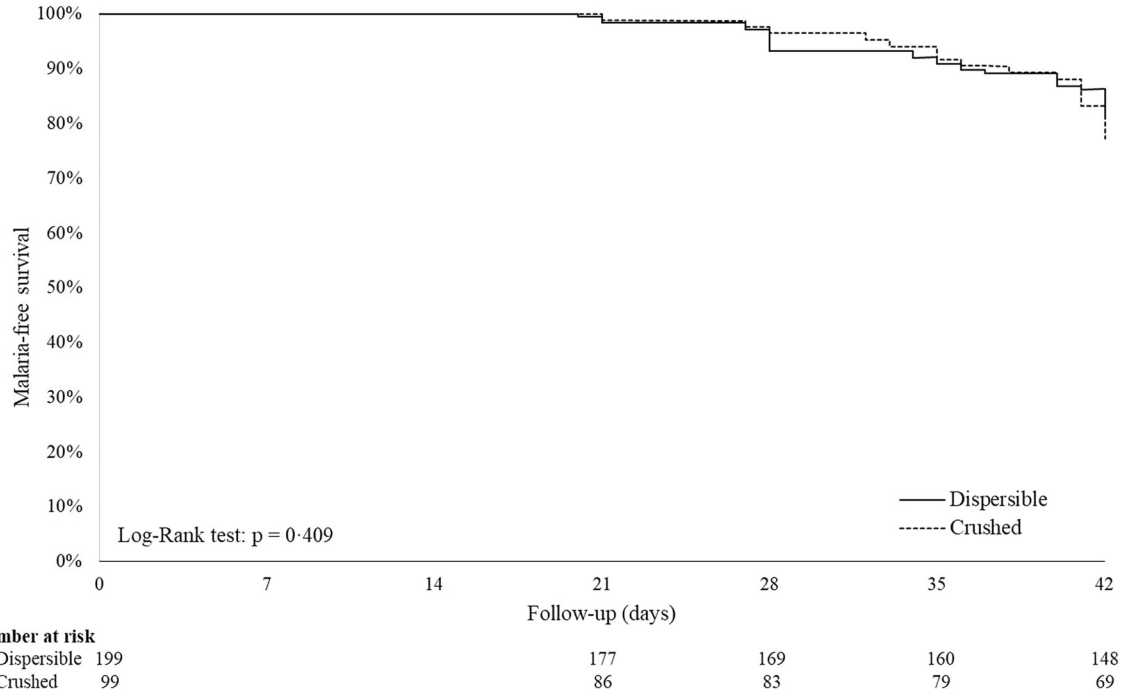

FIG 3 Time to new infections: survival analysis. A Kaplan-Meyer curve shows the cumulative risk of developing new infections in infants (ITT population) treated with either a dispersible- or a crushed-tablet formulation during a 42-day follow-up period.

metocytes did not develop during the course of the study. Due to the quick disappearance of fever and the gametocytes, the corresponding survival analyses were not performed.

Safety results. Both DHA/PQP formulations were well tolerated, with most AEs of mild or moderate severity. The AE profiles for both treatment groups were similar in terms of the type and frequency of events. Totals of 160 (80.4%) and 84 (84.9%) patients, respectively, treated with the dispersible or crushed formulations, had at least one AE during the study. Anemia, vomiting, diarrhea, rhinitis, and cough were the most frequently reported AEs and were often associated with malaria, influenza, and respiratory tract infections. The occurrence of laboratory AEs, e.g., altered liver enzymes (AST), was very rare and was similar between the treatment groups.

The proportion of patients experiencing at least one treatment-emergent adverse event (TEAE), for which the relationship with the study drug was suspected, was slightly more prevalent in patients treated with the crushed-tablet formulation than in patients treated with the dispersible-tablet formulation (42.4% versus 33.7%, $P = 0.139$ [chi-square test]) (Table 6). In particular, gastrointestinal tolerability was better for patients treated with the dispersible-tablet formulation since vomiting occurred less frequently in this group compared to the crushed-tablet group (22.6% versus 31.3%, $P = 0.105$ [chi-square test]). Cutaneous reactions were infrequent, with only one case of rash (crushed group). One patient in the dispersible-tablet group developed moderate anemia, which occurred 1 week after treatment and resolved 3 weeks later. Overall, none of the TEAEs required hospitalization.

There were no significant differences between treatment groups in ECG parameters at baseline and during follow-up. A relevant and comparable reduction of HR (heart rate) in both treatment groups was observed at day 2 and at day 7. Related with this reduction, an increase in PR, QT, and QTcF intervals was observed in both groups with a comparable extension. A change in QTcF interval from baseline was detectable at day 2 predose and increased after drug administration, with 22 patients in the dispersible-tablet group (11.1%) and 14 patients in the crushed-tablet group (14.1%) manifesting a QTcF prolongation over 60 ms, none of which included

TABLE 6 Treatment-emergent adverse events suspected to be related to the study treatment administration (ITT population)

System organ class and preferred term	No. and % of patients in each DHA/PQP treatment group			
	Dispersible tablet (n = 199)		Crushed tablet (n = 99)	
	n	%	*n*	%
Patient with at least one TEAE[a]	67	33.7	42	42.4
Blood and lymphatic system disorders				
Anemia	1	0.5	0	0.0
Thrombocytosis	1	0.5	0	0.0
Gastrointestinal disorders				
Diarrhea	0	0.0	2	2.0
Vomiting	45	22.6	31	31.3
Nausea	1	0.5	0	0.0
Salivary hypersecretion	3	1.5	1	1.0
General disorders and administration site conditions				
Pyrexia	0	0.0	1	1.0
Infections and infestations				
Gastroenteritis	1	0.5	2	2.0
Malaria	1	0.5	0	0.0
Investigations				
Electrocardiogram QTcF prolonged (>60 ms)	23	11.6	15	15.2
Metabolism and nutrition disorders				
Decreased appetite	1	0.5	1	1.0
Respiratory, thoracic, and mediastinal disorders				
Cough	1	0.5	0	0.0
Skin and subcutaneous tissue disorders				
Papulosquamous rash	0	0.0	1	1.0

[a]TEAE, treatment-emergent adverse event.

clinical translation. No cases of QTcF prolongation higher than 500 ms were reported. At day 7, the QTcF prolongation tended to normalization, with only one patient in the dispersible-tablet group and two patients in the crushed-tablet group still maintaining QTcF prolongation. Overall, no arrhythmias or any other cardiovascular AEs were reported.

Two severe adverse events (SAEs) occurred during the study, both judged unrelated to the study treatment. The first SAE involved the death of a 6-month-old male patient enrolled in the crushed-tablet group. He received the full treatment course and was discharged from the hospital after malaria resolution (day 3). At day 27, the child was seen as an outpatient in a peripheral health post, with a history of fever, cough, and diarrhea. After a positive rapid diagnostic test for malaria, he was treated with artemether/lumefantrine. However, a malaria diagnosis was not confirmed by microscopy, and 2 days later the mother reported the child's death. The investigator considered sepsis the most likely cause of death (a diagnosis supported by a verbal autopsy). The second SAE occurred in a male of 11 months enrolled in the dispersible-tablet group. He received the first dose of the study drug, and 20 h later the patient condition deteriorated, with evidence of severe anemia (hemoglobin, 4.9 g/dl) and polypnea. The study treatment was interrupted, and the patient received parenteral quinine for malaria and blood transfusion for anemia. After 4 days of hospitalization, followed by oral treatment with quinine for a further 5 days, the event was resolved.

DISCUSSION

According to guidelines on the clinical investigation of medicinal products in a pediatric population, there is a need to develop new formulations that allow accurate dosing and enhance patient compliance in infants and young children (16). This recommendation becomes even more necessary for the treatment of malaria, as young children and infants are those most affected by the disease, and very few GMP-produced pediatric-friendly formulations are available (15). Artemisinin-based combination therapy (ACT) is the current standard of care for patients with uncomplicated malaria in Africa (4). ACTs are typically provided in tablet form, which can be challenging to administer to young children who are typically unable to swallow whole pills. To facilitate the administration of ACTs to these individuals, tablets can be crushed and mixed with water. However, this process can result in the loss of active ingredients and lead to underdosing. In addition, crushed antimalarial tablets can be unpalatable since they have a bitter taste causing children to spit them out, thus leading to uncertain and/or subtherapeutic dosing (17).

Presently, there is only one ACT reported in the last updated version of the WHO Model List of Essential Medicines for Children (last amended on August 2015), containing arthemether-lumefantrine, which was specifically developed as a dispersible formulation to improve the effectiveness and accuracy of ACT dosing in infants and young children (15). Thus, there is an urgent need to make available on the market other pediatric formulations of different ACTs and, in this context, a new dispersible formulation of the DHA/PQP fixed-dose combination was developed for oral administration.

The dispersible-tablet formulation was similar in tolerability, efficacy, and safety to the standard formulation administered as crushed tablet to infants with uncomplicated *P. falciparum* malaria. In particular, PCR-corrected cure rates at day 28 of follow-up were high for both formulations, and no differences were observed in the response to treatment in terms of clearance of asexual parasites and fever, which were rapid in both treatments groups. Follow-up periods longer than 28 days are currently recommended by the WHO for antimalarial drugs with a long half-life (e.g., piperaquine or mefloquine) to allow drug concentrations in the blood to fall below the minimum therapeutic threshold (25). In fact, short observation periods can yield an underestimation of recrudescence rates. Hence, analysis of cure rates on day 42 showed that they were still high, suggesting a sustained efficacy for both formulations. Furthermore, substantial differences between the efficacies of the two formulations were not observed for the uncorrected ACPRs at day 42. Because the uncorrected cure rate is mainly affected by new infections in high-transmission areas, this finding indicates a similar prophylactic effect exerted by both formulations.

The present study confirmed the satisfactory efficacy of Eurartesim previously obtained in phase III trials performed in Africa and Asia (7). In particular, the PCR-corrected ACPR at day 28 or the incidence of recurrences were similar to that obtained in African children (10).

The dispersible formulation was well tolerated, and its safety profile was comparable to the crushed tablet. No new safety issues arose from the present study, and all findings were in line with the former ones. Most of the commonly reported AEs were symptoms of malaria. Similarly, the pattern of changes in laboratory variables was consistent with acute malaria and its resolution, with no differences between treatment groups. The most common drug-related AE was vomiting, and the corresponding frequency was similar to that previously reported in African children treated with crushed or uncrushed tablets (10). Importantly, no arrhythmias or other cardiovascular events were reported during the study. QTcF interval prolongations (difference from baseline higher than 60 ms) were observed in 11.6 and 15.2% of patients in the dispersible and crushed groups, respectively ($P = 0.381$ [chi-square test]). Prolongation of the QT interval was assessed using both Fridericia's and Bazett's correction methods but, notably, both formulas could be biased by the physiological high HR present in infants and exacerbated by the ill status and fever. In fact, baseline evaluations differed

drastically (by ~60 ms) when the different correction methods were applied. The reduction in HR observed after drug administration could also induce a bias in the estimation of QTc. Accordingly, QTcF recorded at the screening visit was lower than that recorded at day 7 (335 ± 16 ms versus 410 ± 22 ms), when malaria was resolved and the blood level of piperaquine was below the concentration considered relevant for sustaining QT interval prolongation. These considerations suggest that the QTcF assessment in infants by DHA/PQP could be overestimated. Nonetheless, QTcF never exceeded the normal limits (450 to 470 ms), no cases evolved in rhythm disturbance, and all of the changes observed during therapy were never associated with clinical signs of cardiotoxicity.

Dispersible tablets are expected to contribute to ease of drug administration in subjects having difficulties in swallowing whole tablets, i.e., infants and young children. Since the dispersible formulations were similar in efficacy and safety to the conventional formulation in the infant population, we believe that this highly efficacious formulation should be made more readily available to all children below 5 years of age. Cost savings are also likely with its use, with the potential benefit of improving the acceptability of the combination once available on the market. It is noteworthy that a recent cost-effectiveness analysis of DHA/PQP for first-line treatment of uncomplicated malaria in African children showed that the use of this ACT, particularly in areas with moderate to high malaria transmission, is clearly justifiable from both clinical and economic perspectives (18, 19).

The results presented here are part of a large study that was designed to evaluate also the population pharmacokinetics of piperaquine and dihydroartemisinin in African infants, since it appears important to demonstrate bioequivalence of the new formulation on PK grounds once similar efficacy and safety have been confirmed. The new dispersible formulation of Eurartesim is easy and safe to administer and provides better grounds for an enhanced compliance and effective treatment; hence, this should facilitate drug registration by Regulatory Authorities, prequalification by the WHO and, finally, full adoption in malaria control programs. Further studies are needed to clearly establish the improvement of the new formulation on compliance, hoping that the simplicity of its administration will improve substantially the adherence to the full 3-day course of treatment, therefore impacting the reduction of malaria morbidity and mortality in infants and young children and minimizing parasite resistance to ACTs.

MATERIALS AND METHODS

Study design. Between November 2013 and June 2015, a phase II, randomized, open-label, multicenter trial was performed in seven sites of five African countries: Centro de Investigação em Saude da Manhiça, Maputo, Mozambique; Kinshasa School of Public Health, University of Kinshasa, Democratic Republic of the Congo; Centre Muraz Bobo-Dioulasso, Nanoro, Burkina Faso; Centre National de Recherche et de Formation en Paludisme, Ouagadougou, Burkina Faso; Ifakara Health Institute, Bagamoyo, Tanzania; National Institute for Medical Research, Korogwe, Tanzania; and Medical Research Council Unit, The Gambia.

The study protocol was approved by the Institutional Review Board of the Hospital Clínic of Barcelona (Spain) and by the National Ethics Review Committee and/or Institutional Review Board at each trial site. The trial was conducted under the provisions of the Declaration of Helsinki and in accordance with GCP guidelines (20, 21). A data safety monitoring board (DSMB) was established, working independently, to harmonize and monitor patients' safety. The trial was registered in the ClinicalTrial.gov registry under registration no. NCT01992900 on 4 September 2014.

Patients. A targeted number of 300 infants (male and female) with uncomplicated malaria were enrolled in the study. Since the study was designed to evaluate also the population pharmacokinetics of piperaquine and dihydroartemisinin in African infants, the sample size has been estimated on the basis of previous PK studies (22, 23), and all efficacy and safety analyses are descriptive, including the *P* values and confidence intervals. The inclusion criteria were age 6 to 12 months, a body weight of ≥5 kg, a *P. falciparum* monoinfection with asexual parasite densities between 1,000 and 200,000 parasites/μl of blood, fever (axillary temperature of ≥37.5°C), or a history of fever in the preceding 48 h. Exclusion criteria were previous treatment with antimalarials, acute malnutrition, severe malaria, danger signs, moderate/severe anemia (Hb < 7 g/dl), a family history of sudden death or known congenital prolongation of the QT interval, or treatment with QT prolongation inducers or strong cytochrome-P450 inhibitors/inducers or antiretroviral drugs (or lactated by HIV-positive women under antiretroviral therapy). Patients satisfying the inclusion and exclusion requirements were enrolled if the parent or guardian signed a written informed consent.

Randomization and masking. Randomization was centralized using an interactive web-based response system. A randomization list was generated by an independent contract research organization (CROS NT, Italy), with each treatment allocation concealed in sealed opaque envelopes that were opened by the investigators only after patient randomization. An allocation ratio of 2:1 (dispersible versus crushed formulation) was applied, balancing patients for sex, to recruit 200 patients to receive the dispersible DHA/PQP formulation and 100 patients to receive the marketed tablet. No stratification for countries or sites was applied.

Procedures. The DHA/PQP dispersible formulation was a coformulated, water-dispersible flat tablet, provided in two different strength dosages: 10/80 mg and 20/160 mg of dihydroartemisinin/piperaquine tetraphosphate (as cellulose-microencapsulated piperaquine tetraphosphate) and other components (cellulose, starch, croscarmellose, black cherry flavor, saccharine, sucrose, and magnesium stearate). The marketed Eurartesim formulation was a coformulated, film-coated tablet, provided in one strength of 20/160 mg of DHA/PQP (Sigma-Tau, Italy) (12). Both formulations were administered once a day for three consecutive days, according to body weight. Patients weighing 5 to 7 kg received a daily dose of 10/80 mg of DHA/PQP, while patients weighing >7 kg to <13 kg received a daily dose of 20/160 mg of DHA/PQP.

DHA/PQP administration has a food interaction effect resulting in an increase of piperaquine absorption when concomitantly administered with high-fat food (23). To reduce this effect, the first dose was administered as soon as randomization was done, and deliberate efforts were made to ensure that no food was administered in the following 3 h. For the other doses, patients should not have been fed in the 3 h before drug intake and for the following 3 h. However, for infants needing food during the restricted periods, this was limited to breast milk or a low-fat maize porridge.

Patients were kept at the health facility for the 3-day dosing period. Scheduled visits during follow-up were at days 7, 14, 21, 28, and 42 posttreatment. In case of recurrent parasitemia, rescue treatment was performed according to local guidelines.

The diagnosis of *P. falciparum* infection was made at each site by microscopy using standard methods (24). Blood slides collected from screening until parasitemia clearance were also read by a centralized laboratory (Centro de Investigaçao em Saúde da Manhiça, Maputo, Mozambique). If parasitemia recurred during the study, the distinction between recrudescence and new infection was made by PCR genotyping (25). PCR analysis was centralized and performed under blind conditions at the Institute of Tropical Medicine, Antwerp, Belgium. Three polymorphic markers (MSP1, MSP2, and GluRP) were used to distinguish recrudescence from new infections. Recrudescence was defined when at least one identical allele for each marker was detected in the pre- and posttreatment samples. New infections were defined when all alleles for at least one marker differed between the two samples.

Blood samples for hematology and biochemistry were taken at enrollment and at day 7 and then repeated at day 28 if clinically significant abnormalities were detected at day 7 (analysis of main hematological and biochemical parameters, assessed at days 0, 7, and 28 are summarized in Table S1 in the supplemental material).

A 12-lead electrocardiogram (ECG) was recorded for each patient at enrollment (baseline) and then repeated at day 2 before the last drug administration, as well as after 4 to 6 h of drug intake. An ECG was also recorded at day 7 and repeated at day 28 if clinically relevant abnormalities were detected at day 7. All ECGs were digitalized using an ELI-150 cardiograph (Mortara Instruments Europe Srl, Italy), and ECG reading and analysis were centralized by a cardiac laboratory (Cardiabase, Nancy, France).

Outcomes. The main efficacy endpoint was the PCR-corrected adequate clinical parasitological response (ACPR) at day 28. Other efficacy and safety endpoints included the following: (i) day 28, PCR-uncorrected ACPR; (ii) day 42, PCR-corrected and -uncorrected ACPR; (iii) the proportion of patients with early and late treatment failure (ETF and LTF); (iv) asexual parasite density and clearance time; (v) fever clearance time and gametocyte carriage over time; (vi) Kaplan-Maier survival analysis for new infections and recrudescences over time; (vii) AE occurrence; and (viii) changes in hematology, blood chemistry, vital signs, and ECG parameters.

Treatment outcome was assessed according to the study protocol and further revised by the DSMB to complement the WHO *in vivo* efficacy definitions (26). All cases not strictly matching these rules (e.g., patients having taken disallowed drugs or with partially missing data, such as blood parasitemia) were evaluated under blind conditions at the DSMB meetings.

Statistical analysis. All statistical analyses were performed using the SAS system software, version 9.2.

Analyses of efficacy were performed for both the ITT and the PP populations, whereas safety analyses were performed only for the ITT population. The ITT population included all patients taking at least one dose of the study drug. The PP population included all randomized patients who received the full treatment course, completed the day 28 assessment, had an evaluable PCR in case of recurrent parasitemia, and did not meet major protocol violations (e.g., inclusion without a microscopically confirmed diagnosis or the occurrence of severe vomiting or severe malaria). Patients for whom the PCR indicated a new infection were considered failures in the PCR-uncorrected analysis and successes in the PCR-corrected analysis, whereas patients with PCR results indicating a recrudescence were counted as failures in both analyses. Patients for whom the PCR result was not interpretable or missing or not done were imputed as failures in the ITT population and excluded in the PP population. Patients lost to follow up were excluded from the PP population (see Table S2 in the supplemental material).

The two-sided 95% confidence interval (CI) for the treatment difference in the PCR-corrected and uncorrected ACPRs at day 28 (or day 42) were generated using the normal approximation method (27). The limits of the asymptotic CIs were computed. A CI with continuity correction was also computed to

verify the robustness of the conclusions. Whenever the observed counts were small (i.e., not all cells had observed counts of at least five) or there was a disagreement between the results of the two formulas (with or without continuity correction), an exact CI was computed.

The proportion of aparasitemic patients was computed for each study visit by treatment group. The parasite clearance time was defined as the time elapsed between the first study drug intake and the first of two consecutive evaluations with negative parasitemia. The proportion of afebrile patients was computed at each study visit by treatment group. Early and late treatment failures were summarized by proportion per each treatment group.

The cumulative risks of new infections and recrudescences were estimated through survival analysis (Kaplan-Meier). In survival analyses of new infections, patients classified as new infections were considered as having reached the event, whereas patients who did not reach the event (i.e., subjects withdrawing from the study or having a recrudescence or with an uninterpretable or missing PCR or classified as a success) were censored at the relevant time. In survival analyses of recrudescences, patients classified as recrudescence were considered to have reached the event, whereas patients who did not reach the event (i.e., subjects withdrawing the study or with a new infection or with an uninterpretable/missing PCR or classified as success) were censored at the relevant time.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00596-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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N.G., G.V., and Q.B. designed the study protocol, coordinated the study, supervised the enrollment and follow-up of patients, and wrote the manuscript. Q.B. acted also as overall study coordinator and, in collaboration with L.M., as Principal Investigator of the Mozambican site. U.D., T.H., S.S., A.T., A.M., and S.G. were the Principal Investigators of the sites in The Gambia, Nanoro, and Ouagadougou (Burkina Faso), Kinshasa (Democratic Republic of the Congo), and Bagamoyo and Korogwe (Tanzania), respectively, coordinating and supervising trial activities at the site level. S.P. designed the pharmacokinetic section of the study protocol and supervised the related data collection and analysis. L.M., A.S., R.V., H.M., E.S., H.B., S.A., C.O., M.L., T.R., I.V., D.B., J.A., and E.L. contributed to patient enrollment and follow-up, study monitoring activities, data collection and interpretation. In addition, H.B. contributed to the centralized laboratory analysis of parasitemia. All authors critically reviewed the article and approved the final version for journal submission.

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Capítulo X

Malaria

Lola Madrid • Rosauro Varo • Quique Bassat

Introducción. Puntos esenciales

- La malaria o paludismo es la enfermedad parasitaria más frecuente y con mayor impacto en el mundo. Aunque en la última década los casos y muertes por malaria están disminuyendo, todavía cada año ocurren cerca de 212 millones de episodios clínicos y alrededor de 429.000 muertes por malaria. A pesar de ser una enfermedad curable, y no contagiosa de humano a humano, continúa siendo uno de los principales problemas de salud pública a nivel global.
- Es una de las diez principales causas de muerte en países pobres, responsable del 5.2 % del total de muertes globales en menores de 5 años.
- Más del 90 % de las muertes por malaria ocurren en el continente Africano.
- 70 % de todas las muertes por malaria que ocurren en el mundo son en niños menores de 5 años de edad.
- Las medidas implementadas para la prevención y el control de la malaria a diferentes niveles (prevención, diagnóstico y tratamiento) han logrado una reducción drástica en la carga global de malaria pero todavía insuficiente para la eventual eliminación de la enfermedad. En los últimos años se ha desarrollado una vacuna eficaz y segura, llamada RTS,S, la cual ha sido considerada herramienta clave y complementaria a las intervenciones ya establecidas y propuestas por el programa global de control de malaria de la Organización Mundial de la Salud (OMS).

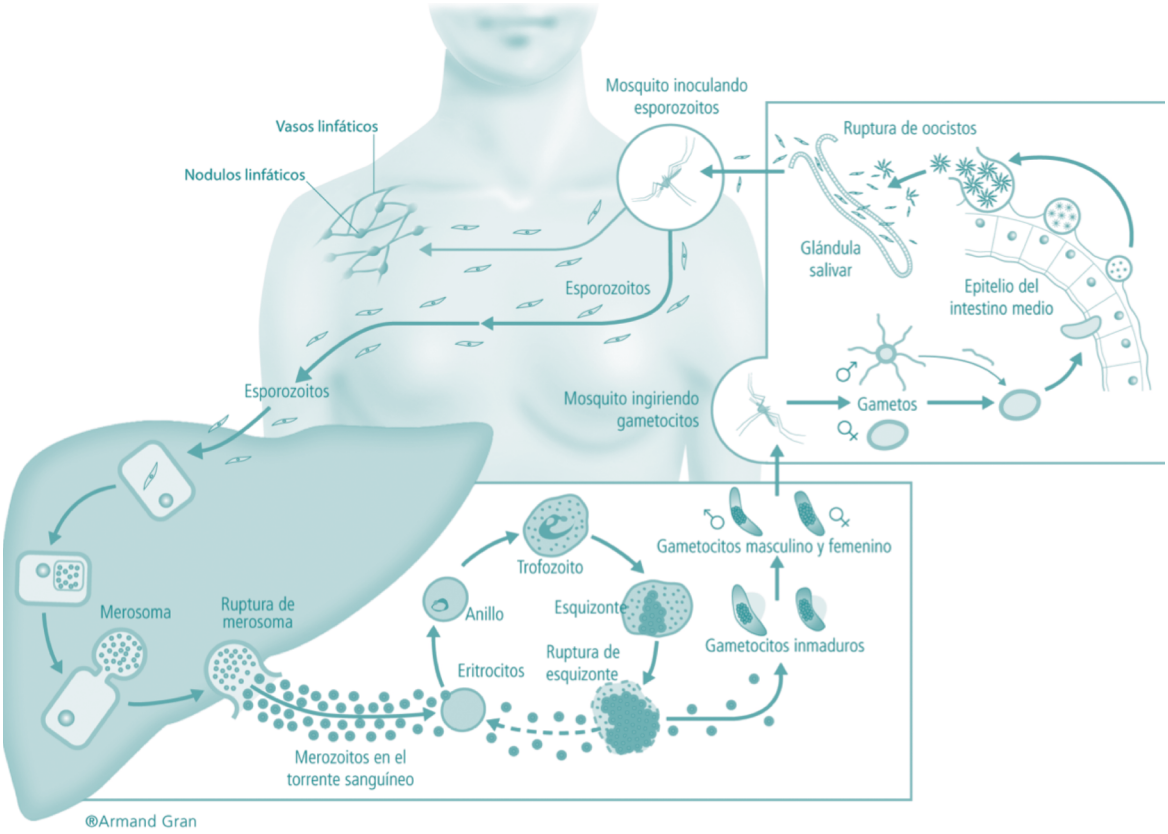
ETIOLOGÍA Y PATOGENIA

Los tres actores necesarios para la transmisión de la enfermedad son:

- El parásito infectante del género Plasmodium;
- El mosquito vector transmisor, del género Anopheles;
- El huésped humano (principal reservorio –aunque no exclusivamente- de la malaria humana)

- Existen 5 especies de *Plasmodium* que afectan al humano: *P. falciparum* (el más grave y responsable de prácticamente la totalidad de las muertes); *P. vivax* (el más extendido, normalmente benigno pero pudiendo complicarse también); *P. ovale, P. malariae y P. knowlesi.*
- En general las malarias animales no afectan al humano, con la excepción de *P. knowlesi,* una zoonosis del mono recientemente descrita en zonas selváticas del sudeste asiático y potencialmente grave. Con la excepción de la malaria congénita, adquirida por transmisión vertical de una madre infectada a su hijo, la enfermedad no se contagia entre humanos sin la mediación del mosquito vector, aunque raramente pueden darse casos de transmisión por vía hematógena (mediante transfusiones o trasplante de órganos de donantes infectados).
- El ciclo vital del plasmodio incluye una fase asexuada en el interior del humano (con múltiples estadíos), y otra sexuada en el mosquito **(Figura 1).**

FIGURA 1. Ciclo vital *Plasmodium falciparum*



EPIDEMIOLOGÍA

- La malaria en el mundo: *Magnitud del problema*
  - La malaria se mantiene como enfermedad endémica en 91 países, y cerca de la mitad de la población mundial vive expuesta al riesgo de contraerla.
  - En 2015, la malaria causó cerca de 212 millones de episodios clínicos y unas 429.000 muertes (datos de la Organización Mundial de la Salud (OMS) del 2016).
  - El 90 % de las muertes suceden en África, concentrándose de forma desproporcionada en niños menores de 5 años de edad. El 80 % de las muertes se concentran mayoritariamente en apenas 15 países.
  - En los países donde la malaria ya no se transmite, pueden observarse esporádicamente casos "importados" en pacientes que se hayan infectado como consecuencia de un viaje o estancia en una zona endémica.

- *Progresos y futuro en la lucha contra la malaria*
  - La incidencia global de malaria muestra una tendencia descendente en la última década, con una reducción en el número de casos y muertes atribuibles a malaria lo que ha tenido una repercusión directa sobre la mortalidad infantil global. Esto se debe en gran medida a la implementación y mayor accesibilidad a estrategias integradas de control basadas en la combinación de herramientas preventivas (uso de redes mosquiteras tratadas con insecticidas, la fumigación de interiores con insecticidas de acción residual; el tratamiento pre-

ventivo intermitente a mujeres embarazadas y en zonas de transmisión estacional, a niños menores de 5 años; la administración masiva de antimaláricos), diagnósticas (pruebas de diagnóstico rápido) y terapéuticas (tratamiento con las combinaciones derivadas de las artemisininas en aquellos casos diagnosticados de malaria).

- Sin embargo, estamos todavía muy lejos de poder garantizar que en los países de mayor transmisión exista una cobertura universal de dichas estrategias.
- En los últimos años, se ha llevado a cabo una transición de la clásica estrategia de prevención y control de la enfermedad al desarrollo de programas de eliminación de malaria en determinadas zonas geográficas. La Estrategia Técnica Mundial para la malaria 2016-2030, tiene como objetivo la reducción de la incidencia mundial de la malaria y de las tasas de mortalidad de al menos el 90 % en 2030, siguiendo el calendario de los Objetivos de Desarrollo Sostenible. Aunque se trata de un reto a largo plazo que requerirá el desarrollo de nuevas medidas de control, supone un cambio de paradigma necesario para acabar con esta enfermedad.

CLÍNICA

- Las características clínicas de la malaria varían ampliamente y pueden simular otro tipo de enfermedades, dependiendo de la especie del parásito presente, el estado de inmunidad del paciente, la intensidad de la infección y la presencia concomitante de otras enfermedades. En zonas donde la malaria se transmite de forma natural, apenas un 1-2 % de los episodios conllevan gravedad, debido a la progresiva adquisición (secundaria a la exposición continuada a las picaduras infectivas de repetición) de una inmunidad parcial hacia la enfermedad clínica. Sin embargo, en los casos de malaria "importada", debido a la ausencia de ningún tipo de inmunidad previa hacia la infección, todo caso es potencialmente grave y debe ser tratado como tal.
- De manera más específica, cada tipo de *Plasmodium* puede presentar una sintomatología más característica:

TABLA 1. Clínica: características diferenciales de la malaria según la especie infectante

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*	*P. knowlesi*
Período de incubación habitual (rango) en días	12 (9-14)	13 (12-17) o más	17 (16-18) o más	28 (18-40) o más	???
Duración de la enfermedad sin tratar	Con frecuencia mortal sin tratamiento	1,5-4 años	1,5-4 años	3-50 años	Pocos días, no recurre
Parasitemia media (por µL)	≥20.000	10.000	9.000	6.000	Potencialmente alta
Predilección por hematíes	Invaden cualquier tipo de hematíe	inmaduros	inmaduros	Maduros o viejos	Cualquier tipo
Gravedad del primer ataque	Grave en no inmunes	Leve a grave	Leve	Leve	Leve (7 % grave)
Periodicidad de los accesos febriles	48 h, si bien errático	48 h	48 h	72 h	24 h
Sensibilidad a cloroquina	Resistencias generalizadas	Focos variables de resistencia	Sensible	Sensible	Sensible
Malaria cerebral	Frecuente	Posiblemente	Nunca	Nunca	Nunca, a pesar de secuestro cerebral
Anemia	Grave y potencialmente mortal	Moderada a grave	Leve	Leve	Leve. Trombocitopenia

1. *P. falciparum:* es la especie que produce enfermedad más grave y con mayor morbi-mortalidad. Se asocia a diversas complicaciones que son descritas más adelante.
2. *P. vivax y ovale:* cuadro de fiebre con sintomatología más leve, que con frecuencia se acompaña de hiperesplenismo. Se relaciona con recaídas hasta 3-5 años tras la infección primaria (por presencia de hipnozoitos hepáticos). En los últimos años se han descrito episodios con morbilidad grave e incluso mortalidad asociada relacionados con el *P. vivax.*
3. *P. malariae:* en relación con parasitemia asintomática crónica.
4. *P. knowlesi:* la única zoonosis de todas las especies con potencial para causar malaria en humanos. Clínica similar a *P. falciparum* con elevada mortalidad y elevada parasitemia. Puede producir insuficiencia hepato-renal severa.

- Malaria no complicada:
  - Tras una picadura infectiva, se produce un período de incubación asintomático (mínimo 7-8 días, habitual entre 2-3 semanas, máximo varias semanas o incluso meses) tras el cual aparecen escalofríos intensos, fiebre alta, sudoración, cefaleas, mialgias, náuseas y, con menor frecuencia, dolor abdominal, vómitos, diarrea y sintomatología respiratoria. La esplenomegalia es frecuente, así como la plaquetopenia.
  - La sintomatología puede manifestarse de forma periódica, coincidiendo con la lisis cíclica de los eritrocitos infectados que tiene lugar al final de la fase sanguínea del ciclo, aproximadamente cada 48 horas.
- Malaria complicada o grave: producida principalmente por *P. falciparum,* aunque *P. vivax* y *P. knowlesi* pueden causar también cuadros graves. En niños, la malaria complicada puede clasificarse según 3 síndromes de gravedad bien definidos (aunque éstos no son mutuamente excluyentes):
  - *Anemia grave:* de etiología multifactorial que incluye: (a) lisis de hematíes infectados y no infectados durante la fase sanguínea del ciclo parasitario; (b) destrucción de eritrocitos parasitados en el bazo mediante fagocitosis; (c) inhibición de la eritropoyesis en la médula ósea. La anemia puede empeorar una vez iniciado el tratamiento por la destrucción de los eritrocitos infectados. A menudo requiere reposición de sangre total o concentrado de hematíes.
  - *Distrés respiratorio:* definido como retracción torácica y/o respiración profunda anormal. Esta alteración normalmente se produce debido a un cuadro de acidosis metabólica y a un incremento de las concentraciones plasmáticas de lactato, y no suele (aunque puede) implicar hipoxemia.
  - *Malaria cerebral:* se puede presentar hasta en un 10 % de los casos en las zonas endémicas y afecta predominantemente a niños entre 2 y 6 años. Tiene una mortalidad de 10-40 % con tratamiento y hasta un tercio de los supervivientes pueden tener secuelas. Suele tratarse de una encefalopatía simétrica y difusa cuya base fisiopatológica es multifactorial y en la que juega un papel muy importante el secuestro del *P. falciparum* y de hematíes (parasitados y no parasitados) a nivel de la microvasculatura cerebral. Suele presentarse en forma de:
    - Alteración del nivel de conciencia no atribuible a otras causas (descartada la hipoglucemia, el periodo post-ictal y meningitis/meningoencefalitis).
    - Coma que se prolonga >6h tras una convulsión generalizada.
    - Más de 2 convulsiones en un periodo menor a 24 h.

    Somnolencia, escala de coma de Glasgow <11 en adultos y niños mayores de 5 años o escala de coma de Blantyre <3 en niños pequeños.
  - Otras complicaciones posibles incluyen la postración, la hipoglucemia (con alta letalidad asociada), o el fallo de otros órganos (síndrome de distrés respiratorio y/o edema pulmonar, insuficiencia renal, insuficiencia hepática fallo multiorgánico, shock,), estos últimos menos frecuentes entre los niños en comparación con los adultos.
- Malaria congénita: es una complicación de la infección malárica de una madre gestante que transmite verticalmente

(intraútero o más frecuentemente intraparto) la infección a su bebé. Puede aparecer pocos días o semanas después del nacimiento y su diagnóstico diferencial incluye a la sepsis neonatal y otras enfermedades infecciosas del período neonatal. La clínica suele ser más larvada y atípica que la de la malaria, en el niño más mayor. Es imprescindible pensar en esta posibilidad diagnostica en caso de exposición de la madre durante el embarazo. El tratamiento debe ser siempre parenteral y bajo supervisión hospitalaria.

- Pronóstico:
  - *P. falciparum:*
    - Sin tratamiento, en un niño sin inmunidad previa, un episodio clínico de malaria puede ser rápidamente mortal. La tasa de mortalidad de la malaria grave oscila entre un 10 y un 20 %.
    - *P. falciparum* es además frecuentemente resistente a varios de los antimaláricos disponibles en la actualidad, lo que condiciona un mayor riesgo de fracaso terapéutico y de aparición de complicaciones.
    - Hasta un tercio de los episodios de malaria cerebral pueden tener secuelas neurológicas de mayor o menor calibre (ceguera cortical, trastornos cerebelares, paresias, trastornos de aprendizaje, epilepsia), aunque en muchos casos son autolimitadas y reversibles.
  - *P. ovale, malariae y vivax:* suelen ser relativamente benignos, aunque en los últimos años la especie *P. vivax* ha sido más frecuentemente implicada en complicaciones.
  - *P. knowlesi:* pueden ser graves o incluso letales, pero de momento su incidencia es prácticamente anecdótica y muy circunscrita geográficamente al sudeste asiático.

DIAGNÓSTICO

Existen diferentes opciones diagnósticas disponibles:

- Microscopía:
  - Técnica de referencia.
  - Identifica de las formas asexuadas intraeritrocitarias del parásito.
  - Permite la correcta identificación de las diferentes especies, así como la cuantificación de la magnitud de la infección y la evaluación de la respuesta al tratamiento.
- Tests rápidos (RDTs)
  - Técnicas inmunocromatográficas que detectan antígenos del parásito en sangre. Para el diagnóstico de *P. falciparum,* los antígenos detectados más comúnmente son enzimas o proteínas del parásito, como la proteína rica en histidina 2 (HRP2), la panlactato deshidrogenasa (pLDH) y la pan-aldolasa.
  - Actualmente, existen en el mercado tests rápidos que detectan exclusivamente *P. falciparum,* tests rápidos que discriminan adecuadamente entre *P. falciparum* y *P. vivax.*, tests rápidos que discriminan *P. falciparum* y otras especies no falciparum sin distinguirlas entre ellas y por último, tests que son capaces de detectar específicamente *P. falciparum, P. vivax, P. ovale and malariae.*
  - Sencillos, altamente sensibles y específicos. La OMS y Foundation for Innovative New Diagnostics (FIND) evalúan anualmente los tests rápidos disponibles en el mercado para monitorear su calidad y asegurar una alta sensibilidad y especificidad.
  - Inconveniente de no poder ser usados para evaluar la respuesta al tratamiento ya que los antígenos parasitarios son detectados durante algunos días o semanas después de la instauración de tratamiento adecuado, manteniéndose el resultado como positivo durante este periodo, aún en ausencia de infección activa.
  - También presentan problemas para detectar parasitemias inferiores a 200 parásitos/µL
- En centros de referencia pueden también usarse técnicas moleculares (PCR) especie-específicas mucho más sensibles.

PREVENCIÓN

Herramientas preventivas:

- Prevención contra el mosquito vector: Las medidas destinadas a eliminar al mosquito vector, o bien a limitar el contacto entre el humano y el mosquito son actualmente consideradas las mejores medidas preventivas contra la malaria. Algunos ejemplos son: el uso de redes impregnadas de insecticida (una de las mejores estrategias para el control de la enfermedad), la fumigación intradomicilairia con insecticidas de larga duración (incluido el DDT), la utilización de larvicidas, o el uso de repelentes de larga duración durante las horas nocturnas (cuando la hembra anofelina tiene predominio por picar).
- Prevención mediante el uso de fármacos:
  - Viajeros y cooperantes: Se recomienda que toda persona, y en especial los niños que viajen a zonas endémicas de malaria realicen quimioprofilaxis antipalúdica. Ésta deberá hacerse con el fármaco o combinación de fármacos más adecuado para la zona a visitar y que mejor se adapte a la idiosincrasia del viajero, y siguiendo las recomendaciones actuales de la OMS (consejos al viajero y mapa de riesgo actualizado disponibles en la siguiente página web: *http://www.who.int/malaria/travellers/en/)* o del CDC americano *(http://www.cdc.gov/malaria/travelers/index.html)*. El fármaco o combinación de fármacos deberán iniciarse unos días antes del viaje, con el objetivo de alcanzar buenos niveles en sangre al llegar al lugar de destino, y se prolongará en general hasta 1-4 semanas después del regreso, para cubrir el período de incubación de una posible picadura infectiva recibida en los últimos días del viaje. El consejo al viajero deberá ser realizado siempre por un especialista e incluir recomendaciones específicas sobre qué fármaco tomar, y las potenciales contraindicaciones. Cabe destacar que un grupo particularmente en riesgo son los inmigrantes que provienen de zona endémica y que ya han pasado una temporada larga en zona donde la malaria no se transmite. Estos pacientes, que debido a la exposición prolongada durante su primera infancia a las picaduras infectivas frecuentes en sus países de origen, han desarrollado un cierto grado de inmunidad parcial frente a la enfermedad, pueden perderla de forma rápida al desaparecer la exposición continuada, volviéndolos de nuevo vulnerables a la enfermedad grave o incluso potencialmente mortal. Será pues obligatorio dar un buen consejo a este tipo de viajeros que incluya la recomendación del uso de profilaxis antimalárica.
  - Tratamiento intermitente preventivo (IPT): se trata de innovadores esquemas preventivos de administración de varias pautas completas de tratamiento antimalárico separadas en el tiempo a grupos de riesgo. El tratamiento preventivo en mujeres embarazadas con sulfadoxina-pirimetamina (SP) es una recomendación implementada hace años en muchos de los países africanos con alta endemicidad para *P. falciparum,* y tiene repercusiones positivas tanto en la madre embarazada (39 % de reducción de anemia), como en el crecimiento fetal (incremento del peso del recién nacido >120gr) y riesgo de supervivencia inicial del recién nacido. Sin embargo, su uso es limitado en zonas de alta prevalencia de VIH, ya que el tratamiento con sulfadoxina-pirimetamina está contraindicado en aquellas mujeres embarazadas en tratamiento con cotrimoxazol. La OMS también recomienda la profilaxis intermitente en lactantes de zonas donde la transmisión de malaria es moderada-alta y la resistencia del *P. falciparum* a la sulfadoxina-pirimetamina es menor del 50 %. Existe también el tratamiento preventivo intermitente "estacional" para proteger a las poblaciones dónde la malaria es altamente estacional y la transmisión sólo ocurre durante unos pocos meses del año, donde se ha demostrado una reducción >80 % en episodios de malaria y casi un 60 % de reducción en todas las causas de muerte.

VACUNAS

Introducción

- Históricamente, las vacunas han sido uno de los mecanismos de control de las enfermedades infecciosas más efectivas y fáciles de administrar, sin embargo, la gran variabilidad antigénica que muestra el parásito de la malaria a lo largo de su ciclo vital ha dificultado enormemente el diseño de una vacuna eficaz. En las últimas décadas, una enorme inversión de recursos, esfuerzo e implicación por parte de la comunidad científica han sido realizados para poder conseguir una vacuna contra *P. falciparum* eficaz y segura. Después de múltiples ensayos pre-clínicos y clínicos en varios países africanos, una vacuna, la RTS,S se ha postulado como la candidata a formar parte de los programas de control de malaria.

- El desarrollo de una vacuna contra *P. vivax,* el segundo parásito que más enfermedad produce, está muy por detrás del desarrollo de vacunas frente a *P. falciparum,* sin ensayos clínicos recientes más allá de fase II.

- Más de 60 estudios pre-clínicos o clínicos han sido o están siendo desarrollados en torno al desarrollo de una vacuna anti-malárica. De estos, 60 estudios están focalizados en vacunas contra *P. falciparum* (30 vacunas pre-eritrocíticos, 17 vacunas eritrocíticas y 13 vacunas de esporozoítos) y un estudio contra *P. vivax*, *(http://www.who.int/immunization/research/development/Rainbow_tables/en/)*

- Aunque posiblemente no se consiga prevenir completamente la infección, las nuevas vacunas serán capaces de disminuir las consecuencias de una enfermedad responsable de una enorme morbi-mortalidad, predominantemente pediátrica.

Composición. Tipos de vacunas

- A la hora de plantear estrategias de diseño de vacunas de malaria, y debido a la ausencia de marcadores serológicos adecuados de inmunidad, sería aconsejable seleccionar antígenos candidatos clave del parásito, inmunogénicos y que ofrezcan la menor diversidad antigénica posible. Esta elección debe basarse en evidencias de que un antígeno juega un papel importante en la patogenicidad del parásito o de que las respuestas inmunes a un antígeno están asociadas a protección en estudios de inmunidad natural adquirida. Durante los últimos años ha quedado claro además, el valor añadido imprescindible que pueden aportar a las vacunas candidatas los potentes adyuvantes en las cuales están formuladas. La identificación de nuevos adyuvantes que sean seguros, eficaces y poco reactogénicos mejorará con certeza las posibilidades de los actuales antígenos candidatos. Diferentes clasificaciones para la vacuna contra la malaria han sido usadas por los diferentes autores.

- **TIPOS DE VACUNAS:** Las vacunas antimaláricas pueden ser diseñadas según diferentes estrategias:
    - Según la población diana: Los malariólogos afirman que pueden ser necesarias vacunas

diferentes para poblaciones diferentes. Una vacuna destinada a proteger a niños que vivan en una zona endémica es conceptualmente muy diferente de una vacuna destinada a proteger a personas sin ningún grado de inmunidad natural adquirida. En el primer caso, la vacuna no necesita proteger totalmente al vacunado, ya que su efecto se añadirá al de la inmunidad natural adquirida. Esta vacuna debería teóricamente imitar esta inmunidad apuntando a los antígenos de la parte asexuada del ciclo del parásito. Sin embargo, una vacuna ideada para proteger a un individuo no inmune (por ejemplo, un viajero) requeriría por el contrario garantizar una eficacia del 100 %, ya que debería neutralizar al parásito antes de que llegue al torrente sanguíneo y pueda causar sintomatología clínica.

- Según el estadio del ciclo vital parasitario contra el que se desee actuar: La complejidad del ciclo vital del Plasmodium implica la posibilidad de establecer diferentes dianas antigénicas para cada uno de los diferentes estadios de la vida parasitaria:
  - Las vacunas pre-eritrocíticas (VPE) están dirigidas contra los esporozoitos o los estadios del parasito intrahepáticos y su objetivo es impedir al parásito alcanzar el torrente sanguíneo e iniciar su estadio eritrocítico. Produce anticuerpos contra la proteína circumsporozoíto (CSP) del parásito.
  - Las vacunas eritrocíticas (VE) o de estadio sanguíneo están dirigidas contra los antígenos sanguíneos del ciclo vital del parásito. Incluyen proteínas de superficie del estadio hematógeno del parásito (merozoíto) que permitiría desarrollar inmunidad natural con menor riesgo de enfermedad. Su función es prevenir la sintomatología clínica al evitar la invasión de los hematíes por los merozoitos post-hepáticos, así como acelerar la destrucción de los hematíes parasitados y por lo tanto evitar su secuestro en la microvasculatura capilar. Este tipo de vacuna no interferiría con la infección, pero disminuiría la severidad de los síntomas.
  - Las vacunas bloqueadoras de la transmisión (VBT) no beneficiarían directamente al individuo vacunado sino que tendrían un efecto más amplio en su comunidad (efecto "altruista"), al bloquear la transmisión de la infección de individuo a individuo. Al estar dirigidas contra estadios sexuales del parásito (usando como antígenos diana aquellos expresados en el intestino del mosquito, y no en el humano) este tipo de vacunas prevendría la aparición de cepas mutantes. Teniendo en cuenta que el mosquito no posee mecanismos inmunes adaptativos, y que los genes del Plasmodium que codifican la parte del ciclo vital del parásito que ocurre en el mosquito se mantienen adecuadamente conservados, parece relativamente sencillo identificar antígenos diana. La combinación de una vacuna de este tipo con otra VPE o VE podría entonces evitar la aparición de mutantes inmunes potencialmente peligrosos.
- Una posible estrategia es combinar antígenos de diferentes estadios del ciclo vital (vacunas multiestadio) para conseguir despertar en el huésped una respuesta inmune intensa y encadenada, ya que

vacunas dirigidas a un estadio inicial de la infección que sean sólo parcialmente efectivas podrían no conseguir evitar la aparición de sintomatología clínica. De forma similar, se pueden combinar también varios antígenos de la misma fase (vacunas multivalentes) para evitar la emergencia de resistencias y aumentar la eficacia de la vacuna candidata. Sin embargo, la inclusión de componentes innecesarios puede aumentar los efectos indeseados y el coste, y de momento ninguna vacuna basada en estas estrategias ha demostrado eficacia e inmunogenicidad prometedoras.

Eficacia

- Idealmente, la vacuna perfecta contra la malaria sería una vacuna 100 % eficaz, barata, fácil de administrar, capaz de conferir una inmunidad duradera y que protegiera a los niños más pequeños. Desafortunadamente, es posible que una vacuna perfecta contra la malaria no sea factible a corto plazo.

Breve historia de la vacuna contra la malaria

- La búsqueda de una vacuna eficaz contra la malaria ha sido un objetivo de la comunidad científica durante muchos años. Los estudios con diferentes especies de malaria en roedores y aves se han estado realizando desde 1910. Sin embargo, los avances más significativos se han producido en los últimos 50 años. Los estudios realizados por Nussenzweig et al en los años 60 mostraron inmunidad protectora en roedores después de la inyección de esporozoitos irradiados. Posteriormente se identificó la proteína del circumsporozoito (CSP), una proteína de la superficie del esporozoito, objetivo importante para los anticuerpos. En los años setenta, Clyde et al. mostraron que era posible obtener la protección contra la infección en humanos, después de la exposición a las picaduras infectivas de múltiples esporozoitos irradiados. Desde la década de 1980, el foco principal ha sido identificar diferentes antígenos de superficie de los esporozoitos, objetivos potenciales de los anticuerpos monoclonales y policlonales. Algunos de ellos fueron candidatos para el desarrollo de vacunas, aunque no demostraron protección significativa. En la década de 1980, investigadores de América del Sur desarrollaron una vacuna a base de péptidos de la fase sanguínea asexual (SPf66), que parecía ser eficaz en monos y en humanos. Sin embargo, estudios posteriores llevados a cabo en diferentes países endémicos de malaria, no confirmaron esos primeros resultados prometedores. De forma simultánea, otros grupos iniciaron nuevos estudios utilizando diferentes péptidos después de la secuenciación de epítopos de anticuerpos protectores dirigidos a la región central de CSP.15 de *P. falciparum.* Estos estudios proporcionaron los mejores resultados en los ensayos preclínicos obtenidos hasta la fecha, utilizando el antígeno de superficie del virus de la hepatitis B, siendo el producto candidato denominado RTS,S. Esta formulación se probó con diferentes adyuvantes innovadores, AS04, AS03 , AS02 y AS01, mostrando este último mayor protección en un estudio multicéntrico en fase III, en diferentes lugares de África. Este ensayo a gran escala ha mostrado protección de hasta aproximadamente el 50 % de episodios de malaria clínica y malaria grave, evaluado a los 14 meses después de la última dosis administrada de vacuna en un amplio grupo de niños de entre 5 y 17 meses. En el grupo de bebés africanos vacunados con la vacuna RTS,S/AS01 de entre 6 y 12 se-

manas, se redujeron las tasas de malaria clínica y complicada en un 31,3 y 36,6 %, respectivamente, durante un periodo de seguimiento de 12 meses. Aunque la efectividad fue inferior a lo inicialmente esperado, es la vacuna más avanzada y con mayor efectividad y la única que ha completado la fase III de evaluación en países endémicos hasta la fecha. Un ensayo clínico en fase II en Gabón, Ghana y Tanzania ha evaluado la administración de la RTS,S/AS01 junto con el resto de vacunas del programa expandido de inmunización (EPI) en dos esquemas diferentes de tratamiento (0, 1 y 2 meses, junto a la administración de DTwP/ HepB/Hib+OPV ; 0,1 y 7 meses, última dosis junto a la vacunación de sarampión a los 9 meses). Este estudio no encontró diferencias en la eficacia de la vacuna RTS,S en los dos esquemas de administración en el primer año después de la última dosis. La Agencia Europea del medicamento evaluó positivamente esta vacuna en Julio 2015 y la OMS anunció en Noviembre 2016 la implementación piloto y a gran escala de dicha vacuna en 3 países del África sub-sahariana. El objetivo de esta implementación piloto será evaluar el efecto protector de la vacuna administrada en niños entre 5 y 9 meses, la factibilidad de la administración de 4 dosis, el impacto en la mortalidad infantil y la seguridad en su uso rutinario en países endémicos.

- En paralelo al desarrollo de las primeras vacunas RTS,S, diferentes estudios evaluaron la respuesta a otras vacunas candidatas basadas en diferentes péptidos y proteínas de la fase sanguínea asexual del parásito, como el antígeno de membrana apical 1 (AMA1), o las proteínas de superficie del merozoito (MSP)1, MSP2, MSP3, entre otros. Ninguna de estas vacunas han mostrado, sin embargo, la protección clínica que ha mostrado la vacuna RTS, S. Al final de la década de los 90, se idearon nuevas vacunas con el objetivo de mejorar la respuesta inmunitaria celular, aunque tampoco demostraron ser eficaces en humanos.

- Otro enfoque en el desarrollo de una vacuna eficaz, consistente en la inoculación de esporozoítos de *P. falciparum* irradiados y por tanto atenuados, está resultando prometedor en los últimos años debido a su alta efectividad. Aunque los primeros estudios basados en este tipo de vacunas se desarrollaron en los años 70 y 80, la complejidad de su administración mediante picaduras de mosquitos, la necesidad de un laboratorio adecuado para la preservación de los mosquitos y la necesidad de múltiples inoculaciones, han hecho que durante décadas, no se haya postulado como una vacuna candidata en países endémicos. Seder et al ha demostrado que la vacuna Sanaria PfSPZ, compuesta por esporozoítos irradiados atenuados, aséptica, purificada y criopreservada, es segura, bien tolerada y fácilmente administrada mediante jeringa usando una variedad de vías y puede inducir una eficacia protectora del 100 % frente a malaria cuando se administra intravenosamente, siendo dosis dependiente. Este tipo de vacuna podría servir como modelo para la inmunidad protectora de alto grado y de resistencia cruzada en animales y seres humanos.
- Los últimos datos sobre esta vacuna parecen mostrar una protección duradera de su efecto. Sin embargo, la aplicabilidad en el terreno de una vacuna basada en un extracto de esporozoítos criopreservados parece por el momento cuanto menos limitada.

Seguridad

Las principales características deseables para una vacuna contra la malaria deben incluir un buen perfil de seguridad, junto con la alta eficacia contra la infección malárica o la enfermedad clínica.

- Las vacunas en ensayos clínicos probadas hasta ahora han mostrado un aceptable perfil de seguridad.
- En el último ensayo clínico con resultados prometedores respecto a eficacia, la vacuna RTS,S/AS01 presentó un desequilibrio en los casos notificados de meningitis, sobretodo en el grupo de edad de 5-17 meses. La relación temporal con la vacunación fue poco clara, y la plausibilidad biológica, baja. La relación causal entre la vacunación y estos casos no puede sin embargo excluirse sin más datos.
- Efectos adversos locales, fiebre o convulsiones febriles fueron similares entre los grupos vacunados y los grupos control.

Indicaciones

El objetivo ideal para una vacuna contra la malaria sería prevenir la enfermedad clínica, los episodios de malaria grave y la transmisión en la comunidad.

Para ello, las poblaciones diana indicadas para ser vacunadas deberían incluir:

- Niños pequeños que viven en zonas endémicas.
- Mujeres embarazadas.
- Personas con inmunodeficiencias u otras comorbilidades
- Viajeros de zonas no endémicas.

Pautas

Se han estudiado diferentes pautas de vacunación en los ensayos clínicos realizados hasta la actualidad. Los resultados más prometedores de los ensayos realizados en fase IIb o fase III, utilizaron las siguientes pautas:

- RTS,S/AS02D (Aponte et al): niños de 10 semanas, pauta de tres dosis a las 10, 14 y 18 semanas de vida.
- RTS,S/AS02D: niños de 8 semanas, pauta de tres dosis a las 8, 12 y 16 semanas de vida.
- RTS,S/AS01 (grupo RTS,S): dos grupos de edad:
    - Grupo de niños de entre 5 y 17 meses de vida: pautas de tres dosis a los 0, 1 y 2 meses desde la primera dosis y booster a los 20 meses.

- Grupo de niños de entre 6 y 12 semanas de vida: pautas de tres dosis a los 0, 1 y 2 meses desde la primera dosis y booster a los 20 meses.

Contraindicaciones

No establecidas en la actualidad.

Marcas

Tal y como he mencionado anteriormente, no existen aún marcas comercializadas de vacunas antimaláricas. La vacuna candidata más avanzada en el proceso de registro es la RTS,S cuyo nombre comercial será "Mosquirix". Existen al menos un centenar más de prototipos de vacuna en varias fases de desarrollo, pero muy pocas de estas moléculas completarán el muy costoso y largo proceso de desarrollo clínico necesario para el registro, siendo la RTS,S la más que probable primera vacuna antimalárica que se comercialice y por tanto, el estandarte frente al cual deberán compararse las futuras vacunas.

Tratamiento

- La malaria es una enfermedad potencialmente letal por lo que su tratamiento es una urgencia y está justificado en caso de sospecha aún en ausencia de confirmación parasitológica.

- El tratamiento de soporte es tan importante como el tratamiento específico antimalárico en los casos de malaria grave.

- Bases del tratamiento específico antimalárico **(Tabla III).**
  - Debe incluir el uso combinado de 2 o más fármacos.
  - Las combinaciones basadas en derivados de las artemisininas (ACTs) son actualmente la primera línea de tratamiento a nivel global para *P. falciparum,* y en algunos casos también para *P. vivax,* debido al aumento de la resistencia por parte del parásito a los fármacos que venían siendo usados de forma habitual (cloroquina, sulfadoxina-pirimetamina, amodiaquina etc…).
  - En casos de malaria por *P. vivax* o *P. ovale,* debe realizarse tratamiento radical con primaquina (excepto en casos de transmisión vertical), debido a la capacidad de formar hipnozoitos silentes en el hígado con riesgo de recidiva semanas o meses más tarde (la periodicidad de las recidivas dependiendo de la procedencia geográfica de las cepas).

- El tratamiento de soporte es fundamental como complemento al tratamiento específico de la infección, y debe incluir un adecuado manejo hidroelectrolítico, anticonvulsivantes para tratar los episodios convulsivos, antibióticos en caso de sospecha de sobreinfección bacteriana o shock, y transfusiones de sangre o hemoderivados en caso de anemia

TABLA III. Tratamiento farmacológico

Malaria no complicada	
Fármaco	**Comentarios**
P. falciparum sensible a cloroquina, P. malariae y P. knowlesi	
Cloroquina	25 mg/kg de cloroquina base repartida en 3 días (10 mg/kg 1era dosis, seguida de 5 mg/kg a las 12, 24 y 48 h). Comprimidos de 250 mg sal equivalen a 150 mg base (en adulto medio 4-2-2-2 comp.).
P. falciparum resistente a cloroquina	
Quinina	10 mg sal/kg/8 h (Cápsulas de 300 mg de sal de quinina) durante 7 días, complementar con un segundo fármaco (doxiciclina 100 mg/12-24 h, tetraciclina 250-500 mg/6 h, clindamicina 150-450 mg/6-8 h ó sulfadoxina-pirimetamina).
Artesunato	Nunca en monoterapia. Dosis 4 mg/kg/día, 3 días. Co-formulado con amodiaquina, o con mefloquina. Co-administrado por separado con Sulfadoxina-pirimetamina o mefloquina.
Arteméter-Lumefantrina	Co-formulado en comprimidos de 20 mg arteméter y 120 mg de lumefantrina. Dosis total en adultos (o niños >34 kg o >14 años) de 24 comprimidos (4 por dosis) a las 0, 8, 24, 36, 48 y 60 h. Dosis pediátricas: 1 comp/dosis si <3 años (5-14 kg); 2 comp/dosis (15-24 kg; 3-8 años); 3 comp/dosis (25-34 kg; 9-14 años). Administrar con comida grasa.
Dihidroartemisinina-piperaquina	Co-formulado en comprimidos de 40 mg (DHA) y 320 mg (PQP). Dosis: 2.25 mg/kg (DHA) y 18 mg/kg (PQP)/24 h/3 días (4 comp/día).
Atovacuona-proguanil	Comprimidos de adulto: 250 mg atovacuona y 100 mg proguanil. (comp. pediátricos 62.5 mg atovacuona; 25 mg proguanil). Dosis total de 1000/400 mg/día durante 3 días (4 comp. en adultos/día). Niños: 5-8 kg: 2 comp. pediátricos/día/3 días; 9-10 kg: 3 comp. pediátricos/día/3 días; 11-20 kg: 1 comp. adulto/día/3 días; 21-30 kg: 2 comp adulto/día/3 días; 31-40 kg: 3 comp. adulto/día/3 días; >40 kg: igual que adultos.
Mefloquina	Comprimidos de 250 mg. Adulto: Dosis total de 1250 mg (5 comp) en dosis única o repartida en 2-3 tomas (cada 6-8 h). Niños 25 mg/kg (máx. 750 mg) en dos dosis separadas por 6-8 h. Sensibilidad reducida en sudeste asiático. Puede combinarse con sulfadoxina-pirimetamina o artesunato.
Sulfadoxina-Pirimetamina	Sulfadoxina 25 mg/kg + Pirimetamina 1.25 mg/kg; dosis única (generalmente 4 comprimidos en el adulto). Sensibilidad parasitaria cada vez más reducida, por lo que cada vez menos usado.
P. vivax y P. ovale	
Cloroquina con Primaquina	Misma pauta de cloroquina que para *P. falciparum,* excepto en zonas de alta resistencia a cloroquina en las que se usarán ACTs. Debe realizarse siempre cura radical de los hipnozoitos hepáticos con fosfato de primaquina (15 mg/día ó 0.3 mg base/kg/día durante 14 días, o en zonas de tolerancia a la primaquina 30 mg/día/14 d). Comprimidos de 15 mg sal que equivalen a 7.5 mg base. La primaquina está contraindicada en déficits severos de G6PD (deben tratarse las recidivas cuando aparezcan), pero puede pautarse de forma semanal en déficits leves-moderados (45-60 mg/dosis única semanal, durante 8 semanas).
Malaria complicada (Generalmente *P. Falciparum,* ocasionalmente *P. vivax* o *P. knowlesi*)	
Fármaco	**Comentarios**
Artesunato	Tratamiento de elección si disponible. Dosis: 2.4 mg/kg (i.v o i.m) a las 0,12, 24 h, seguido de 1 dosis cada 24 h hasta que sea posible pasar a vía oral. Deberá complementarse siempre con una dosis completa de otro antimalárico (ver tratamiento de malaria no complicada en "A").
Arteméter	Por vía i.m, 3.2 mg/kg dosis de carga, seguida de 1.6 mg/kg/12-24 h hasta que sea posible pasar a vía oral. Complementar siempre con un segundo antimalárico oral.

Quinina	20 mg/kg (dosis de carga) seguida de 10 mg/kg/8 h hasta que se pueda pasar a vía oral y completar 7 días. Diluir en 10 mL/kg SG5 % para infusión parenteral lenta (4 h dosis de carga, 2 horas dosis siguientes). Puede usarse vía i.m, rectal o por SNG, pero entonces no es necesaria dosis de carga. Suele administrarse junto a un segundo fármaco (clindamicina o doxiciclina en pacientes >8 años), pero en caso de ser usado como monoterapia, deberá complementarse siempre una vez se tolere la vía oral con una dosis completa de otro antimalárico (ver "A")
Clindamicina	Siempre como tratamiento complementario (en general de quinina). Por vía i.m o i.v, 25-40 mg/kg/día en 3-4 dosis/5 días, diluido en SF o SG5 %
Doxiclina	En niños >8 años de edad. Siempre como tratamiento complementario (en general de quinina). Por vía i.m o i.v 2-4 mg/kg/día/7 días en 1-2 dosis, diluido en SF o SG5 % (proteger de la luz si administrado en infusión)

PREGUNTAS

1. ¿Dónde se concentran la mayorías de los casos de malaria?

Aunque la malaria afecta a la mitad de países del mundo, hasta dos terceras partes de los casos se concentran en únicamente 7 países: República democrática del Congo, Etiopía, Kenia, Nigeria, Sudán, Tanzania y Uganda. El 85 % de los aproximadamente 212 millones de casos anuales en el mundo, y lo que es más importante, hasta un 90 % de las 429.000 muertes anuales causadas por esta enfermedad ocurren en África.

(World Health Organization. The world malaria report 2016. Ginebra; 2016)

2. ¿Qué factores han influido en el descenso total de casos de malaria observados en la última década en África?

Este descenso se ha atribuido principalmente al impacto de la implementación a gran escala de diversas estrategias de control, como las redes mosquiteras impregnadas de insecticida o el cambio de fármacos obsoletos a nuevos fármacos más eficaces, al mismo tiempo que una inyección sostenida de fondos internacionales destinados al control de la enfermedad. En 2015, la financiación total para el control y eliminación del paludismo fue aproximadamente de US$ 2,9 mil millones, US$ 60 millones más que en 2010. Esta cantidad no representa más que el 46 % de la meta fijada por la Estrategia técnica mundial para la malaria 2016-2030. US$ 6,4 mil millones para el 2020.

(World Health Organization: World malaria report 2016. Geneva: WHO; 2016)

3. ¿La infección por *P. vivax* puede considerarse un proceso benigno?

Aunque históricamente se ha asociado a cuadros más leves, recientemente se ha confirmado que *P. vivax,* también puede causar episodios graves o incluso letales (anemia, síndrome de dificultad respiratoria aguda (SDRA), edema agudo de pulmón, convulsiones y coma, insuficiencia renal aguda, rotura esplénica, hemorragias), a pesar de que las complicaciones ocurran con mucho menor frecuencia que en las infecciones por *P. falciparum.* Aunque los mecanismos fisiopatológicos son mal conocidos, se postula un rol importante de la respuesta inflamatoria post-infección.

4. ¿El cuadro clínico producido por la malaria es altamente específico?

No, de hecho la sintomatología de la malaria es con frecuencia difícil de diferenciar de otras infecciones comunes pediátricas.

5. ¿Qué relación existe entre la parasitemia y el cuadro clínico del paciente?

En general, cuanto mayor es la parasitemia, mayor es el riesgo de complicaciones. Sin embargo, en áreas de alta transmisión y como consecuencia del desarrollo de una inmunidad parcial frente a los efectos de la infección, pueden ser bien toleradas parasitemias muy altas. Debido al secuestro en la microvasculatura, la parasitemia periférica puede ser una subestimación importante de la masa parasitaria total, y por tanto hay que ser cautos a la hora de evaluar la gravedad en base a este parámetro.

6. ¿Qué criterios clínicos orientan a un mal pronóstico en la malaria grave?

- Coma (GCS≤8; en niños BCS≤ 2), ≥ 3 convulsiones en 24h.
- Taquipnea, Respiración profunda (Kussmaul) debida a acidosis metabólica, Tensión arterial sistólica <80mm Hg (<50mm Hg en niños) a pesar de infusión de volumen.
- Hemorragias retinianas, retinopatía malárica.

7. ¿Cómo se define anemia grave en el contexto de una malaria en zona endémica?

Hematocrito <15 % o bien hemoglobina<5g/dL. Este umbral es mucho menor del que habitualmente se consideraría como grave en zona no endémica.

8. ¿A qué se debe la hipoglucemia frecuentemente asociada a los casos de malaria grave?

Se define hipoglucemia a valores de glucosa en sangre<45mg/dL. Secundaria a alteraciones en la neoglucogénesis, déficits de aporte externo, y aumento de los requerimientos. La quinina puede empeorarla por su efecto hiperinsulinemiante. Especialmente problemática en niños y embarazadas.

9. ¿Cuál es la recomendación actual de la OMS para el tratamiento de la Malaria no complicada por *P. falciparum*?

Desde el año 2004, la OMS recomienda como tratamiento de elección para la malaria no complicada por *P. falciparum* las terapias combinadas con artemisininas (ACTs). Las artemisininas, derivadas de la planta milenaria china artemisia annua, son en la actualidad el tratamiento más efectivo desarrollado hasta la fecha, actuando de forma más rápida que cualquier otro antimalárico, reduciendo la parasitemia en pocas horas, y tienen un perfil de seguridad excelente. Aunque la aparición de resistencias en esta familia farmacológica ha sido más paulatino, la suceptibilidad a los derivados de artemisinina se ha visto reducida en algunas zonas del Sudeste Asiático.

(*Sinclair D, Zani B, Donegan S, Olliaro P, Garner P. Artemisinin-based combination therapy for treating uncomplicated malaria. Cochrane Database Syst Rev 2009:CD007483.

*Zhou LJ, Xia J, Wei HX, Liu XJ, Peng HJ: Risk of drug resistance in Plasmodium falciparum malaria therapy-a systematic review and meta-analysis. Parasitol Res 2016.)

10. ¿Cuál ha demostrado ser mejor tratamiento en los casos de malaria grave?

El artesunato parenteral, complementado una vez mejore el paciente con un ACT por via oral han demostrado una mayor eficacia en el tratamiento de la malaria grave, disminuyendo de forma significativa la mortalidad asociada a malaria grave, tanto en niños como en adultos.

(Dondorp AM, Fanello CI, Hendriksen IC, et al. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet 2010;376:1647-57)

11. ¿En qué condiciones pueden usarse los ACTs para el tratamiento de *P. vivax?*

Cuando se asocien con un fármaco hipnozoiticida (cura radical), para evitar las recidivas secundarias a los estadios durmientes intrahepáticos. El único fármaco actualmente disponible con este perfil es la primaquina, contraindicado en las embarazadas y en aquellos pacientes con déficits graves del enzima G6PDH debido a sus peligrosos efectos secundarios hemolíticos. La primaquina se administra al mismo tiempo que los otros fármacos antimaláricos, y durante 14 días consecutivos.

(Baird JK, Hoffman SL. Primaquine therapy for malaria. Clin Infect Dis 2004;39:1336-45).

12. ¿Qué ACTs hay disponibles en países endémicos?

Existen pocas marcas de ACTs disponibles y son producidas bajo un estricto proceso de manufactura. Entre ellas se encuentran las combinaciones de: artemether-lumefantrina, dihidroartemisinina-piperaquina, artesunato-mefloquina (usado principalmente en el Sudeste Asiático) o artesunato-amodiaquina. La elección de uno u otro dependerá de la disponibilidad en cada zona, y de las características de sensibilidad del parásito de esa zona a los componentes de cada combinación.

(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).

13. ¿Cuál es el tratamiento de elección para la malaria en el embarazo?

La quinina suele ser el fármaco de elección en caso de malaria sintomática. Tanto ésta, como la cloroquina, proguanil, clindamicina y sulfadoxina-pirimetamina son considerados fármacos seguros durante todo el embarazo. Los derivados de las artemisininas y mefloquina se consideran seguros durante el 2.º y 3er trimestre. No hay datos respecto a atovacuona-proguanil y están contraindicadas primaquina y tetraciclinas en relación a los efectos secundarios que pueden causar en el feto.

(Coll O, Menendez C, Botet F, et al. Treatment and prevention of malaria in pregnancy and newborn. Journal of perinatal medicine 2008;36:15-29).

14. ¿Qué rol tiene los corticoides en el tratamiento de la malaria grave?

Los corticoides no han demostrado su utilidad en el manejo de la malaria grave.

15. ¿Qué medidas preventivas contra la malaria son recomendadas actualmente?

Los métodos preventivos disponibles actualmente se basan en el control vectorial, e incluyen:

- El uso de redes mosquiteras impregnadas de insecticida de larga duración.
- El rociamiento intradomiciliario con insecticidas en el interior de las casas.
- El uso de larvicidas y otras medidas medioambientales para evitar la proliferación de mosquitos en aguas estancadas.

Otras medidas preventivas consisten en el uso de fármacos en un concepto llamado tratamiento preventivo intermitente o la administración única masiva de antimaláricos.

16. ¿En qué consiste el tratamiento preventivo intermitente?

Utiliza los contactos habituales de lactantes o embarazadas con el sistema sanitario para la distribución y administración de dosis curativas de un antimalárico.

17. ¿Qué eficacia tiene el tratamiento preventivo intermitente en la infancia (IPTi)?

Reduce en un 30 % los episodios de malaria clínica en quienes lo reciben.

18. ¿Qué medidas debe adoptar un viajero de Senegal que quiere regresar después de 2 años fuera de su país a casa por vacaciones?

Uso de adecuada profilaxis antimalárica.

19. ¿Si esta misma persona regresa definitivamente a su país, deberá continuar una pauta de profilaxis?

La profilaxis continuada es una estrategia sin sentido en las áreas endémicas, ya que es logísticamente inviable, expone al fármaco utilizado al rápido desarrollo de resistencias, y condiciona invariablemente interferencias en el desarrollo de la inmunidad adquirida frente a esta infección, el único mecanismo protector natural frente a los riesgos de la enfermedad.

20. ¿Por qué es necesaria una vacuna de malaria?

La enorme inversión de recursos e implementación de medidas de control de la malaria, ha conseguido una reducción drástica de la incidencia y casos de muerte en los últimos 15 años. Sin embargo, la carga de enfermedad continúa siendo intolerablemente alta en el mundo y ha de ser afrontada desde un punto de vista multidisciplinario. La vacunación de niños pequeños de áreas endémicas con una vacuna antimalárica eficaz y segura, combinado con el uso de otras medidas de control, podría contribuir de forma decisiva a que la malaria deje de ser una penosa carga para la salud mundial y al desarrollo económico y social de muchos países.

21. ¿Por qué no existe todavía una vacuna comercializada contra la malaria?

Nuestro conocimiento sobre la inmunidad que se desarrolla contra la malaria es muy limitado e incom-

pleto. La gran variabilidad antigénica que muestra el parásito de la malaria a lo largo de su ciclo vital ha dificultado enormemente el diseño de una vacuna eficaz. No se ha encontrado todavía la correlación esperada entre inmunidad clínica o parasitológica y nivel de respuesta inmunitaria a ningún antígeno. Sin embargo, la Agencia Europea del Medicamento y la OMS han evaluado de forma positiva la RTS,S/AS01 y se iniciará una implementación piloto en varios países africanos en los próximos años en el que se evaluará el efecto protector de la vacuna en su uso rutinario.

22. ¿Por qué hay confianza en que una vacuna pueda funcionar?

Un modelo a seguir para conseguir este objetivo es la llamada "inmunidad natural adquirida" (INA), que se desarrolla en los individuos que residen de forma permanente en zonas de endemicidad malárica. Estos individuos adquieren de manera progresiva una inmunidad parcial, que consiste en una protección inicial contra las formas más graves de la enfermedad (muerte y formas severas) y posteriormente una menor incidencia de casos clínicos con una eventual supresión de la parasitemia a niveles bajos o incluso indetectables. Esta protección requiere un efecto recuerdo (booster) continuado, y nunca confiere una inmunidad esterilizante, ya que los individuos pueden seguir infectándose a pesar de no desarrollar la enfermedad clínica. Si se pudiera reproducir este modelo, es decir si se consiguiera acelerar mediante una vacuna la transición de individuo "virgen" a individuo clínicamente inmune, conferiríamos al receptor una protección sólida contra la enfermedad malárica.

23. ¿Una vacuna contra la malaria destinada a proteger a niños que vivan en una zona endémica serviría también para un turista español que quiera viajar a una zona de alta endemicidad malárica?

No. Una vacuna destinada a proteger a niños que vivan en una zona endémica es conceptualmente muy diferente de una vacuna destinada a proteger a personas sin ningún grado de inmunidad natural adquirida. En el primer caso, la vacuna no necesita proteger totalmente al vacunado, ya que su efecto se añadirá al de la inmunidad natural adquirida. Esta vacuna debería teóricamente imitar esta inmunidad apuntando a los antígenos de la parte asexuada del ciclo del parásito. Sin embargo, una vacuna ideada para proteger a un individuo no inmune (por ejemplo, un viajero) requeriría por el contrario garantizar una eficacia del 100 %, ya que debería neutralizar al parásito antes de que llegue al torrente sanguíneo y pueda causar sintomatología clínica.

24. ¿Con qué problemas se enfrentan los malariólogos a la hora de diseñar una vacuna?

Una mezcla de todos los siguientes:

- La enorme variabilidad antigénica del parásito.
- La escasa respuesta inmune inducida por ciertos antígenos.
- Una respuesta inmune que decae con el tiempo.
- Las dificultades a la hora de ensayar una vacuna en el terreno.

25. ¿Una vacuna de bloqueo de transmisión podría ser útil para erradicar la infección en una comunidad pequeña, como por ejemplo una isla?

Sí. Las vacunas bloqueadoras de la transmisión (vacunas "altruistas" o VBT) no beneficiarían directamente al individuo vacunado sino que tendrían un efecto más amplio en su comunidad, al bloquear la transmisión de la infección de individuo a individuo. Al estar dirigidas contra estadios sexuales del parásito (usando como antígenos diana aquellos expresados en el intestino del mosquito, y no en el humano) este tipo de vacunas prevendría la aparición de cepas mutantes.

26. ¿La vacuna RTS,S protege de forma completa frente a los episodios de malaria grave?

No. La vacuna RTS,S ha mostrado protección de hasta aproximadamente el 50% de episodios de malaria clínica y malaria grave, evaluado a los 14 meses después de la última dosis administrada de vacuna en un ensayo clínico en fase III en niños de entre 5 y 17 meses. En el grupo de bebés africanos vacunados con la vacuna RTS,S/AS01 de entre 6 y 12 semanas, se redujeron las tasas de malaria clínica y grave en un 31,3 y 36,6 %, respectivamente, durante un periodo de seguimiento de 12 meses.

(Grupo RTS,S. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet 2015, 386:31-45).

27. ¿Una vacuna eritrocítica parcialmente efectiva podría actuar disminuyendo la morbilidad y mortalidad?

Sí. Las vacunas eritrocíticas o de estadío sanguíneo (VE) están dirigidas contra los antígenos sanguíneos del ciclo vital del parásito. Su función sería prevenir la sintomatología clínica al evitar la invasión de los hematíes por los merozoitos post-hepáticos, así como acelerar la destrucción de los hematíes parasitados y por lo tanto evitar así su secuestro en la microvasculatura capilar. Este tipo de vacuna no interferiría con la infección, pero disminuiría la gravedad de los síntomas.

28. ¿La vacuna RTS,S está indicada para viajeros y mujeres embarazadas?

No. Esta vacuna nunca ha sido experimentada en mujeres embarazadas, aunque sería un grupo poblacional vulnerable y deseable para ser vacunado. Los viajeros a países endémicos necesitarían una vacuna con un 100 % de eficacia, por no tener la inmunidad adquirida que tienen las personas que viven en áreas endémicas.

29. ¿Cuál es la edad ideal para iniciar la vacunación contra la malaria?

La vacuna contra la malaria idealmente debería administrarse en los primeros meses de vida, sin interferir demasiado con el calendario vacunal nacional. De esta forma se protegería contra malaria clínica y grave al grupo etario más afectado por esta enfermedad.

30. ¿Qué anticuerpos deben medirse para evaluar la eficacia en un ensayo clínico de una vacuna de malaria?

Por desgracia, no existe un buen correlato de protección medible en el suero de los pacientes vacunados. Por ese motivo, los ensayos de vacunas de malaria son tan laboriosos y complejos de realizar, ya que requieren para la correcta evaluación de la eficacia comparar la incidencia de infección o enfermedad malárica en el grupo que ha recibido la vacuna y en el grupo control a lo largo de un periodo definido de tiempo.

31. ¿Qué tratamientos se han demostrado efectivos como terapia adyuvante en el tratamiento de la malaria grave?

Desgraciadamente, hasta el día de hoy se han realizado diferentes ensayos clínicos con diferentes fármacos que no han demostrado ser capaces como terapia adyuvante a los antimalaricos de mejorar el pronóstico y la mortalidad de los casos de malaria grave en niños.

32. ¿Pueden ser usados los RDTs para evaluar la respuesta al tratamiento?

No, ya que los antígenos parasitarios son detectados durante algunos días o semanas después del tratamiento adecuado, manteniéndose el resultado positivo durante este periodo aunque la parasitemia esté en descenso.

33. ¿Cuáles son las recomendaciones actuales de la OMS en torno a la vacuna RTS,S/AS01?

En Enero de 2016 la OMS se pronunció oficialmente sobre el uso de la vacuna RTS,S/AS01 recomendando la implementación piloto de la vacuna en 3 países sub-saharianos, con un régimen de 3 dosis para niño entre 5 y 9 meses de edad y con una cuarta dosis entre 15 y 18 meses después. En noviembre de 2016 la OMS aseguro el financiamiento de la primera fase de estos ensayos pilotos (2017-20121) que deberían comenzar en el año 2018.

(WHO: Malaria vaccine: WHO position paper. In *Weekly Epidemiological Report,* WHO, 2016, 91, 33-52)

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