Utilising volunteer infection studies in the characterisation of anaemia in early malaria

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Abstract

The rapid emergence and spread of antimalarial resistance, especially artemisinin resistance in *Plasmodium falciparum*, has led to the use of volunteer infection studies (VIS) in the drug development pipeline. This thesis aims to characterise malaria-associated anaemia using the data from an induced blood stage model (IBSM trial), pooled analysis of existing haematology data and the investigation of the iron-infection axis. Investigation of the iron-infection axis has not been conducted previously in VIS studies.

First, the haematology and parasite dataset were expanded with the execution of two IBSM VIS, one using a new bioreactor manufactured malaria cell bank (MCB) and the other a historical MCB. The clinical studies identified that both MCB were safe to use in malaria-naïve, healthy participants, and that the parasite growth and clearance profiles of the new biomanufactured MCB were comparable to the existing *P. falciparum* MCB used.

Secondly, the haematology and parasite data from 315 participants inoculated with either *P. falciparum* (n=269) or *P. vivax* (n=46) were interrogated in a pooled analysis. The effects of age, sex, drug and parasitaemia and recrudescence were evaluated against the fractional fall in haemoglobin, haemoglobin nadir and the reticulocyte response. The fractional fall in haemoglobin, attributable to malaria was ~4% in *P. falciparum* and ~5% in *P. vivax* after correction for phlebotomy; the contribution of parasitised erythrocytes to the malaria-attributable losses was less than 1% in both species.

Lastly, malaria has recently been described as a cause of iron deficiency, which is a significant public health threat in malaria endemic regions. An exploratory study to characterise the markers of iron metabolism in early malaria was conducted in retrospective samples from previous IBSM VIS. The iron-axis was characterised in *P. vivax* and artemisinin-resistant K13 strain of *P. falciparum*, as well as fully drug-sensitive *P. falciparum*. Those inoculated with *P. falciparum* had depletion of their body iron stores, with a ~23% reduction in their log ferritin/soluble transferrin receptor index. Reduced baseline iron stores were associated with a reduced reticulocyte response ($r=0.39$, $p=0.015$) in those inoculated with *P. falciparum*. Finally, in those inoculated with *P. falciparum*, increased iron stores were associated with increased post-treatment liver transaminases (ALT-$r=0.54$, $p<0.001$; AST-$r=0.31$, $p=0.047$).

These studies have characterised malaria-attributable malaria in experimental and low-level malaria and have extended the existing information on iron-axis in early *P. falciparum* infection, as well a possible mechanism for elevations in liver transaminases in malaria VIS.

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Declaration

Apart from the assistance as outlined in the acknowledgements above, the work described is my own and has not been submitted for a degree or other qualification to this or any other university.

Table of Contents

List of abbreviations

Abbreviation	Definition
ACT	Artemisinin combination therapy
ALT	Alanine aminotransferase
Anti-PS	Anti-phosphatidylserine
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
AUC	Area under the curve
BMGF	Bill and Melinda Gates Foundation
BMI	Body mass index
BWF	Blackwater fever
CI	Confidence interval
Co-I	Co-investigator
CRP	C-reactive protein
CTCAE	Common terminology criteria for adverse events
CTU	Clinical trials unit
DBP	Diastolic blood pressure
DIC	Disseminated intravascular coagulopathy
DILI	Drug induced liver injury
DOD	Department of Defence
ECG	Electrocardiogram
EPO	Erythropoietin
ERFE	Erythroferrone
G6PD	Glucose-6-phosphate dehydrogenase deficiency
GMP	Good Manufacturing Procedures
GMS	Greater Mekong Subregion
HBV	Hepatitis B Virus
HETE	15(S)-hydroxyeicosatetraenoic
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HNE	Hydroxyl-noneal
HR	Heartrate
HRP2	Histidine-rich protein 2
HREC	Human research ethics committee
IBSM	Induced blood-stage malaria
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iPVAC	Indwelling peripheral venous access cannula
IV	Intravenous
LDA	Limiting dilution assay

LLN	Lower limit of normal
MCB	Malaria Cell Bank
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MoD	Ministry of Defence
MMV	Medicines Malaria Venture
NTBI	Non-transferrin bound iron
PADH	Post artesunate delayed haemolysis
PD	Pharmacodynamics
Pf	*Plasmodium falciparum*
***Pf* GLURP**	*Pf* glutate-rich protein
***Pf* MSP-1/2**	*P. falciparum* merozoite surface protein 1/2
PI	Principal investigator
PK	Pharmacokinetics
PMR	Parasite multiplication rate
pRBC	Parasitised red blood cell
PRR	Parasite reduction ratio
Pv	*Plasmodium vivax*
qPCR	Quantitative Polymerase Chain Reaction
RES	Reticuloendothelial system
RNA	Ribonucleic acid
SD	Standard deviation
SBP	Systolic blood pressure
spp.	Subspecies
sTfR	Soluble transferrin receptor
TNF	Tissue necrosis factor
ULN	Upper limit of normal
UNMISS	United Nations Mission in South Sudan
uRNC	Unparasitised red blood cell
VIS	Volunteer infection studies
WGS	Whole genome sequencing
WHO	World Health Organisation

List of figures

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List of publications and presentations

Malaria related publications submitted/published during this Fellowship, which I was either 1st author or co-author:

1. Woolley SD, Fernandez M, Rebelo M, Llewellyn SA, Marquart L, Amante FH, Jennings HE, Webster R, Trenholme K, Chalon S, Moehrle JJ, McCarthy JS and Barber BE. Development and evaluation of a new *Plasmodium falciparum* 3D7 blood stage malaria cell bank for use in volunteer infection studies. Malaria Journal **20**, 93. 2021. https://doi.org/10.1186/s12936-021-03627-z
2. Odedra A, Mudie K, Kennedy G, Watts RE, Rossignol E, Mitchell H, Gower J, Rebelo M, Pava Z, Pawliw R, Woolley SD, Lallo DG, Robinson G, Lynch S, Collins KA, Amante and McCarthy JS. Safety and feasibility of apheresis to harvest and concentrate parasites from subjects with induced blood stage *Plasmodium vivax* infection. Malaria Journal **20**, 43. 2021. https://doi.org/10.1186/s12936-021-03581-w
3. Woodford J, Gillman A, Jenvey P, Roberts J, Woolley SD, Barber BE, Fernandez M, Rose S, Thomas P, Antsey NM and McCarthy JS. Positron emission tomography and magnetic resonance imaging in experimental human malaria to identify organ specific changes in morphology and glucose metabolism: A prospective cohort study. PLOS Medicine. 18(5): e1003567.
4. Barber BE, Kansagra KA, , Fernandez M, Patel HB, Barcelo C, Woolley SD, Patel H, Llewellyn S, Rahman A, Sharma S, Jain M, Ghoghari A, Di Resta A, Fuchs A, Deni I, Yeo T, Mok S, Fidock DA, Chalon S, Moehrle JJ, Parmar D, McCarthy JS and Kansagra K. Safety, pharmacokinetics and antimalarial activity of the novel triaminopyrimidine ZY-19849: a first-in-human, randomised, placebo-controlled, double-blind, single ascending dose study, a pilot food effect study, and a volunteer infection study. Submitted to Lancet ID.
5. Webster R, Sekuloski S, Odedra A, Woolley SD, Jennings H, Amante FH, Trenholme KR, Healer J, Cowman AF, Eriksson, Sathe P, Penington J, Blanch A, Dixon MWA, Tilley MF, Craig A, Storm J, Chan J, Evans K, Papenfuss T, Schofield L, Griffin P, Barber BE, Andrew D, Boyle MJ, Rivera F, Engwerda CR and McCarthy JS. Safety, infectivity and immunogenicity of a genetically attenuated blood-stage malaria vaccine. Submitted to BMC Medicine.

Conferences

1. Woolley SD, Gower J, Marquart L, Amante F, McCarthy JS and Barber BE. Association between iron status and post-treatment liver enzyme elevations in *P. falciparum* volunteer infection studies. Poster presentation, ECCMID 2021.

Chapter 1. Introduction

Malaria is a significant cause of mortality and morbidity globally with over 400,000 individuals dying in 2019, which is also set to increase in response to the SARS-COV-2 pandemic [1,2]. As a military infectious disease specialist, I am specifically concerned on how infectious diseases effect military populations. Malaria has historically been a significant cause of mortality and morbidity for the UK military [3,4]. Military populations are hypothesised to be at a higher risk of developing malaria and other infectious pathogens than conventional tourists due to their unique deployed operational environments [5] . The Ministry of Defence (MoD) has seen 58 cases of malaria in service personnel since 2015, with big outbreaks of malaria amongst UK service people deployed in Sierra Leone (2001) [6]. Recently there were a number of malaria cases in UK service personnel undertaking United Nations (UN) peace keeping operations (UN Mission in South Sudan [UNMISS]) in South Sudan 2017 [7]. The current UK defence priorities have moved away from conventional warfare in Eastern Europe and placed greater strategic importance to Africa and the Indo-Pacific [8]. Of bigger concern to senior UK military medical officers are the increasing use of short training teams, which are small teams of less than 10 people, deployed often in remote locations with no medic and limited medical supplies. There have been numerous cases of malaria in these groups. Lastly, there has been several high-profile medico-legal investigations into antimalarial chemoprophylaxis, in Australian and British service personnel [5,9]. The British medico-legal investigations have focused on mefloquine (Lariam©), which has been claimed to have caused neuro-psychiatric and chronic fatigue-type symptoms in individuals compulsorily made to take the medication in Sierra Leone [5]. As well as medico-legal investigations, there have been subsequent political reviews in both Australia and the UK, with the UK House of Commons Defence Select Committee conducting an inquiry in 2015 [5,9]. This has led to a reliance on atovaquone/proguanil (Malarone©) or doxycycline. Both have significant drawbacks, such as cost, lead-in time and, side effects. Breakthrough malaria infections have also been observed in individuals taking doxycycline due to poor compliance [10]. Finally, antimalarial resistance in both *Plasmodium falciparum* and *Plasmodium vivax* is a major issue. The emerging threat of spread of artemisinin-resistant *P. falciparum* from south-east Asia represents a potential public health emergency and would result in a substantial increase of deaths from untreatable malaria [11].

Agencies such as the Malaria Medicines Venture (MMV) and the Bill and Melinda Gates Foundation (BMGF) have extensively funded the antimalarial drug development pipeline, as part of the global fight against malaria. Novel compounds and dual/triple therapeutic combinations are becoming increasingly required to overcome multi-drug resistance, especially resistance against the artemisinin drug class [12].

Following the outcome of the UK and Australian political and medico-legal investigations into the use of mefloquine and tafenoquine respectively, novel antimalarials and drug combinations are of particular interest to the MoD. New compounds include DSM265, cipargamin, artefenomel, ganaplacide and ferroquine. These novel drugs appear to meet some of the required characteristics for chemoprotective agents, but they have not been trialled in combination.

The original focus for this thesis was to utilise the existing malaria volunteer infection studies using the induced blood-stage model at QIMR Berghofer, Brisbane, to assess the pharmacodynamics and pharmacokinetic (PK/PD) properties of novel antimalarials in combination. The work proposed was going to use existing phase 1b and 2a trials of the single agents, to identify which drugs would be best suited in combination. These combinations would have then been taken forward in IBSM VIS to characterise the parasite reduction ratios (PRR) and PK/PD, to ultimately optimise the dose. The outcome of these trials would be to take forward into phase 3 studies the best combinations.

Unfortunately, due to re-organisational issues and the COVID-19 global pandemic, the focus of this thesis had to change. Consequently, the thesis was refocussed to investigating malaria-associated anaemia, especially in the context of low-level infection. The IBSM VIS potentially are an ideal platform to investigate malaria-associated anaemia, due to the prospective longitudinal parasite, haematological and biochemical sampling. In the QIMR Berghofer IBSM VIS, the parasite sampling is via 18S quantitative polymerase chain reaction (PCR) assay, which targets the *Plasmodium* species specific 18S rRNA gene.

1.1. Research questions

Malaria is a significant cause of mortality and morbidity, of which malaria-associated anaemia is an important cause. I wanted to understand:

1) Can human malaria volunteer infection studies (VIS) be used as a model for malaria-associated anaemia in early malaria?
2) What is the development and progression of malaria-associated anaemia in healthy malaria-naïve individuals in early infection?
3) Is iron metabolism altered in early malaria in VIS subjects?

Major aims

1) Conduct an IBSM malaria VIS utilising a newly developed malaria cell bank, 3D7-MBE-008 to:
    a. Determine the safety of the *P. falciparum* 3D7-MBE-008 malaria challenge agent in healthy subjects.
    b. Characterise the infectivity of the *P. falciparum* 3D7-MBE-008 malaria challenge agent in healthy subjects.
    c. Characterise the parasite clearance profile of the *P. falciparum* 3D7-MBE-008 blood stage parasite after administration of the antimalarial drug artemether/lumefantrine.
    d. Compare the infectivity and parasite clearance profile of the *P. falciparum* 3D7-MBE-008 with the previously developed *P. falciparum* 3D7-V1 and V2 malaria challenge agents.

2) Utilise data from previous IBSM VIS conducted at QIMR Berghofer, including the above mentioned 3D7-MBE-008 IBSM VIS, to investigate the haematological response to malaria, including:
    a. The mean fractional fall in haemoglobin from day of inoculation and day of treatment.
    b. The mean day haemoglobin nadir and mean day of return to baseline.

c. The correlation between haemoglobin nadir and parasite parameters (peak parasitaemia, total parasite burden, parasite multiplication rate and parasite reduction ratio).
d. The effect of recrudescence/treatment failure on haemoglobin nadir.
e. The ratio of pRBC and uRBC to the malaria-attributable haemoglobin loss, and to compare this to previously established data.
f. The progression of anaemia between artemisinin-based compounds and other non-artemisinin antimalarial compounds.

3) Utilise data from previous IBSM VIS to evaluate iron metabolism in participants inoculated with *P. falciparum* and *P. vivax*, including:
    a. Evaluate the longitudinal measurements of erythropoietin, erythroferrone, hepcidin, ferritin, soluble transferrin receptor (sTfR) and sTfR/$\log_{10}$ ferritin index
    b. Correlate iron metabolism markers with haemoglobin and parasitaemia.
    c. Evaluate the effect of iron and hepcidin on parasite multiplication rates.
    d. Evaluate the effect of iron status on post-treatment elevations in liver function tests.

Chapter 2. Literature Review

2.1. Overview of malaria

Malaria is a mosquito-borne parasitic infection that is endemic in tropical and sub-tropical countries globally and has been one of the most significant infectious diseases of humans for millennia [13,14]. Plasmodium is a member of the phylum Apicomplexa, which compromises of the obligate eukaryotic parasites of animals including *Babesia, Plasmodium, Cryptosporidia, Cyclopsora* and *Toxoplasma* [15]. The main species that can cause disease in humans are *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* [13,16,17]. *P. falciparum* and *P. knowlesi*, cause the most serious forms of clinical malaria, although it is becoming increasingly recognised that *P. vivax* causes significant morbidity in endemic regions [18–20]. The transmission vector for all malaria is the female *Anopheles* mosquito [16,21].

2.1.1. Life Cycle

As mentioned above, the *Plasmodium* parasite is an obligate parasite that requires a host to replicate [21]. *Plasmodium* replicates in two hosts, the *Anopheles* mosquito and mammals, with humans being the definitive host for *P. falciparum, P. vivax, P. ovale and P. malariae* [21]. The definitive hosts for *P. knowlesi* and *P. cynomolgi* are macaques, although they cause disease in humans [17,20]. The life cycle starts when an infected female *Anopheles* mosquito takes a blood meal from a human and injects *Plasmodium* sporozoites along with their anti-coagulant saliva [21]. Sporozoites are the infective motile life stage of the *Plasmodium* parasite, which target hepatocytes via the lymphatic and vascular systems [21]. A single sporozoite infects a hepatocyte and undergoes schizogony (multiple asexual replications), generating up to 40,000 merozoites after a period of up to 18 days, although this can be delayed for up to 1 year in *P. vivax* or *ovale* [21,22]. The hepatic schizonts rupture with the released merozoites entering the bloodstream and infecting erythrocytes [21]. The different *Plasmodium* spp. have a predilection for different erythrocyte life stages, and this is discussed below. The merozoites then undergo schizogony within the infected erythrocyte before rupturing into the blood stream and repeating the lifecycle [21]. The chronicity of the erythrocytic schizogony and the number of released merozoites also depends on the *Plasmodium* spp, with *P. falciparum* releasing up to 18 merozoites every 48 hours compared to *P. malariae* which releases only 6-8 merozoites every 72 hours [21,22]. A small number of the merozoites will develop into male or female gametocytes, which are taken into the female mosquito host during the blood meal.

The male and female gametes then fuse forming a zygote and subsequently an ookinete within the midgut of the mosquito [21]. The ookinete then crosses the epithelium of the gut to form an oocyst [21]. The oocyst undergoes further replication cycles resulting in the release of sporozoites, which travel to the salivary gland of the mosquito and recommence the cycle of infection [21]. The mosquito lifecycle is approximately 7-10 days from ingestion of the blood meal to being able to infect a human host [21,22]. *P.vivax* and *P. ovale* are able to form hypnozoites, which are latent and can cause disease weeks or years post inoculation [21–24].

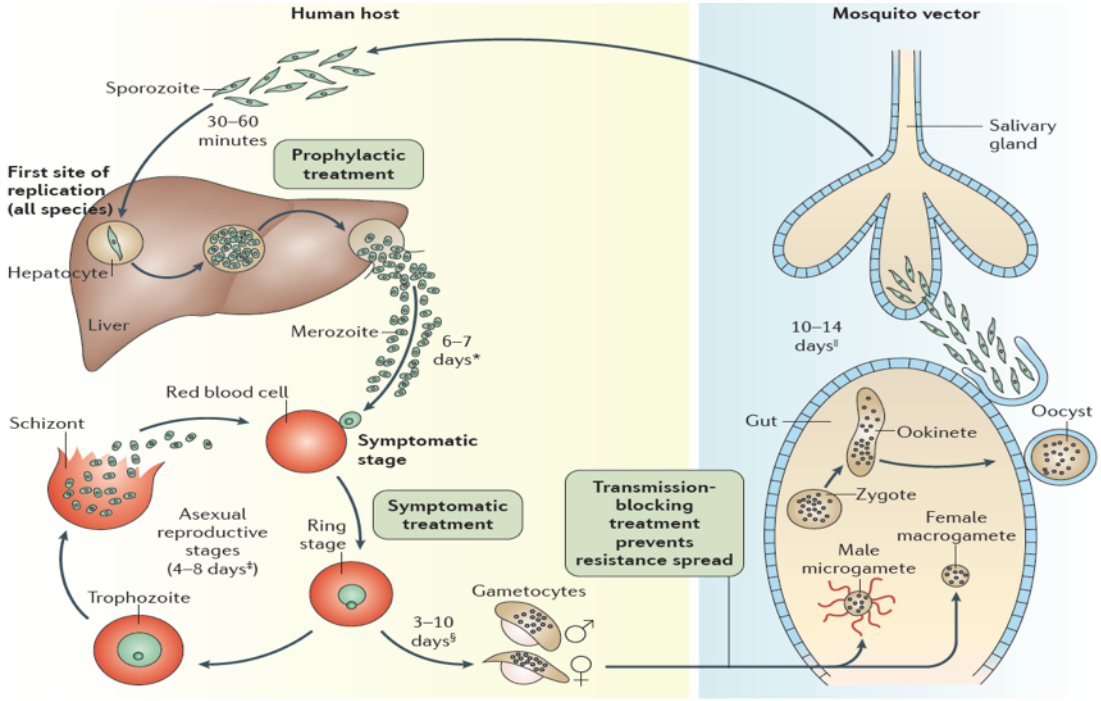


Figure 2.1 Plasmodium life cycle. *Phillips MA et al*[21]

Most drug treatments target the asexual parasites and therefore reduce the circulating parasite count. However, individuals infected with malaria may appear redevelop symptoms post treatment. These terms are defined by the WHO [25] are as follows:

- Recrudescence- recurrence of asexual parasitaemia of the same genotype that caused the original illness, due to incomplete clearance of asexual parasites after antimalarial treatment.
- Relapse- recurrence of asexual parasitaemia in P. vivax or P. ovale infections arising from hypnozoites.

- Reinfection- A new infection that follows a primary infection; can be distinguished from recrudescence by the parasite genotype, which is often difference from that which caused the initial infection

2.1.2. Clinical Syndromes

The clinical syndrome of malaria has common features between species as well as species-specific manifestations. Malaria causes a spectrum of disease ranging from severe life threatening infection to asymptomatic infection [22,26].

Uncomplicated malaria, which is the commonest presentation, is an acute febrile illness with a range of non-specific symptoms including headache, high fever (commonly over 39^{o}C), myalgia, arthralgia, rigors and abdominal cramps [13,22,26,27]. Disease severity is often linked to higher parasitaemia in all *Plasmodium* spp, although this can be variable [13,28]. The gold standard treatment in the UK for uncomplicated malaria is an artemisinin combination therapy (ACT), of which artemether-lumefantrine (AL) or dihydroartemisinin-piperaquine (DHP) is the preferred combination therapy [27]. The alternate options include either atovaquone-proguanil (Malarone ®) or quinine with doxycycline [27].

P. falciparum, *P. knowlesi* and to a lesser degree *P. vivax* are able to cause severe malaria [20,26,27,29]. Severe malaria is a multi-system disease with the main manifestations being cerebral malaria, acute renal failure, acute respiratory distress syndrome (ARDS) and severe malarial anaemia [22,27,30]. Prior to ACT becoming the gold standard treatment, the case mortality rate of falciparum malaria was 1 in 1000, increasing to 1 in 100 where there was poor access to medical treatment and/or increasing antimalarial resistance [26]. In Kennangalem *et al* [31], they observed a 50% reduction in the proportion of malaria cases requiring hospital admission and a 30% reduction in mortality with the introduction of universal artemisinin based therapy.

Non-immune patients are most likely to suffer the severe complications of malaria such as severe anaemia, cerebral malaria, acute respiratory distress syndrome (ARDS), acute kidney injury and hypoglycaemia [27]. Children under 6 months of age who are born in high-endemic areas have passive immunity from their mothers and their foetal haemoglobin (Hb-F) is

resistant to digestion by the malaria parasites [32]. As the child grows older, severe malarial anaemia is the commonest cause of disease in the 1-3 year old age group [13,32,33].

2.1.3. Epidemiology of Malaria

Malaria has been documented within historical archives as one the leading causes of human mortality and morbidity for millennia [14]. As a consequence of this exposure to malaria, it has been hypothesised that the human body has evolved certain genetic polymorphisms that confer protection to the individual against malaria [14]. The prevalence of these evolutionary genetic polymorphisms is highest in geographical regions of their corresponding species of malaria [14]. The genetic polymorphisms include thalassemias, glucose-6-phosphate dehydrogenase (G6PD), sickle cell trait, ovalocytosis and erythrocyte Duffy negativity [14,18]. Sickle cell trait is caused by a single point mutation in gene encoding the beta chain of haemoglobin, resulting in the haemoglobin-S molecule [14]. Heterozygosity confers up to a 50% survival benefit from death resulting from *P. falciparum* infections. This compares to homozygotes who commonly do not live past adolescence in malaria endemic countries due to the consequences of sickle cell disease [14]. In many parts of Africa, the frequency of the sickle cell trait is up to 30%. Duffy coat negativity confers protection to *P. vivax* and is the reason why indigenous populations in West and Central Africa have a degree of protection against infection by *P. vivax* [14].

Malaria was recorded and characterised by ancient European civilisations as the 'benign tertian' and 'quartan' periodic fevers, in reference to their characteristic feature of a fever every 3 and 4 days respectively. 'Tertian' and 'quartan' fevers refer to *P. vivax* and *P. malariae* respectively. *P. falciparum* was classically known as the 'subtertian malignant' fever due to its often-fatal manifestations. *P. vivax* and *P. malariae* traditionally had the widest geographical distribution, however, they have been replaced by *P. falciparum*.

Malaria is still one of the most important infectious diseases and according to the WHO 2020 World Malaria Report, there were an estimated 229 million cases of malaria globally in 2019, which resulted in 409,000 deaths [1]. It is anticipated the COVID-19 pandemic in 2020, could double the number of malaria deaths, due to disruption to the distribution of insecticide-

treated bed-nets and mass-drug administration projects in endemic countries [2]. The major burden of disease is in Sub-Saharan Africa, with the predominating species being *P. falciparum* [16]. *P. falciparum* is present in all areas of Sub-Saharan Africa, with *P. vivax*, *P. malariae* and *P. ovale* species present but in lower numbers [16]. *P. vivax* is more prevalent in South East Asia, East and Southern Africa, with *P. ovale* more prevalent in West and Central Africa [16]. *P. knowlesi* is found in South-east Asia with the exception of Timor-Leste [16,20].

2.1.4. Drug Resistance

Antimicrobial resistance is a significant global threat that is seen in a wide-ranging spectrum of infectious diseases including tuberculosis, Gram-negative bacteria, HIV and malaria. Drug resistance is a significant cause of concern in the malaria community. It first became apparent in 1957 with the observation of chloroquine resistance in *P. falciparum* in Thailand [21,34,34,35]. Chloroquine resistance spread to South America by 1959 and East Africa by the late 1970s [35]. As a consequence, chloroquine is now ineffective globally, with the exception of some areas of Central America [35]. Artemisinin-based compounds are now the gold standard for malaria treatment globally and were developed in the 1970s in response to the spread of chloroquine resistance [11,35,36]. Artemisinin resistance developed in the early 2000s within the Greater Mekong Subregion (GMS) [11,34,37]. There are also reported deaths linked to artemisinin resistance [38].

Drug resistance occurs in 2 phases [35]:

1) An initial genetic event occurs producing a resistant mutant, which consequently provides the parasite with a survival advantage against the drug.
2) The resistant parasites are then selected out from the population and multiply, becoming the dominant population and thus conferring population resistance to the drug.

Some drugs require only a single point mutation for resistance to occur, whereas the rest require multiple point mutations to confer resistance [35]. Poor adherence in drug use and/or single agent use alter the concentration threshold of the drug at which resistance may occur [35]. The mechanisms of drug resistance to antimalarials reflect mutations that affect drug

accumulation or efflux mechanisms (in the case of chloroquine, amodiaquine, quinine and mefloquine), or those that reduce affinity of the drug target for the drug (in the case of pyrimethamine, atovaquone and artemisinin) [35].

Artemisinin resistance is characterised by a slower rate of parasite clearance and when assessed by microscopy, is the failure to become slide negative for parasites in 48 hours of the commencement of treatment in 95% of patients [39]. The parasite killing of artemisinin is related to the cleavage of an endoperoxide bond [40]. It has been suggested that the mechanism by which artemisinin kills parasite is by causing "proteopathy" (degeneration of the parasite's cytoplasm) by alkylating proteins [40]. The gene pfkelch13 (PfK13) is the marker of artemisinin resistance used clinically and *in vivo* [40]. There are 10 genes commonly associated with the pfkelch13 mutation are (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L and C580Y) [41]. The C580Y mutation has emerged in Guyana and Papua New Guinea with other PfK13 mutation reported in India [41]. There are more worrying reports of the emergence of the R561H PfK13 mutation in Rwanda and also A675V and C469Y in northern Uganda [42–44].

2.2. History and development of malaria volunteer infection studies

The deliberate infection of humans with *Plasmodium* spp has been carried out over the last 100 years as not just a research model for the development of therapeutics and vaccinations but also as treatment for neurosyphilis [45].

Prior to the discovery of penicillin in the 1930s, malariotherapy was used in the treatment of neurosyphilis [45]. It was estimated that 5 to 20% of the population had syphilis or had previously contracted the disease [46,47]. The prevalence of syphilis in the UK in 1914 was approximately 7%, with 100 000 new cases being diagnosed in that year [46]. The progression of the disease to neurosyphilis accounted for about 20% of all hospital admissions in sanatoriums [46]. The treatments in the early 20th Century involved either arsenic or mercury based compounds and they failed to prevent the progression of the disease [46,47].

The pioneer of malariotherapy for the treatment of neurosyphilis was an Austrian physician called Julius Wagner-Jauregg who spent his career developing the technique [46,47]. His

hypothesis was that an induced fever could be used to reduce the symptoms or even cure the disease [47]. This hypothesis was based on the theory of pyrotherapy developed by the ancient Greek physicians Hippocrates and Galen [47]. His first trial involved the use of erysipelas to induce fever, but this was met with complications, which he subsequently did not report [47]. As erysipelas is commonly caused by *Streptococcus pyogenes,* the complications in the pre-antibiotic era were likely catastrophic. Other infectious diseases used to induce fever included typhoid, cholera and tuberculin [46,47]. Wagner-Jauregg used tuberculin in his patients with neurosyphilis with good results, but reports of deaths associated with tuberculin use led him to abandon this approach [47].

In 1917 a returning soldier suffering from tertian malaria was admitted to a nearby hospital and Wagner-Jauregg took a sample of his blood and subsequently administered the infected blood to 9 of his patients suffering from neurosyphilis [46,47]. Of the 9 who were inoculated, 1 died, 2 were sent to asylums and 6 demonstrated resolution of their symptoms (4 subsequently had relapses) [47]. The malariotherapy regimen involved 7-12 days of fever followed by administration of quinine bisphosphate and Neosalvarsan, an antimalarial and anti-syphilis drug respectively [47]. These initial results led to Wagner-Jauergg continuing to treat his patients with tertian malaria that was obtained from further returning soldiers. By 1921 he had treated 200 patients with malariotherapy, with 50 reporting sufficient recovery to resume their occupations [47]. This work led to Wagner-Jauregg being the first psychiatrist to be awarded a Nobel Prize for medicine in 1927 [47]. Even with the introduction of penicillin in the middle 1940s, malariotherapy continued to be used up to the mid- 1950s [45–47].

Malaria is implicated in the deaths of several of history's great leaders, including Alexander the Great and Oliver Cromwell [48]. The disease has also had impacts on military conflicts through the ages to the present day. General Douglas MacArthur, Supreme Commander-Southwest Pacific Area in World War 2 is quoted as saying:

"this will be a long war if for every division I have facing the enemy I must count on a second division in hospital with malaria and a third division convalescing from this debilitating disease!" [49]

Malaria had a significant impact on the Allied Far East campaign during World War 2. Field Marshall Sir William Slim, a senior commander for land forces in Burma stated:

"In 1943, for every man evacuated with wounds, we had 128 evacuated sick. The annual malaria rate alone was 84% per annum of the total strength of the army and still higher among the forward troops." [49]

The urgent need for treatments led to the pioneering volunteer infection studies of the Australian physician, Brigadier Hamilton Fairley, and this was instrumental in developing new therapeutics [4]. He developed a human challenge model using malaria-naïve soldiers who were physically and mentally fit, no criminal records, free from venereal diseases, not asthmatic and not had jaundice within the last 12 months [3]. The individuals were infected using known infected *Anopheles* mosquitos, and then given experimental drugs with daily supervision including the recording of daily clinical parameters such as temperature, presence of splenomegaly, hepatomegaly, anaemia, jaundice and herpes [3]. Brigadier Fairley even developed a carrier model, by the production of "artificial carriers" with modified treatment regimens to remove the asexual stages of the parasites, thus leaving gametocytes [3]. A model was developed by Brigadier Fairley and Lt Col Smith, inoculating recipients with 200 mLs of blood from a compatible donor who had not developed malaria following 5-weeks of drug treatment post being bitten by an infected mosquito, in order to detect latent malaria from *P. falciparum* or *P. vivax* [3]. Fairley observed in some of his other experiments, that those individuals inoculated with donor blood infected with *P. falciparum* from the time of the infected bite to day 6, did not develop infection, whereas the recipients developed infection from blood 7 days post infection onwards [3]. Similarly, for *P. vivax* he observed that blood donated 9 days post infection induced infection in the recipients [3].

Malaria volunteer infection studies (VIS) are used in the development of new vaccinations and therapeutics and can truncate the development pipeline. The modern technique of malaria VIS was first taken up in the 1970s, with Clyde and Rieckmann separately demonstrating that using irradiated sporozoites administered through infected female *Anopheles* mosquitoes, provided protection to malaria-naïve individuals from *P. falciparum* [50]. This was then followed by Trager and Jensen who had developed an *in vivo* culture method

for *P*. *falciparum* in 1976 [50]. In 1980, Vanderberg and Gwadz published on the infection of female *Anopheles* mosquitoes on *in vivo* cultures [50–53]. The first VIS using female *Anopheles* mosquitoes infected from *in vivo* culture, was carried out in 1986 by a collaboration between the US Department for Defence (DOD) and the National Institutes of Health (NIH) [50].

The Queensland Institute of Medical Research (QIMR) Berghofer was set up in 1945 by the Queensland government to continue Brigadier Fairley's work in tropical disease research. The induced-blood stage model for malaria was developed in the mid-1990's, following the development of two malaria cell banks (MCBs) [54]. These banks were developed when two volunteers were flown to the University of Edinburgh to be infected with a fully-sensitive *P. falciparum* strain (Pf3D7) [54,55]. These individuals returned to Queensland, when on developing symptoms had 500 mL of their blood harvested, leading to the two MCBs, (3D7-V1 and 3D7-V2) [54,55]. Following the use of the two MCBs in a challenge trial, involving 6 recipients and the re-infection of the 2 donors, it was deemed that 3D7-V2 MCB results were more reproducible and therefore this MCB was subsequently taken forward in the Phase 1 IBSM VIS trials undertaken at QIMR Berghofer [54]. The team at QIMR have now developed three 3D7 MCBs [54,55], an artemisinin resistant K13 *P. falciparum* model [56], a fully sensitive *P. vivax* model [57]and a fully sensitive *P. malariae* model [58]. As of June 2021, over 400 participants have undergone the IBSM VIS at QIMR Berghofer.

2.3. Anaemia in Malaria

Malaria is a significant cause of anaemia within tropical and sub-tropical regions [13,33,59]. The severity of anaemia can range from chronic anaemia of infection to profound acute anaemia requiring blood transfusion [13,18,33]. In high endemic settings (which is defined as receiving up to one infectious bite a day), the more severe forms of anaemia related to malaria are in children under the 5 years of age [13]. This then becomes less of a feature as the child grows older and develops disease-controlling immunity. By end of adolescence/early-adulthood, the prevalence of anaemia declines [13]. In low endemic settings, all age groups can develop anaemia related to symptomatic malaria, however, it is most likely to occur in children and pregnant women [13].

Anaemia in the context of malaria has a wide variety of definitions, although the generally accepted definitions are [13]:

- Mild anaemia $\underline{<}$ 11 g/dL
- Moderate anaemia $\underline{<}$ 8 g/dL
- Severe anaemia $\underline{<}$ 5 g/dL

The reasons why traditionally lower ranges for anaemia are used in malaria studies is because anaemia in tropical and sub-tropical countries is often multi-factorial and include inherited red cell abnormalities, nutritional deficiencies, helminth co-infections and HIV co-infection [13].

Of clinical importance is the acute drop in haemoglobin in individuals who already have a lower baseline haemoglobin (eg 70 g/L vs 130 g/L) especially in vulnerable groups such as children, pregnant women, and immunocompromised individuals [13]. Individuals who have a sub-acute presentation of malaria have a better prognosis due to the body undergoing physiological adaptation to the anaemia such as right shift of the oxygen dissociation curve [60]. A study in Papua demonstrated that individuals with falciparum malaria who had lower admission haemoglobin levels had substantially higher mortality rates, especially when compared to vivax malaria [61]. Severe malaria anaemia is associated with children under the age of 5 in haloendemic areas, with Boyce et al [62] also identifying another peak in the incidence of severe malaria anaemia in the over 50 year olds in rural western Uganda.

The measurements of anaemia used in the malaria literature are either haemoglobin concentrations or haematocrit [13,63,64]. Haematocrit has traditionally been used in field studies because it is easier to measure and does not require substantial equipment or electrical power, whereas measuring haemoglobin requires a portable spectrophotometric device [13,64]. Therapeutic and clinical trials report changes in either haematocrit or haemoglobin, whereas epidemiological/ preventative intervention studies report the incidence of anaemia [13]. Factors that affect the relationship between haemoglobin and haematocrit include acute phase protein concentrations, erythrocyte rigidity and red cell abnormalities such as sickle cell disease and thalassaemia [13,64]. In order to compare between haemoglobin concentration

and haematocrit, there are two conversion factors that have been validated in *P. falciparum* [13,64]:

1. Haemoglobin = Haematocrit/ 3
2. Haematocrit = 5.62 + 2.60 x Haemoglobin

The first conversion factor was thought to produce an overestimation of the haemoglobin concentration, thus underestimating the prevalence of anaemia in field studies [13,64]. Lee *et al* [64] , validated both conversion factors using 3254 measurements from 1810 patients.

2.3.1. Species differences in malaria-associated anaemia

The type and severity of anaemia is dependent on the species of *Plasmodium* parasite, which is related to the red cell populations they have a predilection for as well as their specific growth rates.

P. falciparum is the most studied in part due to its large geographical distribution, but also due to its associated high mortality and morbidity, when compared to the other species. *P. falciparum* has an ability to infect erythrocytes of all ages, including orthochromatic erythroblasts, which are nucleated young erythrocytes [65]. *P. falciparum* has a quick replication cycle which is usually less than 48 hours, and as such, is able to infect a large number of young and old erythrocytes quickly [65]. As a consequence of these high parasitaemias, complicated severe malaria often occurs, especially in non-immune patients with *P. falciparum* infection [65].

P. vivax was traditionally thought to cause only benign disease when compared to *P. falciparum* infection, however, the recent literature challenges this [18]. Douglas *et al* [18], commented that anaemia is the most frequently encountered severe complication of *P. vivax* infection. Erythrocytes that are less than 14 days old and especially reticulocytes are the cell types that *P. vivax* has a strong predilection for, when compared to *P. falciparum* [18]. As a consequence, parasitaemias greater than 2% are considered rare in *P. vivax* infections [18]. *P. ovale* also only infects young and developing erythrocytes, and as a consequence, the peripheral parasite counts are lower than in both *P. falciparum* and *P. vivax* [66].

A great deal of the information on *P. malariae* is historical due to its use in early volunteer infection studies around World War 2 [4]. There are a number of factors that limit the severity of disease in *P. malariae* infections and these factors are [67]:

- Longer development cycle (72 hours vs 48 hours for *P. falciparum* and *vivax*)
- Lower number of merozoites released per erythrocyte cycle
- Predilection for older erythrocytes
- Quicker immune responses compared to other *Plasmodium* species

P. knowlesi, which was previously thought only to infect monkeys, is now increasingly recognised as an important emerging infectious disease, especially in Indonesia and Malaysia [20,68]. Morphologically it appears similar to *P. malariae*, however *P. knowlesi* is able to cause severe complicated malaria similar to *P. falciparum* [68]. The erythrocytic life cycle of 24 hours is shorter than the other species known to cause disease in humans and Grigg *et al* [68], hypothesise that the low detected parasitaemias may indicate variable efficiency in infecting erythrocytes.

2.3.2. Clinical features of malaria associated anaemia

Anaemia is one of the first features of acute malaria, with most symptomatic patients losing > 1 g/dL of haemoglobin before receiving medical treatment [63]. This was demonstrated by Price *et al* [63], who showed the development of anaemia in patients with acute *P. falciparum* malaria on the Thailand-Myanmar border, which is a lower transmission area. These patients had little or no immunity to malaria.

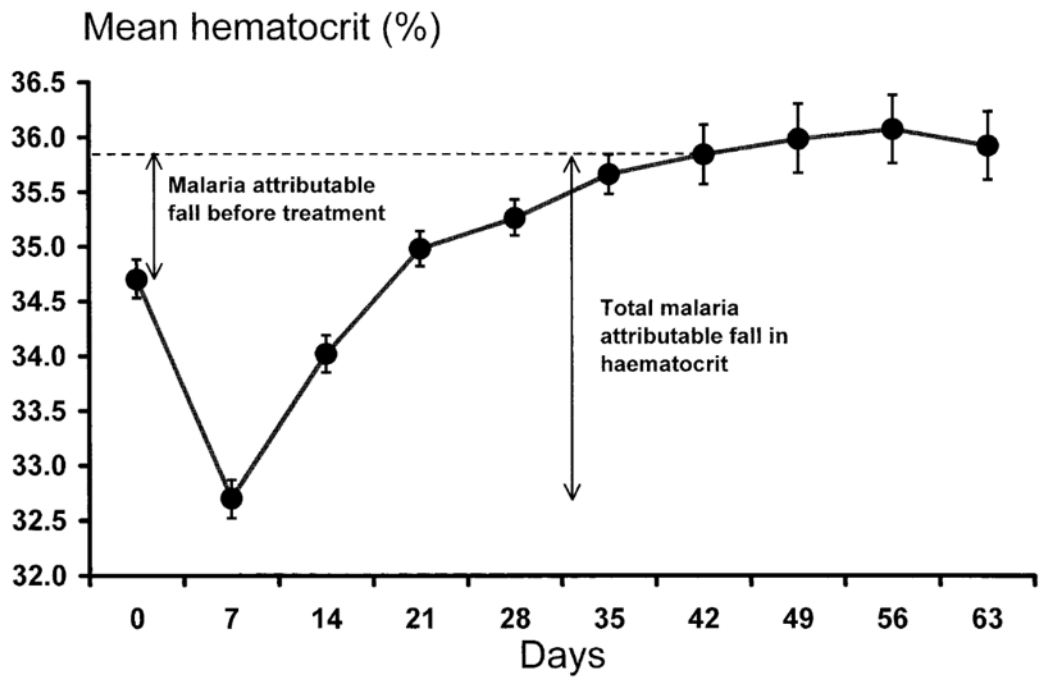


Figure 2.2. The haematological recovery after an episode of falciparum malaria. *Price RN et al* [63]

Figure 2.2, demonstrates that the nadir haematocrit is 7 days after presentation and treatment, which is thought to be explained by the dyserythropoiesis occurring in the bone marrow [63]. The resolution of the anaemia can take between 4-6 weeks [63]. Drug resistance is a factor associated with increased anaemia because of the slow initial drug response and the subsequent risk of recrudescence [13,63].

Anaemia can be a feature of asymptomatic or submicroscopic malaria. Children presenting with severe anaemia may have a blood film negative for parasites, but their anaemia may respond to antimalarial therapy [69]. Sen *et al* [69], commented that Indian children with asymptomatic *P. falciparum* infection who had moderate to severe anaemia and hepatosplenomegaly were noted to have increasingly deranged haematology markers (neutropenia, atypical lymphocytes and thrombocytopenia) at lower levels of parasitaemia when compared to similar children with acute symptomatic *P. falciparum* infection. A cause for the increase in haemolysis could be the increased splenic clearance of both parasitised red blood cells (pRBC) and unparasitised red blood cells (uRBC) with associated white cell abnormalities and hypersplenism [13]. Abnormal iron metabolism is also associated with the

anaemia of chronic disease and is considered in the literature as a possible factor in anaemia in asymptomatic malaria [13,70].

2.3.3. Pathophysiology of malaria-associated anaemia

Anaemia in malaria is multi-factorial and not just related to the haemolysis of pRBC [13]. All *Plasmodium* species are obligate organisms within a vertebrate host and all have an intra-erythrocyte step in their life cycles [21].

Bone marrow dyserythropoiesis occurs during and after the infection [13]. Dyserythropoiesis is hypothesised to be related to the intramedullary production of suppressors of erythropoiesis, such as proinflammatory cytokines, nitric oxide, lipoperoxides and bioactive aldehydes, which also cause red cell precursor cell apoptosis [71,72]. Hemozoin, a malaria pigment produced during the digestion of haemoglobin by the *Plasmodium* parasite, is expelled during the rupture of schizonts from the pRBC [71,72]. The toxic haem is processed inside the developing parasite's food vacuole and hemozoin is formed. It is a crystalline dimer of α-haematin complexed with lipids and proteins [71]. Hemozoin crystals are similar to β-haematin, consisting of a ferric ion (Fe^{3+}) with a protoporphyrin IX ring structure [71].

Hemozoin is found in peripheral blood or bone marrow smears after being phagocytosed by neutrophils and monocytes/macrophages of the reticulo-endothelial system (RES) [71]. High circulating serum concentrations of hemozoin and hemozoin containing monocytes are associated with severe anaemia in children, in haloendemic *P. falciparum* regions [73].

The additive effect of TNF-α on hemozoin has been demonstrated to inhibit erythropoiesis [72]. IFN-γ is also implicated in severe malarial anaemia. It has been hypothesised that high levels of TNF-α may directly suppress burst-forming unit erythroid cells (BFU-e) through a variety of mechanisms such as expression of TNF p55 and p75 receptors and TNF-related apoptosis-inducing ligand or through accessory cells [72]. Hemozoin stimulates monocytes to secrete proinflammatory cytokines and produce endoperoxides, such as 15(S)-hydroxyeicosatetraenoic (HETE) and hydroxyl-noneal (HNE) that impair erythroid growth [72].

These effects of TNF-α and hemozoin appear to be independent, but their effects on erythroid growth are additive [72].

Parasitaemia is commonly different between malaria species. For example parasitaemia is lower in *P. vivax* compared to *P. falciparum* [18]. However, the number of erythrocytes cleared from the circulation is considerably higher in *P. vivax* than in *P. falciparum* and can be explained by the removal of 34 uRBC for every 1 pRBC in *P. vivax* [74] compared to 8:1 in *P. falciparum* [75]. The mechanisms of these differences are not completely understood.

There is emerging evidence of the greater role that antibodies against erythrocytes play in the pathophysiology of malaria-associated anaemia. One antibody of note that has been observed to play a role in the progression of malaria-associated anaemia are the anti-phosphatidylserine (anti-PS) antibodies, which target the phosphatidylserine in the phospholipid bilayer of erythrocytes [76]. Phosphatidylserine is exposed in both pRBC and uRBC, likely secondary to oxidative stress [76,77]. In a *P. yoelli* mouse model, Fernandez-Arias *et al* [77], demonstrated malaria infection produced anti-PS IgM and IgG antibodies, with subsequent blocking leading to a faster recovery from anaemia. Barber *et al* [76], observed that both anti-PS IgM and IgG were increased in *P. falciparum* and *P. vivax* infection in both natural malaria and VIS, with higher antibody titres observed in those infected with *P. vivax* compared to *P. falciparum*. This could also account for the increased clearance of uRBC vs pRBC in *P. vivax*, compared to *P. falciparum*.

Rosetting, the process where pRBC adhere to uRBC, occurs in both *P. falciparum* and *P. vivax* although, *P. falciparum* pRBC are also able to adhere to endothelial cells, causing sequestration of the parasitised cells within microvascular structures; an important factor in the pathogenesis of *P. falciparum* [18]. The sequestration may account for the lower number of red cells lost in *P. falciparum* compared to *P. vivax* [18]. A recent publication by Kho *et al* [78] observed an increase in CD71+ reticulocytes and the various asexual stages of *P.vivax* erythrocytes in the spleens of 22 untreated individuals living in an endemic setting, with non-phagocytosed parasite densities 3,590 times greater than in the peripheral circulation. This suggests *P. vivax* has adapted to replicate and survive longer in the spleen [78]. Kho et al [79] also observed there were non-phagocytosed erythrocytes infected with *P. falciparum*, although

the median spleen to blood ratio was smaller than observed in *P. vivax* (289 [range 18-1530] vs 3590 [range 2300-4210]). The work by Kho et al [78,79] supports the hypothesis that may have developed a cryptic intrasplenic parasitic life-cycle [80,81], thus acting as a sanctuary site in chronic carriage.

Red cell deformability is another mechanism to help the *Plasmodium* spp evade the spleen [18,82]. It is hypothesised that *P. falciparum* has evolved to make the erythrocytes they parasitise less deformable than unparasitised cells, which means the pRBC have a reduced ability to pass through the spleen, and causing them to accumulate in the microvasculature [18]. In *P.* vivax, the pRBC are more deformable and are therefore able to evade being cleared by the spleen [83].

Blackwater fever (BWF) is a condition compromising sudden massive haemolysis, haemoglobinuria and its associated acute kidney injury [84]. Disseminated intravascular coagulation (DIC) and RBC fragmentation often accompany BWF. BWF is associated with G6PD and high quinine use in Africa and South East Asia, and a reduction in BWF was associated with the switch from quinine to chloroquine [84]. Other antimalarial drugs that have been implicated in BWF include halofantrine and mefloquine [84]. Artemisinin-based compounds are associated with delayed-onset haemolysis and this is most often seen in patients with high parasite counts treated with intravenous (IV) artesunate [27,36].

Post-artesunate delayed-haemolysis (PADH) is another cause of malaria-associated malaria. Since the AQUAMAT and SEAQUAMAT studies which demonstrated artesunate was superior to quinine, artemisinin based therapeutics have been the treatment of choice for severe *P. falciparum* and chloroquine-resistant *P. vivax* [85–87]. However post-artesunate delayed haemolysis (PADH), is a being recognised clinically as a drug-induced cause of malaria associated anaemia. The mechanism of PADH is not completely understood but it is likely to be related to increased splenic clearance of pitted erythrocytes and uRBC [88]. Pitting is the process where artesunate exposed trophozoite rings are expelled from the host erythrocytes in the spleen, with the now uninfected erythrocytes returned to the circulation albeit with a reduced lifespan and therefore at risk of haemolysis [88]. For some individuals, blood transfusion was required. Those individuals who had higher median parasitaemias (>8%) were

more at risk of developing haemolysis [88–90]. PADH appears to develop from day 8 up to 21 days post the commencement of the parenteral artesunate [88–90], with Roussel *et al* [90] reporting a frequency of occurrence in 42% (42/98) in a sub-group analysis, with no fatal outcomes or serious adverse events.

2.4. Iron deficiency in malaria

2.4.1. Iron metabolism

Iron is important for the development of almost all organisms and is essential for the synthesis of haem [91,92]. In humans, enterocytes, erythrocyte precursors, macrophages and hepatocytes play particularly important roles in iron homeostasis [92–94]. Their main roles are as follows [95]:

- Enterocytes in the proximal small bowel absorb dietary iron.
- Erythrocyte precursors utilise iron for haem, and subsequently haemoglobin production.
- Macrophages in the RES recycle iron from senescent erythrocytes and store iron.
- Hepatocytes store iron and are the main source of the iron regulatory hormone hepcidin.

The average human has approximately 4 g of iron, which is distributed as follows [91,93,94]:

- 2.5g is bound in haemoglobin.
- 1.0 g is stored in hepatocytes and the cells of the RES.
- 0.5 g is found in myoglobin, cytochromes and other ferroproteins.

The body typically loses 1-2 mg of iron per day through desquamation of epithelia and minor blood loss, which in a healthy individual is easily replaced through intestinal absorption [94]. A typical Western diet provides about 15mg of iron per day, with approximately 10% of this being absorbed [94]. This dietary iron can be in both haem and non-haem forms.

2.4.2. Regulation of iron homeostasis

The major controller of body iron metabolism is the hormone hepcidin, which is produced predominantly by hepatocytes [92]. Hepcidin acts by binding to ferroportin, a transmembrane

iron transporter, and inducing its degradation, therefore inhibiting cellular iron efflux [92,94,96]. As a consequence, hepcidin controls systemic iron by inhibiting iron export from macrophages, enterocytes, hepatocytes and other body cells and induces a hypoferraemia [94]. Hepcidin expression is low in the presence of anaemia and increased by elevated iron stores and inflammation [92,94].

Hepcidin is upregulated by Interleukin-6 (IL-6) in response to inflammation and this response is observed in acute infection as well as in chronic diseases such as chronic renal disease and inflammatory conditions such as rheumatoid arthritis [92,94]. There have been several murine models [97,98], experimental infections [99] and natural infections [100,101] which have demonstrated hepcidin is upregulated in the acute phase of malaria (up to day 7) before normalisation of the levels post-treatment.

Hepcidin action is also regulated by the recently discovered hormone erythroferrone (ERFE) [102–104]. ERFE is produced by erythroblasts in response to erythropoietin (EPO) [104]. Hypoxia, oxidative stress and the resulting anaemia cause EPO to be produced by the kidney [104]. This increase in EPO causes erythroferrone to be secreted into the systemic circulation and it acts on hepatocytes to decrease the production of hepcidin which in turn increases the dietary absorption of iron and systemic iron release for erythropoiesis [104] (**figure 2.3**).

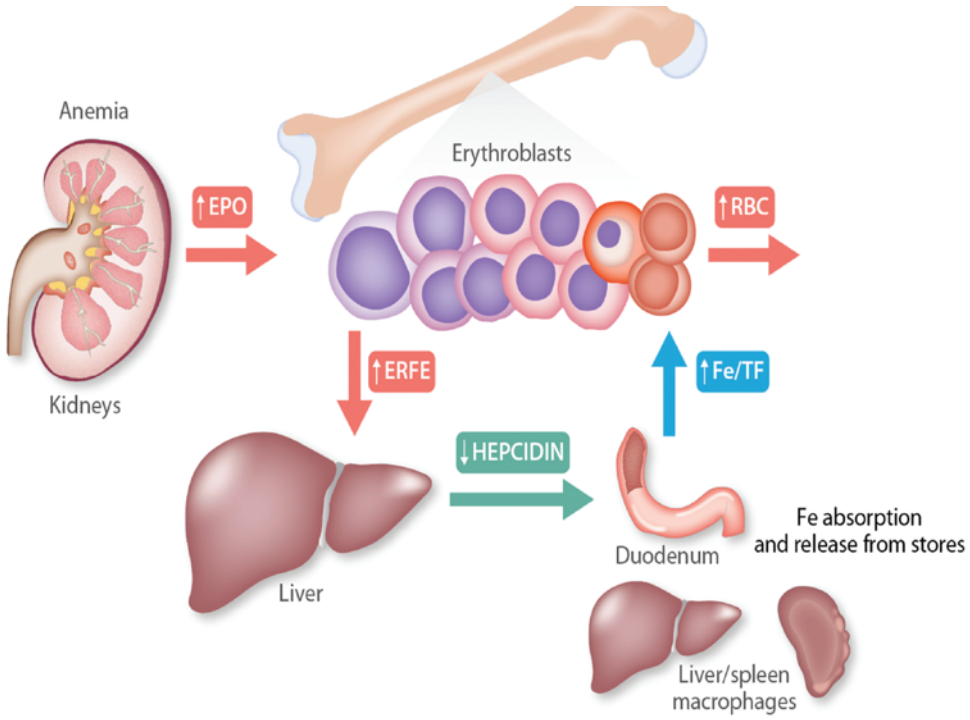


Figure 2.3. Erythroferrone in systemic iron homeostasis. *Coffey R et al* [104]

In the context of anaemia with ineffective erythropoiesis the production of erythroblasts is increased however many of the undifferentiated cells undergo apoptosis [104] (**figure 2.4**). The increased erythroblast population causes further production of ERFE that then further suppresses hepcidin [104]. This reduction of hepcidin causes the hyperabsorption of iron across the gut and an increase in iron release from macrophages [104]. The non-transferrin bound iron (NTBI) is then taken up by the liver, where it can contribute to end organ failure if the iron load is very high [104]. This is one of the mechanisms that occur in the iron overload conditions such as haemochromatosis and severe thalassaemias [104]. The effect of malaria on the EPO-EFRE-hepcidin axis is not known.

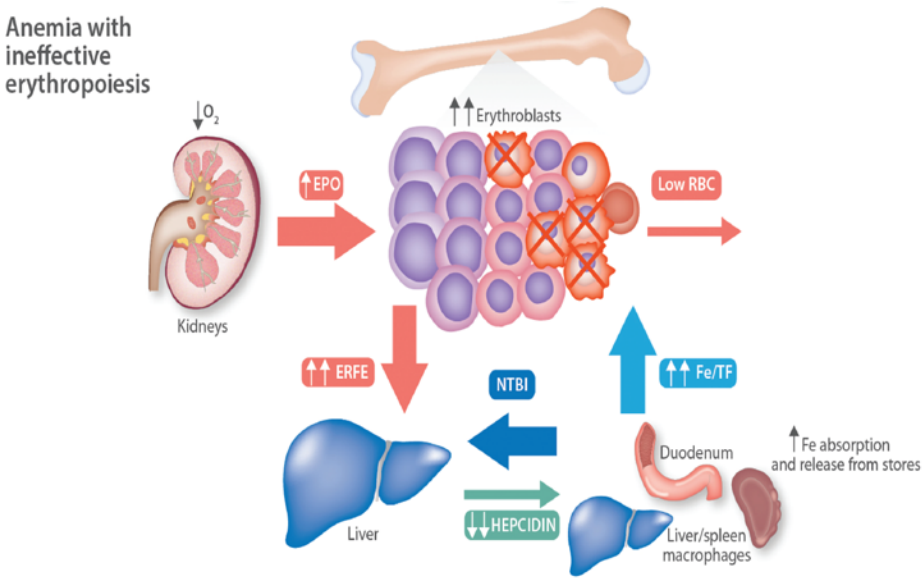


Figure 2.4. Erythroferrone in ineffective erythropoiesis. *Coffey R et al* [104]

2.4.3. Iron deficiency

Anaemia was estimated to affect up to 800 million women and children in 2011, with the highest prevalence in children aged 6-59 months and pregnant women, aged 15 to 49 [105]. According to regional estimations in 2011, the greatest burden of anaemia was in Africa and South-East Asia. Iron deficiency is the leading cause of anaemia globally, accounting for up to 60% of cases [105]. It affects up to 2 billion people worldwide, of which a quarter develop anaemia [106]. The WHO estimated in 2004 that iron-deficiency anaemia was a risk-factor for early death and caused 726,000 deaths in late pregnancy and children [107].

Iron deficiency is defined as the inadequate mobilisation of iron stores caused by long-term negative iron balance and depleted ferritin and haemosiderin stores [105]. Iron deficiency causes the erythrocytes to become hypochromic and microcytic [105]. Iron is also required for the development and cell growth of the nervous system and muscles [106]. Iron is also needed for the development of peroxidase and nitrous oxide-generating enzymes that form an integral part of the host-immune response to infection, and for the regulation of cytokine regulation and action in response to infection [106]. Hassan *et al* [108], investigated the effect of iron deficiency on the immune system of children with iron deficiency compared to healthy children and noted that the iron deficient group had lower serum immunoglobulin G (IgG), IL-6, reduced phagocytic activity and reduced oxidative burst of neutrophils.

A developing neonate will usually deplete their iron stores, acquired during the last month of pregnancy, in 4-6 months post-delivery [106]. If the infant's diet does not contain sufficient iron they then risk developing iron deficiency anaemia, and those who have low total body iron are most at risk of iron deficiency anaemia [106]. The WHO also reported in 2004 that cereal-based weaning foods may exacerbate iron deficiency due to poor absorption of the iron [107]. Infections, in particular soil transmitted helminths, schistosomiasis and malaria, are another important risk factor in the development and progression of iron deficiency anaemia [106]. Iron deficiency has a significant impact on child development, including cognitive, emotional and motor development, growth, immune function and increased risk of infection [106]. Several neurotransmitters are affected by iron status (dopamine, adrenaline and serotonin) and low iron is also associated with disrupted sleep cycles, abnormal motor control, delayed learning and poor memory [105].

In 2011 the WHO identified that a total 528 million women of child-bearing age globally were anaemic, of which 20 million were severely anaemic [109]. Women of child-bearing age are a higher risk group for the development of iron-deficiency especially there is insufficient dietary iron to replace normal daily losses with menstrual losses [110]. The recommended daily iron intake for women of child-bearing age is 18 mg, although the minimum median daily intake requirement to meet losses from menstruation is 12 mg [111]. Therefore, women who have diets with low iron content such as vegan/vegetarian diets or do not have regular access to food are especially vulnerable to iron-deficiency [111].

To diagnose iron deficiency and iron deficiency anaemia, there must be haemoglobin, iron and ferritin measurements [106]. Haemoglobin alone is not sufficiently sensitive or specific for the diagnosis, due to other causes of anaemia. Ferritin is the most commonly accepted marker for iron deficiency, although it can be difficult to interpret because it is also an acute phase protein and often elevated in inflammation/infection [105,106]. The WHO recommendation for iron status assessment of populations is to include serum ferritin and soluble transferrin receptor (sTfR) and at least one acute phase protein i.e. CRP or alpha-1 acid glycoprotein [105]. Measuring an acute phrase protein alongside the ferritin, allows for a more accurate assessment of ferritin as a marker of iron status [105,106]. Therefore, more

accurate assessments of iron status have been investigated, although are challenging as the traditional reference ranges for ferritin and sTfR are used to distinguish iron-deficiency anaemia rather than iron status [112]. sTfR is a useful marker in determining the progression from iron-deficient erythropoiesis to iron-deficiency anaemia, as it does rise in response to acute inflammation like ferritin, although it is not useful in determining the start of the iron depletion [112]. Figure 2.5 from Suominen *et al* [112], illustrates the progression of iron deficiency anaemia using markers and the sTfR/log ferritin index is able to identify those individuals who are developing iron deficiency through the loss of stored iron. The sTfR/ log ferritin index has been demonstrated to be effective in determining iron status in infants by Park *et al* [113]. Skikne *et al* [114] observed the sTfR/ log ferritin index was superior to sTfR (AUC 0.87 vs 0.74, p<0.0001) in diagnosing iron deficiency anaemia. Skikne et al[114] recruited 145 anaemic patients with common conditions associated with iron-deficiency anaemia and anaemia of chronic disease from multiple centres. They evaluated sTfR, the sTfR index and ferritin doubled the detection of iron deficiency when compared to just using ferritin alone [114].

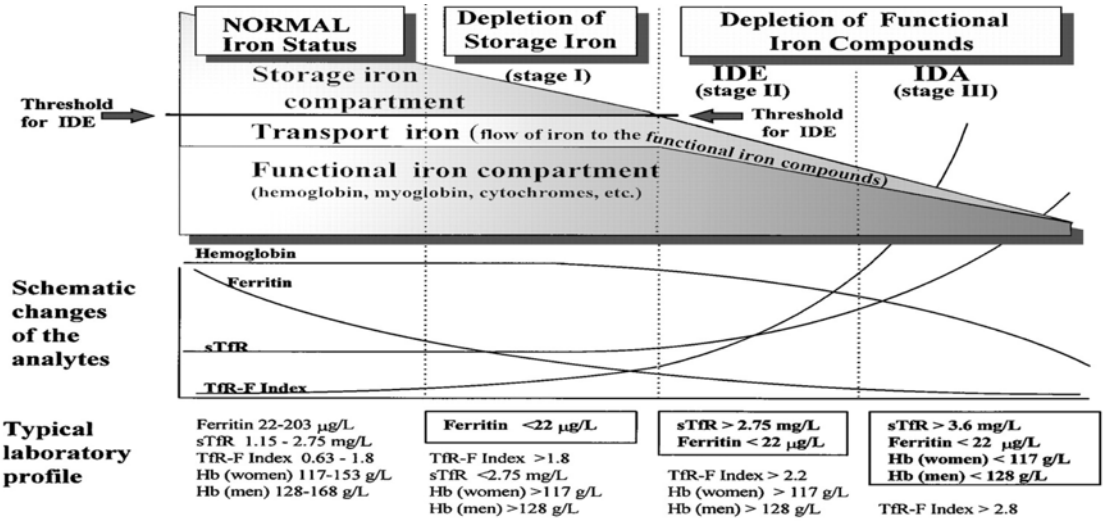


Figure 2.5. Phases of iron-deficiency. IDE- iron-deficient erythropoiesis; IDA- iron-deficient anaemia; sTfR- soluble transferrin receptor; sTfR-F – sTfR/ log ferritin index. Adapted from Suominen *et al* [112].

Frosch *et al* [115], compared the markers of iron status (ferritin, sTfR, and hepcidin) and red cell indices (haemoglobin and mean corpuscular volume (MCV)) in 138 HIV positive subjects and 52 healthy adults. The authors' conclusion was that sTfR was the most reliable marker of iron

status in HIV infected individuals due to the rise of ferritin associated with inflammation [115]. Prentice *et al* [116], investigated a series of markers of iron status to determine which most accurately reflected the incorporation of iron into erythrocytes in anaemic Gambian children (either post malaria treatment or non-malaria associated anaemia). The authors concluded that hepcidin was the best marker of intra-erythrocytic iron incorporation and suggested that a point of care of assay could assist with iron supplementation programmes in low resource settings [116].

2.4.4. Iron deficiency in malaria

Malaria is prevalent in the same geographical regions that anaemia and iron deficiency are, especially Africa and South-East Asia. The relationship between iron deficiency and malaria is complex and the debate on whether to give supplemental iron in malaria endemic settings is ongoing. Previous studies have suggested that giving iron supplementation in malaria endemic regions increases the risk of malaria and also increased mortality [105,106,117]. However, the latest Cochrane review by Neuberger *et al* [106] argues that iron supplementation in malaria endemic regions does not increase the risk of malaria in the context of effective malaria-control programmes, via readily available malaria prevention interventions and treatment services. The WHO currently do not recommend screening for anaemia or iron deficiency before universal iron supplementation in malaria endemic regions [105]. Muriuki *et al* [118] investigated whether malaria leads to iron deficiency, by conducting a Mendelian randomisation analysis using children with sickle cell trait (SCT) (HbAS, rs334); SCT was used as SCT is protective against malaria. The authors found that HbAS was associated with a 30% reduction in iron deficiency in children living in malaria-endemic countries in Africa (n=7453), but not among individuals living in malaria-free areas (n=3818). They observed an odds ratio of 2.65 (95% confidence interval 1.64-4.26) of developing iron deficiency per unit increase in the log incidence rate of malaria, suggesting that reducing the incidence of uncomplicated malaria by half would reduce the prevalence of iron deficiency in African children by 49% [118]. The authors hypothesised that hepcidin mediated block of iron absorption across enterocytes, with retention in the RES and hepatocytes, was the likely mechanism for the observed iron deficiency [118].

The other debate in the literature is whether iron deficiency is protective against malaria and whether hyperferraemia increases the risk of malaria, especially severe malaria. The removal of iron from the systemic circulation is increasingly recognised as an important response to infection in humans, as iron is required by many pathogens for their growth, development and pathogenicity [119]. This is certainly true in malaria, as the erythrocytic form of the *Plasmodium* parasite requires free iron for its development, however in iron deficiency there is hypoferraemia [119]. This hypoferraemia is described as functional iron deficiency and is regulated by hepcidin [119].

Murray *et al* [120] , commented first on the association of supplemental iron and the increased risk of malaria. This observational study reviewed the effect of refeeding on patients and relatives admitted to an Eastern Niger hospital during a drought on the frequency of malaria, whilst also measuring serum-iron and transferrin saturation [120]. It was noted that hyperferraemia which was associated with refeeding, led to an increased frequency of malaria [120]. The other sentinel paper by Sazawal *et al* [121] was a randomised placebo-controlled trial of dietary supplementation (iron, folic acid and zinc) in children under the age of 5 living on Pemba Island, Zanzibar. It was observed by the authors that in those who received iron and folic acid +/- zinc were 12% (CI 2-23, p=0.03) more likely to die or need treatment in hospital due to an adverse event and were 11% (1-23, p=0.03) more likely to be admitted to hospital, compared to the placebo group [121]. The conclusion was similar to Murray *et al* [121] in that iron and folic acid supplementation in areas with high rates of malaria can result in an increased risk of severe disease and death. The study was terminated early by the study data safety and management board following the above results, and further sub-group analysis highlighted that the adverse outcome of hospitalisation and/or death occurred in those children who were iron deficient when the iron supplementation was commenced [106,121]. Their recommendation was that iron supplementation should only be given in those areas where there were active malaria detection and treatment programmes [121]. The findings of this trial changed WHO policy from the general recommendations on dietary supplementation in 2003, to restricting it to only areas where there active prevention and treatment programmes in endemic regions [106,121]. The Cochrane review in 2016 by Neuberger *et al* [119] does appear to have a significant limitation, in that there were no standardised

markers for iron status across the analysed studies, therefore making it difficult to interpret the effect of iron supplementation on infection risk.

Clinical and field studies have observed that iron deficiency appears to be protective against malaria, with studies in children (Gwamaka *et al* [122], Nyakeriga *et al* [123], and Jonker *et al* [117]) and also in pregnant women (Kabyemela *et al* [124], and Senga *et al* [125]). It was also noted that iron deficiency reduced the risk of placental malaria, when compared to multiparity [124].

Iron deficiency appears to be protective against malaria through functional hypoferraemia and also because of the physiological differences between an iron-deficient and iron-replete erythrocytes. Functional hypoferraemia is upregulated by hepcidin in response to infection, in order to restrict systemic iron that can be utilised by pathogens [119]. The downside to the upregulation of hepcidin is the retention of iron within macrophages, which is beneficial to intracellular pathogens, such as *Salmonella* spp [119]. Ferroportin is the transmembrane that exports iron under the regulation of hepcidin, and the ferroportin Q248H mutation is partially resistant to hepcidin-induced degradation and is possibly associated with protection from the *Plasmodium* parasite [126].

Iron deficient erythrocytes appear to be harder for the *Plasmodium* parasite to invade [127]. The physiological differences between iron-deficient and iron-replete erythrocytes could account for this observation and these differences include greater osmotic fragility and membrane rigidity, accelerated ageing *in vivo*, lower haemoglobin concentration and microcytosis [127]. Clark *et al* [127] noticed that *in vitro* experiments, the parasitised erythrocyte multiplication rate (PEMR) was lower in *P. falciparum* infected erythrocytes that were from iron-deficient donors. They also observed that there were fewer merozoites when examined by microscopy, in the iron-deficient erythrocytes compared to the iron-replete erythrocytes [127]. Lastly, the authors also observed that *P. falciparum* growth was increased in iron-supplemented iron deficient erythrocyte group [127]. The increase in *in vitro* parasite growth with iron supplementation was also demonstrated by Goheen *et al* [128]. Goheen *et al* [128] observed that the population of reticulocytes was increased at day 49, which was when the increase in the parasite growth was observed. The author's explanation for this increase in parasite growth is that iron supplementation drives erythropoiesis releasing reticulocytes and younger

erythrocytes into the circulation and it is well documented that *P. falciparum* has a predilection for reticulocytes and younger erythrocytes [128]. Goheen *et al* [128] also confirmed the work by Clark *et al* [127] that erythrocytic invasion of merozoites was reduced in iron deficient erythrocytes and that iron supplementation increased invasion susceptibility.

2.4.5. Iron homeostasis in malaria

Most human pathogens require iron for their growth and have developed many strategies for competing with their human host for iron [92,129]. The mechanisms of how certain bacteria scavenge iron are well known. Examples include the highly pathogenic strains of Yersinia, which have developed a common high-pathogenicity island in their genome that codes for the siderophore yersiniabactin [92,129]. Bacterial siderophores are high-affinity iron-chelating compounds that compete for free iron in the human circulation [92].

The mechanisms by which protozoan species acquire iron are less well known and this is true of malaria. It is hypothesised that intra-erythrocytic parasites acquire iron from serum transferrin, the breakdown of haemoglobin or from free intra-cellular iron [70]. It is less well known how the liver or mosquito life stages of the parasite acquire iron. In response to malaria, hepcidin is upregulated, leading to an accumulation of iron within macrophages and a decrease in serum iron, which in turn contributes to dyserythropoiesis and subsequently malaria-associated anaemia [70]. The accumulation of iron within macrophages may also explain bacterial co-infection with non-typhoidal *Salmonella* and *Mycobacterium tuberculosis* [70].

Latour *et al* [130] investigated the contribution of erythroferrone to hepcidin repression in a murine model of malarial anaemia [130]. They inoculated mice with *Plasmodium berghei* K173 and observed that hepcidin levels were raised in the acute phase of infection (days 1 to 5), although they did not observe an associated rise in IL-6 or activin B mRNA expression [130]. IL-6 and activin B are known activators of hepcidin expression in inflammation [130,131]. Latour *et al* [130] also noted that, as the anaemia continued to develop in the mice, hepcidin concentration fell below baseline levels on day 13 to 18 [130]. The nature of hepcidin expression in malaria is also commented upon by Casals-Pascual *et al* [132]. They measured serum hepcidin levels on

100 Kenyan children presenting to a clinic and noted a raised serum hepcidin concentration on admission, followed by a drop up to a month later [132].

Latour *et al* [130] also noted that in their murine malaria model, EPO mRNA expression in the kidneys was upregulated on days 7 to 18 of infection compared to the control group. Other markers of erythropoiesis were also increased at the same time. The markers that were increased were spleen index (spleen weight relative to body weight) and glycophorin A [130]. Glycophorin A mRNA expression was reduced between days 2 and 5, which Latour *et al* hypothesised is related to the inhibition of erythropoiesis by the increase in circulating cytokines. ERFE mRNA expression was also raised with EPO [130]. Hepcidin levels were elevated in both wild-type and ERFE deficient mice up to day 7, however, hepcidin was supressed below baseline in the wild-type mice only with no significant blunting of the hepcidin suppression in the ERFE deficient mice [130]. There was an initial higher haemoglobin concentration in the ERFE deficient mice compared to the wild-type at day 10, although the subsequent drop in haemoglobin was most significant in the ERFE deficient group at day 13 [130]. Lastly the authors noted that EFRE deficient mice had lower parasitaemia when compared the wild-type group at day 13 [130]. The authors speculated that by preventing the complete suppression of hepcidin, the ERFE leads to transient restriction in iron during *P. berghei* infection and also impacts on parasitaemia [130]. They also hypothesise that ERFE deficiency is protective against the parasite invasion of erythrocytes during the early infection [130].

2.4.6. Liver function abnormalities in malaria and VIS and the role of iron metabolism

Raised liver enzymes have been observed in both *P. falciparum* and *P.vivax* IBSM VIS [133,134]. In the IBSM VIS, the parasites bypass the liver stage, due to the direct inoculation of erythrocytes infected with asexual-stage parasites. A common hypothesis for these observed elevations was thought to be drug-induced liver injury (DILI), although in Chughlay *et al* [133], other factors for these raised liver enzymes have been raised such paracetamol administration as an anti-pyretic, a pro-inflammatory response increasing a participants susceptibility to DILI and also the study therapeutics [133]. Odedra *et al* [134], also comment on the parasite clearance burden as a factor in developing an ALT of greater than double the upper limit of normal (ULN), with higher ALT values observed in those who had rapid parasite clearance and also high parasite

clearance burden. Odedra *et al* [134] also investigated changes in the liver enzymes in a malaria-endemic setting, with data from a previous study from patients in Thailand and Malaysia [68,134–136]. Odedra *et al* [134] found that raised liver enzymes following antimalarial treatment was less common in these studies, suggesting that previous exposure to malaria may modulate the host immune response and lead to a reduced inflammatory response, which may explain a lower frequency of raised liver enzymes compared to that observed in malaria-naïve individuals. Another hypothesis suggested by Odedra *et al* for increased susceptibility to liver enzyme elevations could genetic polymorphisms, such polymorphisms in haptoglobin heme-oxygenase-1, or possibly iron deficiency [134,137,138].

In natural infection, one study by Reuling *et al* [139] reported raised liver enzymes in 69% of imported uncomplicated malaria cases, with 64% under 2.5x ULN. In those observed cases, the raised liver enzymes peaked quickly after drug administration, returning to normal in 3-6 weeks post treatment. In clinical infection, Reuling *et al* [139], state the observed liver injury appears to have an hepatocellular origin due to the disproportionate rise in liver transaminases compared to alkaline phosphatase. with Woodford *et al* [140] observed peak ALT occurred 4-11 days post treatment. Bilirubin appears to peak prior to treatment in natural infection compared to VIS [139,140], with Woodford *et al* [140] further commenting that the burden of haemolysis does not appear to modulate the timing of transaminase elevations. The choice of treatment or when treatment commenced post diagnosis did not appear to be correlated with the timing of peak elevations in the liver transaminases. With elevations in the liver enzymes occurring 6-7 days post-treatment in both natural infection and VIS participants, who do not undergo the liver stage of infection, the contribution of the liver stage to the observed elevations in liver enzymes is likely to be small [139,140]. In uncomplicated *P. falciparum* infection the mechanism is likely to be related to tissue hypoxia and deranged metabolic processes related to parasite sequestration [139], whereas with low parasitaemia in VIS the pathogenesis of liver injury is likely to be driven by systemic inflammation [139].Systemic inflammation due to an increase in oxidative stress and heme-mediated damage, promoted by tumour necrosis factor have been hypothesised as likely causes of the rise in liver transaminases [134,140]. In Odedra *et al* [134] , they illustrated that a higher maximum CRP was associated with an increased risk of a peak ALT 2.5xULN [134].

A possible mechanism for elevation in liver transaminases in malaria could be the intrahepatic storage of iron due to the upregulation of hepcidin. Hepcidin is increased in the early stages of malaria, corresponding to the increase in CRP [94]. The hepcidin binds to ferroportin and prevents the absorption of iron from the gut via the enterocytes, and also leads to the retention of iron in the reticuloendothelial system and hepatocytes [94]. As a consequence, iron is retained in the liver and spleen, causing inflammation and local tissue destruction [141,142]. This could also be compounded by the scavenging of necrosed hepatocytes by Kuppffer cells, with these iron-loaded macrophages and causing secondary iron loading in the liver thus exacerbating the local liver injury [141,142]. Once hepcidin levels decrease in response to the reduction in parasites and the reduction in pro-inflammatory cytokines such as IL-6, iron is released from the reticuloendothelial system, liver and enterocytes to drive an increase in erythropoiesis. This release of iron from the liver to drive erythropoiesis, reduces the iron-overload in the liver and subsequently the reduction in liver enzymes.

Iron-overload in tissues was traditionally measured via a liver biopsy, however non-invasive methods can be used such as magnetic resonance imaging [143]. Cippa *et al* [143], investigated iron overload in patients with suspected liver disease in the presence of hyperferritinaemia (>300 μg/L), and observed that the ferritin/AST ratio was a good estimator of hepatic iron levels. The ferritin/AST ratio was strongly correlated with hepatic iron concentration ($r= 0.47$, $p<0.001$), with a cut off of 17μg/U giving a sensitivity of 83.3% and specificity of 78.6% [143]. On receiver operator curve analysis between the ferritin/AST ratio and transferrin saturation, the ferritin/AST ratio had a significantly larger AUC than the transferrin saturation (0.83 vs 0.62; $p=0.001$) [143]. The sample population in Cippa *et al* [143] was low, n=147 [143], however this estimator of hepatic iron concentration could be used in the VIS population, as ferritin and liver transaminases are routinely collected in the IBSM participants.

Chapter 3. Development and evaluation of a new *Plasmodium falciparum* 3D7 blood stage malaria cell bank for use in malaria volunteer infection studies

3.1. Introduction

Due to diminishing supplies of the existing *P. falciparum* 3D7-V2 malaria cell bank (MCB), there was an urgent requirement to develop a new MCB. In the original development of the induced blood-stage model (IBSM) of malaria volunteer infection studies (VIS) in 1995, two MCBs were developed, however only Pf3D7-V2 was taken forward to higher parasitaemia levels [54]. This new MCB was manufactured using a bioreactor as has been previously been described [56,144]. The development and evaluation of this novel MCB increases our knowledge about safety of the IBSM VIS and adds data to the haematology database which will be used in the retrospective analysis, as well as the pilot study evaluating markers of iron metabolism.

3.2. Declaration

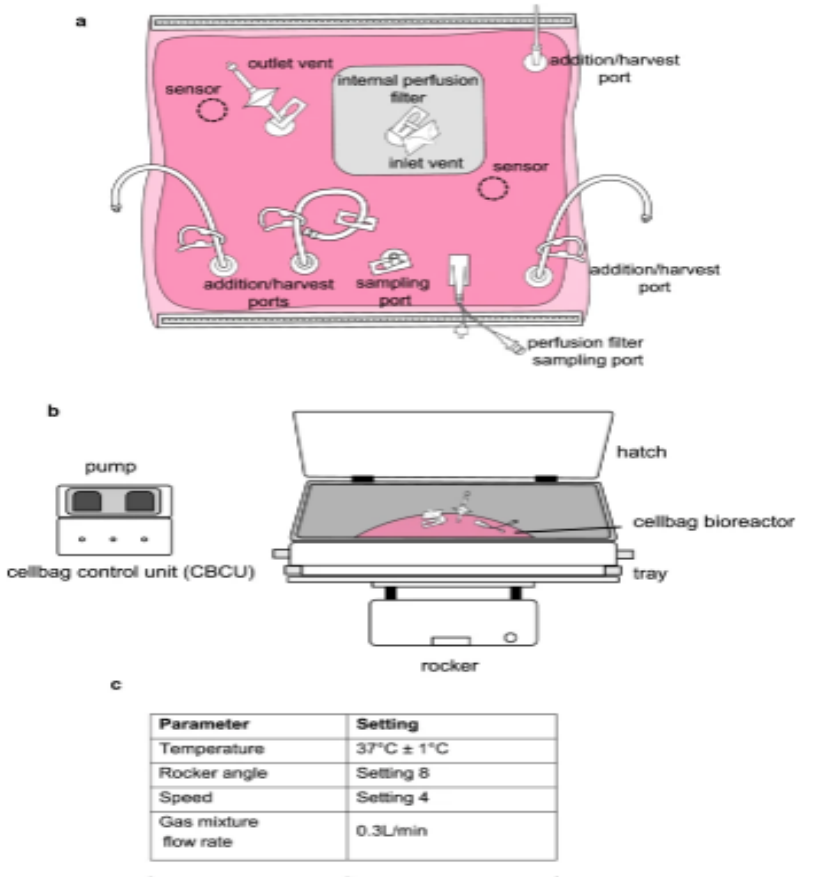
The study was designed by Stephen Woolley (author), Melissa Fernandez, Rebecca Watts, Maria Rebello, Katharine Trenholme, Louise Marquart, James McCarthy and Bridget Barber. Bridget Barber was the principal investigator, with James McCarthy as co-investigator and Stephen Woolley as sub-investigator. Maria Rebello, Fiona Amante, Helen Jennings were responsible for the production of the inocula. The data analysis was conducted by Stacey Llewellyn, Louise Marquart, Bridget Barber and Stephen Woolley. Joerg Moehrle and Stephan Chalon provided expert opinion and sat on the study safety committee. All other authors contributed and participated in the manuscript preparation, with Stephen Woolley writing and co-ordinating the final manuscript.

3.3 Bioreactor

In this study the Ready to Process WAVE 25^{TM} system, a system which had been used in a previous study was utilised [144]. A vial of the 3D7-V2 bank was thawed and subsequently cultured under standard malaria culture conditions in an atmosphere of 5% CO2, 5% O2 and 90% nitrogen at 37^{o}C with the media changed every 24 hours. The parasite culture was then expanded to a volume of 100 mL, subsequently then being transferred to the 2 L cell bag of

the WAVE 25^{TM} bioreactor after the parasites were synchronised using a single treatment of 5% sorbitol.

The Ready to Process WAVE 25^{TM} system is a system that contains a pre-sterile single use cultivation cell bag, with inlets and outlets to allow for addition of media and extraction of samples. The cell bag is then placed on a temperature-controlled rocking platform, which induces a wave motion in the cell bag.



c

Parameter	Setting
Temperature	37°C ± 1°C
Rocker angle	Setting 8
Speed	Setting 4
Gas mixture flow rate	0.3L/min

Wave bioreactor configuration. **a** Each cellbag has built-in inlet and outlet air filters, and ports that allow the addition of culture medium and extraction of samples. **b** The Ready to Process WAVE 25™ system (GE Healthcare) was used for this study. **c** Temperature, rocking speed, rocking angle and atmospheric conditions are monitored and controlled by the cellbag control unit (CBCU)

Figure 3.1. Wave 25TM bioreactor system. Pawliw et al [144]

3.4 Alloimmunisation

In the IBSM trials an important risk for volunteers to understand is the risk of red cell alloimmunisation, as the inoculum is a blood product. This is the process when an individual is lacking a certain blood group antigen is exposed during transfusion or pregnancy [145]. The formation of new red cell antibodies maybe clinically significant, potentially leading to delayed haemolysis, serological transfusion reactions or haemolytic disease of the foetus and newborn. Factors that may trigger this event include donor factors (ethnicity, inflammation and good vs poor storer), recipient factors (age, ethnicity, antigen recognition, immune status, inflammation and prior antigen exposure), erythrocyte unit factors (storage age and solution, component modifications, and cellular damage) and finally intrinsic erythrocyte antigen factors (immunogenicity and density/copy number) [145,146].

Over 400 individuals have undergone the IBSM VIS at QIMR, with only 3 individuals developing erythrocyte alloimmunisation. One individual developed alloimmunisation, although following the data and safety monitoring board (DMSB) in conjunction with two independent clinical haematologists, concluded there was no evidence it was caused by the inoculum. The two other individuals were part of a vaccine trial utilising a genetically modified IBSM inoculum as a possible vaccine and had received a higher inoculum dose which contained a higher volume of erythrocytes. These two individuals developed minor antibody class reactions as determined by the DMSB and independent haematologists. The trial was subsequently terminated early due to the issue of red cell alloimmunisation. Those individuals were reviewed by a transfusion haematologist, given warning cards and notes were placed via the primary care physician and onto the Australian Red Cross Blood Transfusion system.

The team have several mitigations in place to reduce the risk of red cell alloimmunisation. These measures are as follows:

- The major malaria cell banks used are O rhesus negative
- The dose of erythrocytes in a 2 mL dose of *P. falciparum* 3D7-V2 parasites was $2.6x10^8$, which 4 log lower than in a standard unit of packed red cells used in transfusion

- The inoculum is leucodepleted to reduce the risk of a major histocompatibility complex (MHC) reaction
- In the development of new malaria cell banks all participants are followed with red cell antibody testing up to 90- days post the administration of the inoculum
- All volunteers are informed of the risk of red cell alloimmunisation in the consenting process.

3.5 Paper

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Development and evaluation of a new *Plasmodium falciparum* 3D7 blood stage malaria cell bank for use in malaria volunteer infection studies

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Abstract

Background: New anti-malarial therapeutics are required to counter the threat of increasing drug resistance. Malaria volunteer infection studies (VIS), particularly the induced blood stage malaria (IBSM) model, play a key role in accelerating anti-malarial drug development. Supply of the reference 3D7-V2 *Plasmodium falciparum* malaria cell bank (MCB) is limited. This study aimed to develop a new MCB, and compare the safety and infectivity of this MCB with the existing 3D7-V2 MCB, in a VIS. A second bank (3D7-V1) developed in 1995 was also evaluated.

Methods: The 3D7-V2 MCB was expanded in vitro using a bioreactor to produce a new MCB designated 3D7-MBE-008. This bank and 3D7-V1 were then evaluated using the IBSM model, where healthy participants were intravenously inoculated with blood-stage parasites. Participants were treated with artemether-lumefantrine when parasitaemia or clinical thresholds were reached. Safety, infectivity and parasite growth and clearance were evaluated.

Results: The in vitro expansion of 3D7-V2 produced 200 vials of the 3D7-MBE-008 MCB, with a parasitaemia of 4.3%. This compares to 0.1% in the existing 3D7-V2 MCB, and < 0.01% in the 3D7-V1 MCB. All four participants (two per MCB) developed detectable *P. falciparum* infection after inoculation with approximately 2800 parasites. For the 3D7-MBE-008 MCB, the parasite multiplication rate of 48 h (PMR_{48}) using non-linear mixed effects modelling was 34.6 (95% CI 18.5–64.6), similar to the parental 3D7-V2 line; parasitaemia in both participants exceeded 10,000/mL by day 8. Growth of the 3D7-V1 was slower (PMR_{48} of 11.5 [95% CI 8.5–15.6]), with parasitaemia exceeding 10,000 parasites/mL on days 10 and 8.5. Rapid parasite clearance followed artemether-lumefantrine treatment in all four participants, with clearance half-lives of 4.01 and 4.06 (weighted mean 4.04 [95% CI 3.61–4.57]) hours for 3D7-MBE-008 and 4.11 and 4.52 (weighted mean 4.31 [95% CI 4.16–4.47]) hours for 3D7-V1. A total of 59 adverse events occurred; most were of mild severity with three being severe in the 3D7-MBE-008 study.

Conclusion: The safety, growth and clearance profiles of the expanded 3D7-MBE-008 MCB closely resemble that of its parent, indicating its suitability for future studies. *Trial Registration:* Australian New Zealand Clinical Trials registry numbers: P3487 (3D7-V1): ACTRN12619001085167. P3491 (3D7-MBE-008): ACTRN12619001079134

Keywords: *Plasmodium falciparum*, Induced blood-stage malaria, CHMI, VIS, Malaria

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Background

Malaria continues to cause major morbidity and mortality worldwide, with current control measures being threatened by the spread of artemisinin-resistance in the Greater Mekong Subregion [1–3]. New anti-malarial drugs and vaccines are, therefore, urgently required. The current anti-malarial drug pipeline has been accelerated by the use of human volunteer infection studies (VIS) [4–8], particularly the induced blood-stage malaria model (IBSM) [5, 6]. In these studies, healthy, malaria-naïve participants are inoculated with *Plasmodium*-infected erythrocytes, enabling the assessment of the blood stage schizont activity of antimalarial drug candidates [5, 6, 9–11]. As of March 2020, 401 volunteers have been inoculated with the *Plasmodium falciparum* 3D7 clone, most at QIMR Berghofer in Brisbane, Australia (n = 335), but some at sites in the Netherlands and UK (n = 66) [6, 12–16].

The current QIMR Berghofer malaria cell bank (MCB) used to inoculate volunteers with *P. falciparum*, termed 3D7-V2, was produced in 1995 [17–19]. At that time, two volunteers were experimentally infected by mosquito bite with *Plasmodium falciparum* 3D7, and 500 mL of blood was collected from each volunteer six hours following the onset of fever [19]. Although two MCBs were produced (3D7-V1 and 3D7-V2), the higher parasitaemia in the 3D7-V2 bank (0.1% compared to < 0.01% of erythrocytes parasitized, respectively) has led to this bank being utilized in subsequent malaria VIS. The 3D7-V1 has been utilized only once, for re-inoculation into the original donor [19].

Stocks of the *P. falciparum* 3D7-V2 MCB are limited, therefore, further MCBs are required to ensure an ongoing supply of this valuable resource. The development of further banks can be undertaken by collection of samples from malaria-infected patients or experimentally infected volunteers [17]. An alternative approach is the in vitro manufacture of banks using a bioreactor, such as the Wave ™ 25 bioreactor system [17]. This method has been used previously to produce and test in vivo two cell banks, a genetically modified *P. falciparum* blood stage-cell bank [17], and an artemisinin-resistant *P. falciparum* cell bank [20]. This proved to be a cost-efficient method for the production of a MCB for use in IBSM studies [17, 21]. This method also allows for blood group selection of the MCB.

The development of a new MCB, 3D7-MBE-008 (MBE-008), using this biomanufacture process, and the clinical evaluation of this MCB is reported. Safety, infectivity and parasite growth and clearance of the 3D7-MBE-008 and the previous 3D7-V1 were compared to the existing data on the 3D7-V2 bank.

Methods

Development of 3D7-MBE-008 Master Cell Bank

The 3D7-MBE-008 MCB was manufactured in accordance with Good Manufacturing Practice standards [22] in 2015 using the previously described method [17]. In brief, a single vial of the 3D7-V2 MCB was thawed and expanded using the bioreactor. Erythrocytes used in the production of the MCB were from a single blood group O Rh (D) negative donor, provided by Lifeblood (formerly Australian Red Cross Blood Service). The donor was screened in accordance with TGA regulatory requirements for donation of blood for transfusion. Pooled, heat inactivated serum collected from donors by Key Biologics (Memphis, Tennessee, U.S.) used in the manufacturing process was also extensively screened. The final 3D7-MBE-008 culture was cryopreserved with Glycerolyte 57 in 1:2.2 ratio, and aliquoted to produce 200 1 mL cryovials, which were stored between −140 and −196 °C in secure, monitored vapour phase liquid nitrogen tanks at Q-Gen Cell Therapeutics, Brisbane, Australia.

Laboratory testing of the Master Cell Banks

The percentage of parasitized erythrocytes and the percentage of ring-stage parasites were determined via thin film microscopy for 3D7-MBE-008 and thick film microscopy for 3D7-V1. Testing for microbial contamination was performed in line with the British Pharmacopoeia Appendix XVI E- microbial contamination of cellular products [23].

Parasite viability of 3D7-MBE-008 was determined using flow cytometry as previously described [20] at the time of manufacture and then in an ongoing stability and sterility program, with testing every 12 months. The viability of the parasites in the 3D7-V1 bank was determined at the time of manufacture by limiting dilution assay followed by PCR as previously described [19].

For confirmation of parasite identity, the DNA sequence of three widely used hypervariable genes (*P. falciparum* merozoite surface protein-1 [*Pf* MSP-1], *Pf* MSP-2 and *Pf* glutate-rich protein [*Pf* GLURP]) from 3D7-MBE-008 were compared to 3D7-V2. In vitro drug sensitivity testing to nine antimalarials was also undertaken as previously described [19].

Inoculum preparation

The viability of ring stage parasites in the MBE-008 MCB was assessed by flow cytometry as previously described [20], to identify the dilution required to achieve an inoculum dose similar to 3D7-V2 MCB. To prepare the inoculum, one or more vials of the MCB were thawed, with the resulting red cell pellet washed and resuspended in 0.9% sodium chloride. The washed cell suspension was then

diluted with 0.9% sodium chloride to achieve the target number of viable ring-stage parasites in each 2 ml inoculum, taking into account the characteristics of each MCB including the percentage of parasitized erythrocytes, the percentage of ring-stage erythrocytes, and parasite viability. The number of parasites in the final inoculum was verified by 18S quantitative PCR targeting the *P. falciparum* 18S rRNA gene (qPCR) [24] with results available after inoculation.

Clinical study design

Two concurrent IBSM studies were conducted, one with the 3D7-V1 MCB and the other with 3D7-MBE-008. Each study consisted of two single-participant cohorts, with a 5-week period between cohorts. The primary objective of both studies was safety. Secondary objectives included infectivity, parasite growth and clearance, the latter following administration of artemether-lumefantrine.

The studies were conducted at Q-Pharm Pty Ltd, Brisbane, Australia. Ethical approval was given by QIMR Berghofer Human Research Ethics Committee (HREC), and by Lifeblood HREC for *P. falciparum* 3D7-MBE-008. All participants gave written informed consent before enrolment. Both studies were registered with the Australian New Zealand Clinical Trials registry; 3D7-V1 (ACTRN12619001085167) and 3D7-MBE-008 (ACTRN12619001079134).

Participants

Participants were eligible if they were aged 18–55 years, healthy and malaria-naïve (see Additional file 1: Appendix for inclusion and exclusion criteria). For the 3D7-V1 study, only males who were blood group Rh(D) positive were eligible, due to the fact that the 3D7-V1 MCB donor was Rh(D) positive.

Procedures

All participants were inoculated with approximately 2800 viable infected erythrocytes on Day 0. Parasitaemia was monitored by 18S qPCR daily [24] from Day 4 until parasites were detected, then twice daily until artemether-lumefantrine (20 mg artemether/ 120 mg lumefantrine; Novartis Pharmaceuticals Pty Ltd) was given, and then at specified timepoints post treatment until qPCR was negative (Additional file 1: Table S1). Figure 1a illustrates the study design for 3D7-V1 MCB and Fig. 1b illustrates the study design for 3D7-MBE-008 MCB. The protocol specified that a curative course of artemether-lumefantrine, consisting of 6 doses of 4 tablets over a 60-h period, would be given when the participants' parasitaemia exceeded 10,000 parasites/mL, or the participants' malarial clinical score was ≥ 6 (see Additional file 1 for calculation of malaria clinical score). However, due to slower than expected parasite growth in the first subject inoculated with 3D7-V1, and lack of any clinical symptoms in the participant at a parasite count of 10,000 parasites/mL., the 3D7-V1 protocol was amended to change the parasite treatment threshold to 100,000 parasites/mL.

Safety assessments

Safety was evaluated by recording all adverse events as well as any abnormal laboratory results. Investigations were performed at the timepoints specified in Additional file 1: Table S1. During every outpatient visit and during confinement, a malaria clinical score for each participant was generated. A graded assessment of symptoms and laboratory results was used (see Additional file 1).

Parasite growth and clearance

The parasite multiplication rate of 48 h (PMR_{48}) for each MCB was calculated by applying the pre-treatment qPCR data to a sine-wave growth model, estimated using a non-linear mixed effects model in R Statistical package 3.6.1, as previously reported [25]. The PMR_{48} for each MCB was then presented as an estimate with a 95% CI. To determine if there were any significant differences between the growth model parameter estimates from the new MCBs and the previously used 3D7-V2 MCB, an omnibus test for between-group differences was used [26]. The sine- wave growth model estimated using a non-linear regression model was also used to retrospectively calculate the parasite growth characteristics of 3D7-V1 in the initial volunteer re-infected with his isolate (3D7-V1) in 1995. The parasite clearance profiles post artemether-lumefantrine treatment for each participant were estimated from the slope of best fit of the parasite clearance rate and transformed to estimate the parasite reduction ratio (PRR) per 48 h in the logarithmic-scale ($\log_{10}PRR_{48}$) and the parasite clearance half-life as previously reported [27], using R Statistical package 3.6.1. Parasite clearance parameters for each bank are summarized as a weighted mean and corresponding 95% CI estimated using the inverse variance method as detailed in [27].

Results

MCB characteristics

The blood used for the biomanufacture of 3D7-MBE-008 tested negative for microbial contamination and for serologic evidence of infective agents. Manufacture was completed in November 2015 and produced 200 vials. The analysis of the three genetic markers (*Pf msp*-1, *Pf msp*-2 and *Pf glurp*) showed that no changes had taken place between the starting 3D7-V2 MCB and resulting 3D7-MBE-008 MCB, ruling out high level genetic change during the biomanufacturing process. The in vitro drug sensitivity of the 3D7-MBE-008 MCB showed the same

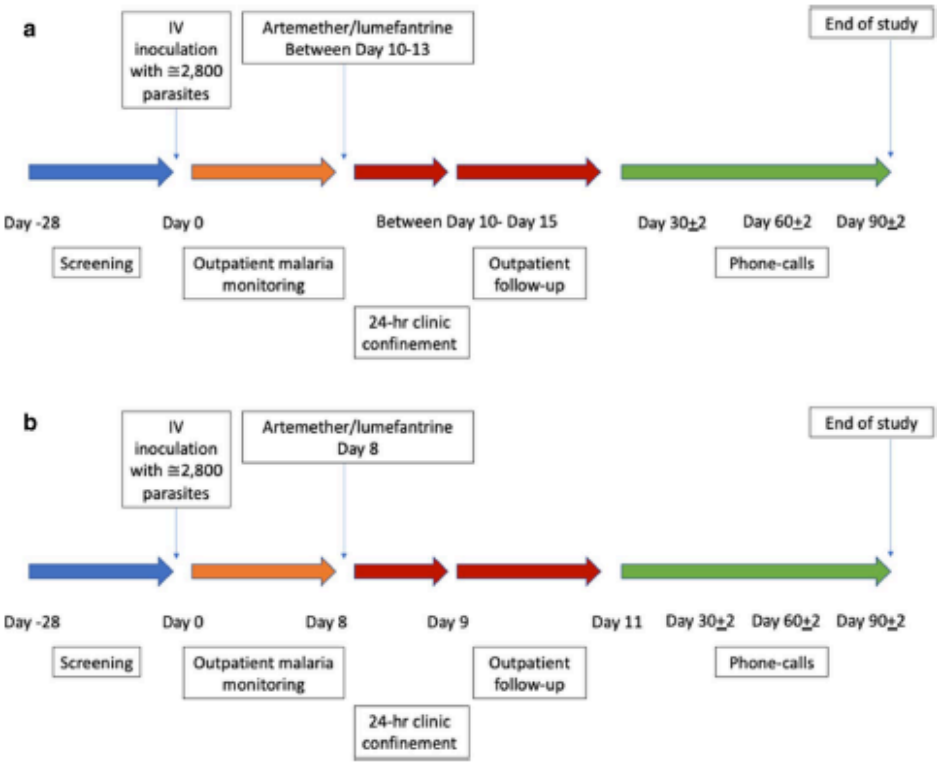

Fig. 1 Study Design. In both studies, participants were inoculated with approximately 2800 infected erythrocytes on Day 0, followed by artemether-lumefantrine treatment when threshold parasitaemia was reached (> 10,000 parasites/mL). The participants were confined for 3 days, during which they were treated with artemether-lumefantrine. The last study visit occurred on Day 90 ± 2. For the 3D7-V1 study, the confinement period occurred between Day 10–13 (Panel **a**). For the 3D7-MBE-008 study, the confinement period commenced on Day 8 (Panel **b**)

drug sensitivity profile as the established parental line to nine antimalarials (sensitive to amodiaquine, atovaquone, artemisinin, chloroquine, lumefantrine, piperaquine, pyronaridine and quinine; resistant to mefloquine). The parasite concentration of 3D7-MBE-008 MCB was 4.3%, with 96% of parasites in ring-stage.

Viability of the 3D7-MBE-008 MCB at 12, 24 and 36 months (prior to use) was 83%, 71% and 63%, respectively. Microbial contamination testing at these time points was negative. The parasitaemia of 3D7-V1 at the time of collection was 0.01%. The viability as measured by limit dilution and PCR [19] at the time of manufacture was approximately 34%.

Study participants

The studies were conducted between August 2019 and December 2019.Two participants were enrolled into each study. A total of 22 potential volunteers were screened for the 3D7-MBE-008 study and 19 for 3D7-V1. All four inoculated participants completed the study and are included in the safety and parasite profile analysis. The participants for the 3D7-MBE-008 study were a 32-year-old white male and a 31-year-old white non-pregnant female. The participants for the 3D7-V1 study were both 19-year-old white males.

Inoculation and parasite growth

In the 3D7-MBE-008 study, the number of parasites in each of the inocula, determined retrospectively by 18S qPCR, was 18,700 and 23,100. Both participants had parasitaemia detectable by 18S qPCR on Day 4 and reached threshold parasitaemia (> 10,000 parasites/mL) on Day 8, when artemether-lumefantrine was commenced. The parasite counts prior to treatment were 141,416 parasites/mL and 480,871 parasites/mL, with peak parasitaemia reached for both subjects at 2 h post administration of artemether-lumefantrine (239,278 parasites/mL and 563,886 parasites/mL) (Fig. 2). Using the non-linear mixed effects model estimates, the estimated PMR_{48} was 34.6 (95% CI 18.5–64.6) (Table 1), similar to

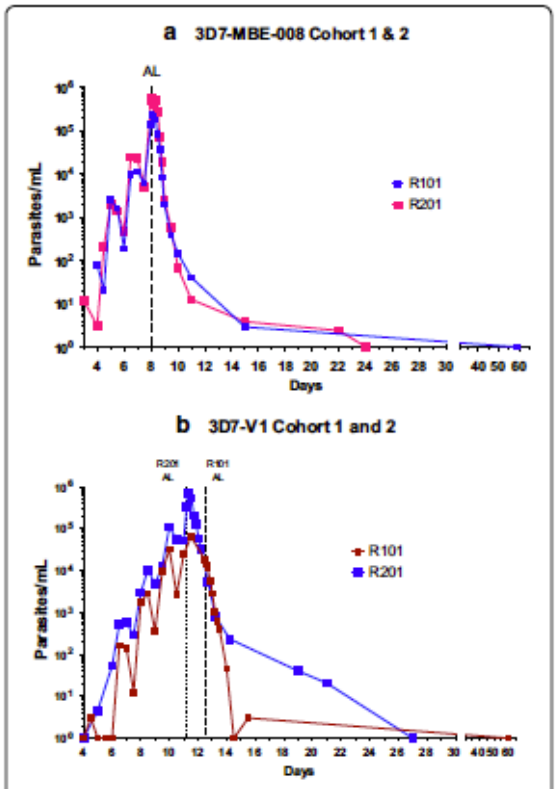

Fig. 2 Parasite growth and clearance after artemether-lumefantrine (AL) treatment Parasitaemia from Day 4 to Day 60 for the 3D7-MBE-008 study (Panel **a**). Parasitaemia from Day 4 to Day 60 for the 3D7-V1 study (Panel **b**). The vertical dashed lines represent the administration of artemether-lumefantrine treatment

that reported in a large meta-analysis of the growth rate of 3D7-V2 in previous VIS, which is 31.9 (95% CI 28.7–35.4) [25]. There were no significant differences between the growth parameters of 3D7-MBE-008 (n = 2) and 3D7-V2 (n = 177) (see Additional file 1: Table S2).

In the 3D7-V1 study, the number of parasites in each inocula, determined retrospectively by qPCR, was approximately 3050 and 2694 respectively. Participant one developed detectable parasitaemia on day 5, whereas the second participant had detectable parasitaemia on day 4. The parasitaemia in both participants increased more slowly than those inoculated with 3D7-MBE-008, despite them being derived from the same parental 3D7 clone; on day 8, when treatment was expected to be required, the parasitaemias of the two participants were only 1776 parasites/mL and 2982 parasites/mL, respectively, with malaria clinical scores of zero in both participants. An urgent protocol amendment was approved by the HREC to allow treatment to be administered at a parasite threshold of > 100,000 parasites/mL, and the two participants were, therefore, treated on days 12 (first participant) and day 11 (second participant). For the first participant the pre-treatment parasite count was 17,699 parasites/mL, and the peak parasite count (day 11) was 64,786 parasites/mL. For the second participant, the pre-treatment parasite count was 340,789 parasites/mL, and the peak parasite count (occurring 4 h post artemether-lumefantrine) was 742,813 parasites/mL. The estimated PMR_{48} for 3D7-V1 was 11.5 (95% CI 8.5–15.6) (Table 1). The primary parasitaemia data from the original 3D7-V1 subject that had been calculated using a different method [19] was retrieved. Using these data in the sine wave growth model, the PMR_{48} for 3D7-V1 in the initial volunteer re-infected in 1995 was 6.4 (95% CI 4.6–8.8) (Table 1). Due to the heterogeneity in the method of calculation of parasitaemia between these studies, a combined analysis was not performed. The tests of heterogeneity of the individual growth parameters of the 3D7-V1 and existing 3D7-V2 MCB showed a significant difference between the parasite growth rate ($p < 0.001$)

Table 1 Summary of parasite growth and clearance characteristics

Parameter	3D7-V1 (n = 2) Estimate (95% CI)	3D7-MBE-008 (n = 2) Estimate (95% CI)	3D7-V1 1995 (n = 1) Estimate (95% CI)	3D7-V2 (n = 177) Estimate (95% CI) [25]
PMR_{48}	11.5 (8.5–15.6)	34.6 (18.5–64.6)	6.4 (4.6–8.8)	31.9 (28.7–35.4)
Lifecycle (hours)	36.5 (35.0–38.0)	38.8 (36.7–40.8)	42.5 (39.2–45.8)	38.8 (38.3–39.2)
Growth rate (log_{10} parasites/day)	0.52 (0.30–0.74)	0.80 (0.54 –1.10)	0.35 (0.13–0.58)	0.63 (0.59–0.66)
PRR_{48}	2254 (1705–2979)	3806 (1446–10018)	_	_
log10 PRR_{48}	3.35 (3.23–3.47)	3.58 (3.16–4.00)	_	_
Parasite clearance half life	4.31 (4.16–4.47)	4.04 (3.61–4.57)	_	_

n, number of participants; CI, 95% confidence intervals; PMR, parasite multiplication rate; PRR, parasite reduction ratio. Growth parameter estimates are calculated from a non-linear mixed effects model. Clearance parameter estimates (PRR_{48}, log_{10} PRR_{48} and parasite clearance half-life) are calculated from a weighted mean of individual clearance parameters estimates

and parasite lifecycle ($p \leq 0.001$) (Additional file 1: Table S3).

Parasite clearance

Parasitaemia cleared in all four participants following the administration of artemether-lumefantrine. In the 3D7-MBE-008 study, the parasite clearance half-lives in the two participants were 4.01 and 4.06 (weighted mean 4.04, 95% CI 3.61–4.57) hours, and the $\log_{10}PRR_{48}$'s were 3.60 and 3.56 (weighted mean 3.58, 95% CI 3.16–4.00) h. In the 3D7-V1 study, the parasite clearance half-lives in the two participants were 4.11 and 4.52 (weighted mean 4.31, 95% CI 4.16–4.47) hours and the $\log_{10}PRR_{48}$'s were 3.52 and 3.20 (weighted mean 3.35, 95% CI 3.23–3.47) h in the 3D7- V1 study (Table 1).

Adverse events

There were 35 reported adverse events in the 3D7-MBE-008 study and 24 in the 3D7-V1 study (see Table 2). In the 3D7-MBE-008 study the majority of the adverse events were mild or moderate (32/35, 91.4%) and attributable to early malaria (25/35, 71.4%); three were graded as severe (two episodes of lymphopenia; see below) and one of raised alanine transaminase [ALT]; see below). In the 3D7-V1 study, the majority of adverse events were mild (14/24, 58.3%), with the remainder being moderate; nearly all were attributable to early malaria (23/24, 95.8%). There were no serious adverse events reported in either study. The most common adverse events reported across both studies were chills (n = 6), headaches (n = 6) and myalgia (n = 7). One of the participants in the 3D7-MBE-008 study had a maximum malaria clinical score of 9 (8 h post artemether/lumefantrine) (see Table S4). One of the participants in the 3D7-V1 study had a maximum score of 8 (36 h post artemether/lumefantrine) (see Additional file 1: Table S5).

One participant in each study developed a raised ALT. One participant in the 3D7-MBE-008 study had a peak ALT on Day 11 of 191 U/L (4.8 × ULN [upper limit of normal]) which normalized by Day 59. The peak aspartate aminotransferase (AST), also on Day 11, was 122 U/L (3.1 × ULN) and it normalized on Day 15. The bilirubin was normal. In the 3D7-V1 study, one participant had a peak ALT on Day 14 of 128 U/L (3.2 × ULN) which normalized by Day 27. The AST and bilirubin were not significantly raised.

Three participants developed transient falls in white cell counts that were classified as moderate or severe: lymphopenia (3D7-V1, nadir of 0.42×10^9/L, lower limit of normal [LLN] = 1.0×10^9/L]; 3D7-MBE-008, nadir of 0.34×10^9/L and 0.39×10^9/L); neutropenia (3D7-MBE-008 n = 1, 1.35×10^9/L, LLN = 1.5×10^9/L). These transient reductions were attributed to malaria.

Table 2 Adverse events reported during the studies

Adverse event	3D7-V1 (N = 2) n (M)	3D7-MBE-008 (N = 2) n (M)
Systemic		
Arthralgia	2 (2)	0 (0)
Chills	2 (3)	2 (3)
Decreased appetite	1 (1)	1 (1)
Fatigue	1 (2)	0 (0)
Feeling hot	1 (1)	1 (1)
Headache	2 (3)	2 (3)
Lethargy	0 (0)	1 (1)
Malaise	1 (1)	0 (0)
Myalgia	2 (3)	2 (4)
Pyrexia	2 (2)	2 (3)
Sweating	2 (2)	1 (3)
Tachycardia	1 (1)	1 (2)
Laboratory abnormalities		
ALT increased	1 (1)	1 (1)
AST increased	0 (0)	1 (1)
Lymphocyte count decreased	1 (1)	2 (1)
Neutrophil count decreased	0 (0)	1 (1)
Gastrointestinal		
Abdominal pain	0 (0)	2 (2)
Constipation	0 (0)	1 (1)
Diarrhoea	0 (0)	2 (2)
Other		
Back pain	0 (0)	1 (1)
Erythema (from tape)	0 (0)	1 (1)
Pain (venepuncture site)	0 (0)	1 (1)
Ulcer (lip)	0 (0)	1 (1)
Upper respiratory tract infection	1 (1)	0 (0)

N, total number of participants in each cohort; n, number of participants reporting the adverse event; M, number of occurrences of adverse events. Adverse events were coded to System Organ Class and Preferred Term using MedDRA Version 20.1

Discussion

Here we report the manufacture and evaluation of a new *P. falciparum* MCB that can be utilized in future IBSM studies. In vivo testing of 3D7-MBE-008 MCB, and the previously manufactured 3D7-V1 MCB, indicated that they were well tolerated in healthy, malaria-naïve participants. The PMR_{48} for the 3D7-MBE-008 MCB was comparable to the existing 3D7-V2 MCB. In contrast, the 3D7-V1 MCB had a slower PMR_{48}, with the parasitaemia of one participant not exceeding 10,000 parasites/mL until Day 10, two days later than generally occurs with 3D7-V2.

The parasite growth parameters of the 3D7-V1 in this study were similar to those obtained when the same non-linear growth model was applied to the data from the initial donor re-infected with 3D7-V1 in 1995 [19, 25]. The estimated PMR_{48} in the two subjects in this trial was 11.5 (95% CI 8.5–15.6) compared to 6.4 (95% CI 4.6–8.8) in the original volunteer inoculated in 1995. This suggests that loss of viability of the parasites after cryopreservation for over twenty years was not the reason for the slower PMR.

One possible explanation for slower than expected growth of the 3D7-V1 MCB was the lower number of infected red cells in this inoculum. Because the parasite concentrations in the individual MCBs were substantially different (<0.01% vs 4.3% for 3D7-V1 MCB and 3D7-MBE-008, respectively), the 3D7-MBE-008 participants likely received a higher mean dose of erythrocytes infected with ring stage parasites. Alternatively, host factors may have played a factor. The participants in the 3D7-V1 study were younger than those in the MBE-008 study; however, analysis of data from previous studies has not found an association between age and PMR_{48} [25].

In addition to the slower growth rate of the 3D7-V1, another limitation for the 3D7-V1 MCB is that the original donor is Rh(D) positive, thus precluding Rh negative females from enrolling in studies using this isolate due to the risk of red cell allo-immunization. As a consequence of this and the associated slower PMR, it is likely that the 3D7-V1 MCB will be less suitable for use in further IBSM studies.

The adverse events reported for both studies are in keeping with previous IBSM studies [9–11, 28, 29]. The asymptomatic raised liver enzymes, with no associated significant rise in bilirubin, have been reported in previous IBSM studies [30, 31], sporozoite VIS [32] and in naturally occurring malaria [30, 33]. Similarly, the reduction in white cell counts, especially lymphopenia and neutropenia have previously been reported in IBSM VIS, sporozoite VIS and clinical malaria [12, 16, 34–36].

Both MCBs had similar parasite clearance profiles, with no recrudescence, further confirming that both MCBs are safe to use in malaria VIS. With the development of the biomanufactured MCB, there are now two further *P. falciparum* 3D7 MCBs that can be used in IBSM VIS. These are in addition to previously developed MCBs of non-falciparum species including *Plasmodium vivax* and *Plasmodium malariae* [11, 37, 38]. The development of these MCBs in falciparum and non-falciparum species may in future also enable IBSM VIS to be conducted in malaria endemic populations, to gain further understanding of host-immune response and to evaluate antimalarial drug efficacy in participants who are regularly exposed to natural malaria.

A limitation of this study was that the MCBs were each evaluated in only two participants. There is a need to, therefore, be cautious in comparing the parasite growth rates of the new MCBs against the established 3D7-V2 MCB, especially as there is variability within the observed growth rates and individual growth parameters within the 3D7-V2 MCB [25]. However, when comparing the growth rates of both 3D7-MBE-008 and 3D7-V2, the individual parameters are similar (Additional file 1: Table S2).

Conclusion

A newly developed *P. falciparum* new MCB which is safe to use in healthy, malaria-naive participants is reported. The growth characteristics of the bioreactor-expanded *P. falciparum* 3D7-MBE-008 MCB have been demonstrated and are comparable to the existing 3D7 MCB, hence this new bank is suitable for use in future studies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-021-03627-z.

Additional file 1: Table S1. Schedule of events for both studies. **Table S2.** Growth parameters of the 3D7-MBE-008 pilot bank (n = 2) compared to 3D7 bank using historical data (n = 177). **Table S3.** Growth parameters of the 3D7-V1 pilot bank (n = 2) compared to 3D7-V2 bank using historical data (n = 177). **Table S4.** Overall clinical score recorded for each participant during 3D7-MBE-008 study. **Table S5.** Overall clinical score recorded for each participant during 3D7-V1 study.

Abbreviations

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; Co-I: Co-investigator; DBP: Diastolic blood pressure; ECG: Electrocardiogram; GMP: Good Manufacturing Procedures; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; HIV: Human Immunodeficiency Virus; HR: Heart rate; HRP2: Histidine-rich protein 2; HREC: Human research ethics committee; IBSM: Induced blood-stage malaria; LDA: Limiting dilution assay; LLN: Lower limit of normal; MCB: Malaria Cell Bank; MMV: Medicines for Malaria Venture; Pf: *Plasmodium falciparum*; *Pf* GLURP: *Pf* Glutamate-rich protein; *Pf* MSP-1/2: *P. falciparum* Merozoite surface protein 1/2; PI: Principal Investigator; PMR: Parasite multiplication rate; PRR: Parasite reduction ratio; qPCR: Quantitative Polymerase Chain Reaction; RNA: Ribonucleic acid; SBP: Systolic blood pressure; spp.: Subspecies; ULN: Upper limit of normal; VIS: Volunteer infection studies; WGS: Whole genome sequencing.

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Authors' contributions
SDW, MR, MF, RW, KT, JSM and BEB contributed to the design of the study. SDW, MR, MF, SL, LM, FA, HJ, SC, JJM, JSM and BEB participated in the implementation of the study and/or analysis of results. SDW, MR, MF, SL, KT JSM and BEB participated in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Ethical approval was given by QIMR Berghofer Human Research Ethics Committee (HREC), with further ethical approval given by Lifeblood (formerly Australian Red Cross Blood Service) HREC for *P. falciparum* 3D7 MBE-008. All participants gave written informed consent before enrolment.

Consent for publication
Not applicable.

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

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Publisher's Note

The development and evaluation of a novel *P. falciparum* blood stage malaria cell bank for use in malaria volunteer infection studies- Supplementary Appendix

Eligibility criteria for both studies

Inclusion criteria

Demography

1. Male aged 18 to 55 years inclusive who will be contactable and available for the duration of the trial and up to 2 weeks following the EOS visit.
2. Total body weight greater than or equal to 50 kg, and a body mass index (BMI) within the range of 18 to 32 kg/m^2 (inclusive). BMI is an estimate of body weight adjusted for height. It is calculated by dividing the weight in kilograms by the square of the height in metres.

Health status

3. Certified as healthy by a comprehensive clinical assessment (detailed medical history, complete physical examination and special investigations).
4. Vital signs at screening and pre-inoculation (measured after 5 minutes in the supine position):
    - Systolic blood pressure (SBP) - 90-140 mmHg,
    - Diastolic blood pressure (DBP) - 40-90 mmHg,
    - Heart rate (HR) - 40-100 bpm.
5. At screening and pre-inoculation: QTcF ≤450 ms, QTcB ≤450 ms (male participants); PR interval ≤210 ms.
6. Male participants must agree to use a double method of contraception including condom plus diaphragm, or condom plus intrauterine device, or condom plus stable oral/transdermal/injectable hormonal contraceptive by the female partner, from the time of informed consent until the end of artemether-lumefantrine treatment. Abstinent male participants must agree to start a double method if they begin sexual relationships during the study, and until the end of artemether-lumefantrine treatment. Male participants with female partners that are surgically sterile, or male participants who have undergone

sterilisation and have had testing to confirm the success of the sterilisation, may also be included.

Regulations

7. Completion of the written informed consent process prior to undertaking any study-related procedure.
8. Must be willing and able to communicate and participate in the whole study.

Exclusion criteria

Participants fulfilling any of the following criteria are not eligible for inclusion in this study:

Medical history and clinical status

1. Participant with Rhesus (D) Negative blood group (For 3D7-V1 only).
2. Participant lives alone (at any stage from inoculation day until the end of the artemether-lumefantrine treatment).
3. Any history of malaria or participation in a previous malaria challenge study or malaria vaccine trial.
4. Must not have travelled to or lived (>2 weeks) in a malaria-endemic region during the past 12 months or planned travel to a malaria-endemic region during the course of the study. Must not have lived for >1 year in a malaria-endemic region in the past 10 years. Must not have ever lived in a malaria-endemic region for more than 10 years inclusive. For endemic regions see https://map.ox.ac.uk/country-profiles/#!/. Bali is not considered a malaria-endemic region.
5. Has evidence of increased cardiovascular disease risk (defined as >10%, 5-year risk for those greater than 35 years of age, as determined by the Australian Absolute Cardiovascular Disease Risk Calculator (http://www.cvdcheck.org.au/)). Risk factors include sex, age, systolic blood pressure (mm/Hg), smoking status, total and HDL cholesterol (mmol/L), and reported diabetes status.
6. History of splenectomy.

7. Participant unwilling to defer blood donations to the Blood Service for at least 6 months after the End of Study visit.

8. Participant who has ever received a blood transfusion.

9. Any recent (<6 weeks) or current systemic therapy with an antibiotic or drug with potential antimalarial activity (e.g. chloroquine, piperaquine phosphate, benzodiazepine, flunarizine, fluoxetine, tetracycline, azithromycin, clindamycin, doxycycline etc.).

10. Known hypersensitivity to artesunate or any of its excipients, artemether or other artemisinin derivatives, proguanil/atovaquone, primaquine, or 4-aminoquinolines.

11. Haematology, clinical chemistry or urinalysis results at screening or at the Day -3 to -1 eligibility visit that are outside of Sponsor-approved clinically acceptable laboratory ranges or are considered clinically significant by the Sub-Investigator.

12. Participation in any investigational product study within the 12 weeks preceding inoculation with the malaria challenge agent.

13. Symptomatic postural hypotension at screening (confirmed on two consecutive readings), irrespective of the decrease in blood pressure, or asymptomatic postural hypotension defined as a decrease in systolic blood pressure ≥20 mmHg within 2-3 minutes when changing from supine to standing position.

14. History or presence of diagnosed (by an allergist/immunologist) or treated (by a physician) food or known drug allergies (including but not limited to allergy to any of the antimalarial rescue medications), or history of anaphylaxis or other severe allergic reactions. Participants with seasonal allergies/hay fever or allergy to animals or house dust mite that are untreated and asymptomatic at the time of dosing can be enrolled in the study.

15. History of convulsion (including intravenous drug or vaccine-induced episodes). A medical history of a single febrile convulsion during childhood is not an exclusion criterion.

16. Presence of current or suspected serious chronic diseases such as cardiac or autoimmune disease (HIV or other immuno-deficiencies), insulin-dependent and non-insulin dependent diabetes (excluding glucose intolerance if exclusion criterion 5 is met), progressive neurological disease, severe malnutrition, acute or progressive hepatic disease, acute or progressive renal disease, porphyria, psoriasis, rheumatoid arthritis, asthma (excluding

childhood asthma, or mild asthma with preventative asthma medication required less than monthly), epilepsy, or obsessive-compulsive disorder.

17. History of malignancy of any organ system (other than localised basal cell carcinoma of the skin or in situ cervical cancer), treated or untreated, within 5 years of screening, regardless of whether there is evidence of local recurrence or metastases.

18. Participants with history of schizophrenia, bi-polar disease, psychoses, disorders requiring lithium, attempted or planned suicide, or any other severe (disabling) chronic psychiatric diagnosis.

19. Participants who have received psychiatric medications within 1 year prior to enrolment, or who have been hospitalised within 5 years prior to enrolment for either a psychiatric illness or due to danger to self or others.

20. History of more than one previous episode of major depression, any previous single episode of major depression lasting for or requiring treatment for more than 6 months, or any episode of major depression during the 5 years preceding screening.

    The Beck Depression Inventory will be used as an objective tool for the assessment of depression at screening. In addition to the conditions listed above, participants with a score of 20 or more on the Beck Depression Inventory and/or a response of 1, 2 or 3 for item 9 of this inventory (related to suicidal ideation) will not be eligible for participation. These participants will be referred to a general practitioner or medical specialist as appropriate. Participants with a Beck score of 17 to 19 may be enrolled at the discretion of the Principal investigator (PI) or Co-investigator (Co-I) if they do not have a history of the psychiatric conditions mentioned in this criterion and their mental state is not considered to pose additional risk to the health of the participant or to the execution of the study and interpretation of the data gathered.

21. History of recurrent headache (e.g. tension-type, cluster or migraine) with a frequency of ≥2 episodes per month on average and severe enough to require medical therapy, during the 2 years preceding screening.

22. Presence of clinically significant infectious disease or fever (e.g. sublingual temperature ≥38.5°C) within the 5 days prior to inoculation with the malaria challenge agent.

23. Evidence of acute illness within the 4 weeks prior to screening that the Sub-Investigator deems may compromise participant safety.

24. Significant inter-current disease of any type, in particular liver, renal, cardiac, pulmonary, neurologic, rheumatologic, or autoimmune disease by history, physical examination, and/or laboratory studies including urinalysis.

25. Participant has a clinically significant disease or any condition or disease that might affect drug absorption, distribution or excretion (e.g. gastrectomy, diarrhoea).

26. Blood donation of any volume within 1 month before inclusion, or participation in any research study involving blood sampling (more than 450 mL/unit of blood), or blood donation to Australian Red Cross Blood Service (Blood Service) or other blood bank during the 8 weeks prior to the reference drug dose in the study.

27. Medical requirement for intravenous immunoglobulin or blood transfusions.

28. Any vaccination within the last 28 days.

29. Any corticosteroids, anti-inflammatory drugs (excluding commonly used over-the-counter anti-inflammatory drugs such as ibuprofen, acetylsalicylic acid, diclofenac), immunomodulators or anticoagulants within the past 3 months. Any participant currently receiving or having previously received immunosuppressive therapy (including systemic steroids, adrenocorticotrophic hormone or inhaled steroids) at a dose or duration potentially associated with hypothalamic-pituitary-adrenal axis suppression within the past year.

30. Use of prescription drugs or non-prescription drugs or herbal supplements (such as St John's Wort), within 14 days or 5 half-lives (whichever is longer) prior to or inoculation with the malaria challenge agent. As an exception, ibuprofen (preferred) may be used at doses of up to 1.2 g/24 hours or paracetamol at doses of up to 4 g/24 hours after discussion with the PI or Co-I. Limited use of other non-prescription medications or dietary supplements, not believed to affect participant safety or the overall results of the study, may be permitted on a case-by-case basis following approval by the PI or Co-I. Participants are requested to refrain from taking non-approved concomitant medications from recruitment until the conclusion of the study.

General conditions

31. Any participant who, in the judgment of the PI or Co-I, is likely to be non-compliant during the study, or is unable to cooperate because of a language problem or poor mental development.
32. Any participant in the exclusion period of a previous study according to applicable regulations.
33. Any participant who is the PI, Co-I or any Sub-Investigator, research assistant, pharmacist, study coordinator, or other staff thereof, directly involved in conducting the study.
34. Any participant without a good peripheral venous access.

Biological status

35. Positive result on any of the following tests: hepatitis B surface antigen (HBs Ag), anti-hepatitis B core antibodies (anti-HBc Ab), anti-hepatitis C virus (anti-HCV) antibodies, anti-human immunodeficiency virus 1 and 2 antibodies (anti-HIV1 and anti-HIV2 Ab).
36. Positive urine drug test. Any drug listed in Section 8.2 in the urine drug screen unless there is an explanation acceptable to the Sub-Investigator (e.g., the participant has stated in advance that they consumed a prescription or over-the-counter product which contained the detected drug) and/or the participant has a negative urine drug screen on retest by the pathology laboratory. Any participant testing positive for acetaminophen (paracetamol) at screening and/or inoculation day may still be eligible for study participation, at the PI or Co-I's discretion.
37. Positive alcohol breath test.

Specific to the study

38. Cardiac/QT risk:
    - Family history of sudden death or of congenital prolongation of the QTc interval or known congenital prolongation of the QTc interval or any clinical condition known to prolong the QTc interval.
    - History of symptomatic cardiac arrhythmias or with clinically relevant bradycardia.

- Electrolyte disturbances, particularly hypokalaemia, hypocalcaemia, or hypomagnesaemia.
- ECG abnormalities in the standard 12-lead ECG (at screening and prior to inoculation with the malaria challenge agent) which in the opinion of the Sub-Investigator is clinically relevant or will interfere with the ECG analyses.

39. History or presence of alcohol abuse (alcohol consumption more than 40 g/4 units/4 standard drinks per day), or drug habituation, or any prior intravenous usage of an illicit substance.
40. Tobacco use of more than 5 cigarettes or equivalent per day, and unable to stop smoking for the duration of the clinical unit confinement.
41. Ingestion of any poppy seeds within the 24 hours prior to screening (participants will be advised by phone not to consume any poppy seeds in this time period).
42. Excessive consumption of beverages or food containing xanthine bases including Red Bull, chocolate, coffee etc. (more than 400 mg caffeine per day, equivalent to more than 4 cups of coffee per day).
43. Unwillingness to abstain from consumption of quinine containing foods/beverages such as tonic water and lemon bitter from inoculation day until the end of the artemether-lumefantrine treatment.
44. Unwillingness to abstain from consumption of grapefruit or Seville oranges from inoculation day until the end of the artemether-lumefantrine treatment.

Table S1. Schedule of events for both studies

Procedures	Screening	Safety Visit required)[a]	Challenge Inoculation	Malaria Monitoring		Drug Treatment (Confinement)			Post-treatment Follow-up (outpatient)			Safety Assessment		EOS
Day	-Day 28 to Day -1	Day -3 to Day -1	Day 0	Phone Contact Day 1 – Day 3	Day 4 until artemether lumefantrine treatment[b]	First dose (0hrs)	Second dose (12hrs)	Third dose (24hrs)	36hrs	48hrs	72hrs	Day 30±2	Day 60±2	Day 90±2
Eligibility Assessments														
Informed consent	X													
Beck Depression Inventory	X													
Demography	X													
Medical history, inc/exc. criteria, & prior medications	X		X											
Drug & alcohol screen	X		X											
Body weight	X										X			
Height	X													
Serology	X													X
RBC alloantibody	X													X
Coagulation profile	X													
G6PD testing	X													
Safety Assessments														
Complete Physical exam	X										X			
Abbreviated physical exam			X			X								
Symptom-directed physical exam					Throughout the period when clinically indicated									
ECGs	X		X			X		X			X			

Procedures	Screening	Safety Visit required)[a]	Challenge Inoculation	Malaria Monitoring		Drug Treatment (Confinement)			Post-treatment Follow-up (outpatient)			Safety Assessment		EOS
Vital signs	X		X		Throughout the period									
Haematology & Biochemistry	X	X				X		X			X			
Urinalysis	X	X				X					X			
Safety serum storage			X								X			
Diary card			X	X	X				X	X	X			
Adverse Events			Throughout the period									X	X	X
Malaria Clinical Score[d]			X		Throughout the period									
Phone Call												X	X	
Other														
Blood Stage Challenge Agent			X											
Artemether-lumefantrine Treatment						X	X	X	X	X				
Artemether-lumefantrine Treatment phone call/text[e]										X				
Malaria 18S qPCR blood sampling[f]			X		X	X	X	X	X	X	X			

ECG: Electrocardiograph; G6PD: Glucose 6-phosphate dehydrogenase; RBC: Red blood cell; AEs: Adverse events; qPCR: quantitative polymerase chain reaction; qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction; EOS: End of Study.

[a] This visit is not required in the event that the screening visit is conducted within this period.

[b] Daily visits until qPCR Positive, then twice-daily visits until antimalarial treatment.

[d] Record malaria clinical score 3 times a day whilst confined prior to each dose of artemether-lumefantrine. During the out-patient monitoring phase the malaria clinical score will only be recorded if vital signs are abnormal.

[e] Participant will be contacted via phone call/ txt to ensure the final dose of artemether-lumefantrine is taken at home.

[f] qPCR blood samples will be taken at the following times in relation to the first dose of artemether-lumefantrine: pre-dose, 2, 4, 8, 12, 16, 20, 24 36, 48 and 72 hours. Alternate sampling time points may be used at the Investigator's discretion based on the progression of parasitemia or participant safety

Table S2. Growth parameters of the 3D7-MBE-008 pilot bank (n=2) compared to 3D7 bank using historical data (n=177)

Parameter	3D7-MBE-008 Bank (n=2) Estimate (SE)	3D7-V2 Bank (n=177) Estimate (SE)	Q_B	p value
Growth rate (log_{10} parasites/day)	0.770 (0.073)	0.752 (0.012)	0.058	0.81
Amplitude ^ (log_{10} parasites/day)	0.804 (0.144)	0.629 (0.018)	1.44	0.23
Life- cycle (days)	1.615 (0.046)	1.615 (0.009)	0.00	1.00

^absolute value of amplitude

Q_B, test statistic from the omnibus test for heterogeneity

SE, Standard error

Non-linear mixed effect model parameter estimates from the 3D7-MBE-008 bank and from historical 3D7 bank are given in Table S2, along with the results from the tests of heterogeneity of the parameter estimates. The results from the heterogeneity test suggests that there is no evidence of a difference in any of the growth parameters between the banks.

Table S3. Growth parameters of the 3D7-V1 pilot bank (n=2) compared to 3D7-V2 bank using historical data (n=177)

Parameter	3D7-V1 Bank (n=2) Estimate (SE)	3D7-V2 Bank (n=177) Estimate (SE)	Q_B	p value
Growth rate (log_{10} parasites/day)	0.530 (0.035)	0.752 (0.012)	35.3	<0.001
Amplitude ^ (log_{10} parasites/day)	0.521 (0.118)	0.629 (0.018)	0.8	0.37
Life- cycle (days)	1.522 (0.033)	1.615 (0.009)	7.5	0.006

^absolute value of amplitude

Q_B, test statistic from the omnibus test for heterogeneity

SE, Standard error

Non-linear mixed effect model parameter estimates from the 3D7-V1 bank and from historical 3D7-V2 bank are given in Table S3, along with the results from the tests of heterogeneity of the parameter estimates. Evidence of a difference in the growth rate was noted between the banks.

Table S4. Overall clinical score recorded for each participant during 3D7-MBE-008 study

Days post-inoculation	Overall clinical score	
	R101	R201
0	0	0
3	-	1
4AM	0	2
4PM	0	1
5AM	0	0
5PM	0	0
6AM	0	0
6PM	1	1
7AM	1	0
7PM	3	1
8 Pre-dose	1	1
Adm +8h	9	1
Adm +12h	4	1
Adm + 24h	3	1
Adm + 36h	2	2
Adm + 48h	1	-
Adm + 72h	0	-

Table S5. Overall clinical score recorded for each participant during 3D7-V1 study

Days post-inoculation	Overall clinical score	
	R101	R201
0	0	0
3	0	0
4AM	0	0
4PM	0	-
5AM	0	0
5PM	1	-
6AM	0	0
6PM	0	0
7AM	0	0
7PM	0	0
8AM	0	0
8PM	0	0
9AM	0	0
9PM	1	0
10AM	0	0
10PM	0	2
11AM	0	3
11PM	0	0
12	1	-
Adm +8h	1	1
Adm +12h	1	4
Adm + 24h	0	1
Adm + 36h	3	8
Adm + 48h	2	-
Adm + 72h	1	-

3.6 Significance of this paper

The key findings of this study are as follows**:**

- **Participant Safety.** In this study the new bioreactor produced MCB, 3D7-MBE-008, had a similar safety profile to the established 3D7-V2 MCB [54]. The most frequently reported adverse events in the 3D7-MBE-008 participants were chills (n=2), headaches (n=2) and myalgia (n=2), which was similar to the 3D7-V1 participants. The majority (32/52) of the adverse events were mild or moderate as per CTCAE Vers 5.0 criteria [147], with 3 severe adverse events. Each participant developed a transient episode of lymphopenia and one participant developed raised liver transaminases (day 11 ALT 191 U/L [4.8x upper limit of normal (ULN)] and day 11 AST 122 U/L [3.1x ULN]). Occurrence of lymphopenia and raised liver transaminases have been reported in both clinical and VIS malaria infections [133,134,139,140,148–151]. These findings suggest the safety profile of the new MCB is in keeping with existing MCBs.

- **3D7-MBE-008 parasite growth and clearance profiles.** The growth rate in the new MCB, 3D7-MBE-008 was similar to the existing 3D7-V2 MCB which has been used in the last ten years of IBSM VIS at QIMR Berghofer. A parasite count >10,000 parasites per mL was reached on day 8 which is also in keeping with the 3D7-Vs 0MCB. The clearance profile with artemether-lumefantrine, again was similar to the 3D7-V2 MCB.

- **MCB availability**. By using the bioreactor method, the new 3D7-MBE-008 MCB will have an enduring supply, and thus enabling the use of IBSM VIS in malaria-endemic regions.

In summary, the 3D7-MBE-008 MCB which was produced in the bioreactor is safe to use in malaria-naïve participants, with similar parasite growth/clearance rates to the established 3D7-V2 MCB. Whilst this study does not directly investigate malaria-associated anaemia in these two clinical studies, the haematology and parasite data has been used in the retrospective pooled analysis of haematology and parasite data (**chapter 4**) and in the markers of iron metabolism study (**chapter 5**)

Chapter 4. Haematological response in experimental human *P. falciparum* and *P. vivax* malaria

4.1. Introduction

In infants and children living in malaria endemic setting, malaria is an important sequelae of malaria infection [1,13]. It is also of significance for malaria-naïve individuals who are susceptible to all the sequelae of malaria, unlike individuals residing in endemic settings, where the more serious forms of *P. falciparum* such as cerebral malaria or anaemia are age linked [13]. Over the last ten years, the IBSM model has been used in Phase 1 drug development studies conducted at QIMR Berghofer, with over 400 individuals being inoculated with *P. falciparum*, 15 with the K13 artemisinin-resistant strain of *P. falciparum*, 46 with *P. vivax* and 2 with *P. malariae* [55,58]. There is an extensive dataset containing the longitudinal 18S qPCR parasite data, as well serial haematology and biochemistry samples that has not been reviewed previously and could provide further insight into the development of anaemia in early and also low-level infection.

4.2. Declaration

The study was designed by Louise Marquart, James McCarthy, Bridget Barber and I. Louise Marquart provided statistical support to me throughout the analysis. John Woodward was a sub-investigator in some of the trials that contributed to this pooled analysis. Joerg Moehrle and Stephan Chalon provided external advice, safety data and review team input and sponsors for the majority of the studies used. All other authors contributed and participated in the manuscript preparation, with me writing and co-ordinating the final manuscript.

4.3 Statistics analysis plan

A brief statistical analysis plan was devised by Bridget Barber, Louise Marquart, and I prior to commencing this pooled analysis. The data used for the pooled analysis were retrospective data from 26 IBSM trials conducted at QIMR over the previous 10 years. This incorporated the data over 315 participants, with 269 being inoculated with *P. falciparum* and 46 with *P. vivax*. There were two individuals inoculated with *P. malariae* and 8 inoculated with a genetically attenuated *P. falciparum* strain, however they were excluded from the analysis due to a low sample size and the genetically attenuated parasite not being a clinical strain.

The primary objective of this pooled analysis was to investigate the haematological response in experimental malaria using haematology, parasite and clinical data stored in the Clinical Tropical Medicine Group's VIS database. Secondary endpoints were as given in Chapter 1.

Primary endpoints

- The fractional fall in haemoglobin from the day of inoculation until day of haemoglobin nadir
- The day of haemoglobin nadir and day of haemoglobin normalisation
- The corelations between haemoglobin nadir and fractional fall in haemoglobin with parasite parameters (peak parasitaemia, total parasite burden, parasite multiplication rate and parasite reduction ratio) unadjusted and adjusted for co-variables such as age/day of treatment
- The ratio of pRBC and uRBC to the malaria-attributable loss

Secondary

- The effect of parasite recrudescence/treatment failure on the haemoglobin nadir, fractional fall in haemoglobin, day of haemoglobin nadir and day of return to haemoglobin baseline
- The effect of sex and age on the haemoglobin nadir, fractional fall in haemoglobin, day of haemoglobin nadir and day of haemoglobin normalisation
- The haemoglobin nadir, fractional fall in haemoglobin, day of haemoglobin nadir and day of haemoglobin normalisation baseline by drug/drug-combination

Definitions

- *Fractional fall in haemoglobin*- difference between baseline and nadir haemoglobin as a percentage of the baseline haemoglobin
- *Haemoglobin nadir*- the lowest haemoglobin from the day of first antimalarial treatment (termed 'day of treatment') onwards
- *Day of haemoglobin nadir*- the day at which the nadir haemoglobin occurred post-treatment

- *Day of haemoglobin normalisation*- the earliest day post-treatment at which the haemoglobin was equal to or greater than the baseline haemoglobin
- *Peak parasitaemia*- the highest parasitaemia within 24 hours of treatment
- *Total parasite burden*- The total parasite burden as determined by area under the curve of the 18S qPCR data from day 4 until the time of treatment
- *Malaria-attributable loss*- the proportion of the total red cell loss attributable to malaria
- *Parasite multiplication rate* and *parasite reduction ratio* will be conducted using variables generated using the method by Wockner et al[152,153]

Analysis

Baseline characteristics were analysed according to whether they are parametrically or non-parametric, with parametric data being described using mean and standard deviation and non-parametric data being described using median and interquartile range. Parametric data were compared using the Students' t-test or ANOVA if multiple groups and if non-parametric, the data will be compared using the Mann-Whitney, Wilcoxon matched pairs signed-rank and Kruskal-Wallis tests.

For correlation analyses, after testing for normality, Pearson's correlation was used for parametric data or a Spearman's correlation for non-parametric data. Linear regression was used to evaluate the association between haematology and parasite factors, adjusting for any variable found to be significant in the prior analysis.

4.4 Paper

This manuscript has been accepted for publication in the Malaria Journal.

Haematological response in experimental human *Plasmodium falciparum* and *Plasmodium vivax* malaria

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Abstract

Introduction

Malaria-associated anaemia, arising from symptomatic, asymptomatic and submicroscopic infections, is a significant cause of morbidity worldwide. Induced blood stage malaria volunteer infection studies (IBSM-VIS) provide a unique opportunity to evaluate the haematological response to early *P. falciparum* and *P. vivax* infection.

Methods

We analysed the haemoglobin, red cell counts, and parasitaemia data from 315 participants enrolled in IBSM-VIS between 2012 - 2019, including 269 participants inoculated with the 3D7 strain of *P. falciparum* (Pf3D7), 15 with an artemisinin-resistant *P. falciparum* strain (PfK13) and 46 with *P. vivax*. We evaluated factors associated with the fractional fall in haemoglobin (Hb-FF), and estimated the malaria-attributable erythrocyte loss after accounting for phlebotomy-related losses. We also estimated the relative contribution of parasitised erythrocytes to the malaria-attributable erythrocyte loss.

Results

The median peak parasitaemia prior to treatment was 10,277 parasites/ml (IQR 3566 – 27,815), 71,427 parasites/ml [IQR 33,236 – 180,213], and 34,840 parasites/ml (IQR 13,302 – 77,064) in participants inoculated with Pf3D7, PfK13, and *P. vivax,* respectively. The median Hb-FF was 10.3% (IQR 7.8 – 13.3), 14.8% (IQR 11.8 – 15.9) and 11.7% (IQR 8.9 – 14.5) in those inoculated with Pf3D7, PfK13 and *P. vivax*, respectively, with the haemoglobin nadir occurring a median 12 (IQR 5 – 21), 15 (IQR 7 – 22), and 8 (IQR 7 – 15) days following inoculation. In participants inoculated with *P. falciparum*, recrudescence was associated with a greater Hb-

FF, while in those with *P. vivax*, the Hb-FF was associated with higher pre-treatment parasitaemia and later day of antimalarial treatment. After accounting for phlebotomy-related blood losses, the estimated Hb-FF was 4.1% (IQR 3.1 – 5.3), 7.2% (IQR 5.8 – 7.8), and 4.9% (IQR 3.7 – 6.1), in participants inoculated with Pf3D7, PfK13, and *P. vivax*, respectively. Parasitised erythrocytes were estimated to account for 0.015% (IQR 0.006 – 0.06), 0.128% (IQR 0.068 – 0.616) and 0.022% (IQR 0.008 – 0.082) of the malaria-attributable erythrocyte loss in participants inoculated with Pf3D7, PfK13, and *P. vivax*, respectively.

Conclusion

Early experimental *P. falciparum* and *P. vivax* infection resulted in a small but significant fall in haemoglobin despite parasitaemia only just at the level of microscopic detection. Loss of parasitised erythrocytes accounted for <0.2 % of the total malaria-attributable haemoglobin loss.

Introduction

Malaria remains a major cause of mortality and morbidity worldwide, with 229 million cases and 409,000 reported deaths in 2019 [1]. Furthermore, it is likely that the disruption of services due to the current COVID-19 pandemic may significantly increase the number of malaria cases and malaria deaths [2]. Malaria-associated anaemia is one of the most important complications of malaria [3–9]. Hospital-based studies have demonstrated that severe anaemia can occur in patients infected with *P. falciparum*, *P. vivax* and *P. malariae*, and in all three species increases the risk of death [3]. More recently, cross-sectional community surveys have demonstrated that asymptomatic and sub-microscopic infections are also associated with a high risk of anaemia, contributing significantly to the overall burden of malaria associated anaemia [10,11].

Malaria associated anaemia is multi-factorial. Although occurring in part due to the rupture of parasitised red blood cells (RBCs), the major contributor to malaria is the loss of unparasitised RBCs, with previous studies estimating that the ratio of the loss of parasitised to unparasitised RBCs is 1:8 in *P. falciparum* [12,13] and 1:34 in *P. vivax* [14]. Potential contributors to this loss of unparasitised cells include increased free radical damage [15], production of anti-phosphatidylserine antibodies [16], and the loss of erythrocyte surface complement regulatory proteins [17]. Dyserythropoiesis caused by bone marrow suppression secondary to the direct effects of the parasites as well as the effect of cytokines [18–21], and inflammation-induced iron deficiency [22], are other key contributors to malaria-associated anaemia. Shortened red cell lifespan [23] following artesunate therapy [24] may also be contributory.

The induced blood-stage malaria (IBSM) volunteer infection model developed at QIMR Berghofer in 1995 has recently assumed a key role in antimalarial drug development [25]. To date, over 400 participants have been enrolled in these studies at QIMR Berghofer, including 342 inoculated with the fully sensitive *P. falciparum* 3D7 strain, 15 with the K13 artemisinin resistant stain, and 46 with *P. vivax* [26,27]. In these studies parasitaemia is closely monitored with a highly sensitive quantitative PCR targeting the 18S rRNA gene, and frequent blood sampling occurs to monitor haematological parameters throughout the course of infection [27]. Therefore, these studies provide a unique opportunity to investigate the haematological response that occurs in early *P. falciparum* and *P. vivax* blood-stage infection.

In this study we analysed haematology data from 26 IBSM volunteer infection studies undertaken at QIMR Berghofer over the last ten years, aiming to describe the haematological response to *P. falciparum* and *P. vivax* infection, and to evaluate factors associated with the fractional fall in haemoglobin. We also used the haematological and parasitaemia data to estimate the relative contribution of the loss of parasitized and unparasitised cells to the total malaria-attributable haemoglobin loss.

Methods

Study design and participants

Data were retrieved from the records of 315 participants enrolled in 26 IBSM studies conducted at QIMR Berghofer between 2012 and 2019 (**supplementary table 1**). Details of these studies have been published previously [26,28–50]. In brief, malaria-naïve participants were included if they were aged 18 – 55 years, had no significant co-morbidities or concurrent illness, and had haematology and biochemistry results at baseline that were within the

protocol specified range. Participants were inoculated (day 0) with red blood cells parasitised with either *P. falciparum* 3D7 (Pf3D7; n=254), an artemisinin-resistant strain of *P. falciparum* with a defined mutation on the K13 propeller gene (PfK13; n=15), or *P. vivax* (n=46). Parasitaemia was closely monitored at specified time-points throughout the studies by quantitative PCR (qPCR) targeting the species specific 18S ribosomal ribonucleic (rRNA) gene [51]. The studies involved evaluation of 18 different investigational antimalarial drugs, either alone or in combination (**supplementary table 1**). The day on which antimalarial treatment was administered ranged from day 7 to 8 for Pf3D7, day 9 for PfK13 and day 8 to 14 for *P. vivax*. For many of the studies, a sub-curative dose of the investigational drug was administered, resulting in recrudescence of parasitaemia to facilitate calculation of the drug minimum inhibitory concentrations. In all studies, participants were treated with a registered antimalarial drug, typically artemether-lumefantrine, either at the time of recrudescence, or at the end of the study if recrudescence did not occur.

All studies were conducted in accordance with the Declaration of Helsinki and the International Committee of Harmonisation Good Clinical Practice guidelines. Ethical approval for all studies was granted by the Human Research Ethics Committee at QIMR Berghofer. All participants provided informed written consent.

Haematology measurements

Blood was taken for standard haematology laboratory testing during screening, prior to inoculation, prior to antimalarial treatment, and then at protocol defined time-points throughout the study. The maximum volume of blood taken within a 30-day period was 400mL. For the purposes of analysis, baseline haemoglobin was defined as the last value prior

to inoculation. Nadir haemoglobin was defined as the lowest haemoglobin from the day of first antimalarial treatment (termed 'day of treatment') onwards. The fractional fall in haemoglobin was the difference between baseline and nadir haemoglobin as a percentage of the baseline haemoglobin. The day of haemoglobin normalisation was defined as the earliest day post-treatment at which the haemoglobin was equal to or greater than the baseline haemoglobin. The reticulocyte difference was defined as the difference between the baseline reticulocyte count prior to inoculation (day -1) and the final reticulocyte count at the end of study.

Statistical analysis

Data was analysed using Stata V.16.0 and GraphPad Prism V.8.1. For categorical variables the number and frequency (%) were reported and differences between groups compared using Pearson Chi-squared test or Fisher's exact test. For normally distributed continuous variables the mean and standard deviation (SD) were reported and differences between groups were compared using the student's t-test or analysis of variance (ANOVA). Non-normally distributed continuous variables were summarised using the median and interquartile range (IQR) and differences between groups were compared using the Mann-Whitney, Wilcoxon matched pairs signed-rank and Kruskal-Wallis tests. In participants inoculated with *P. vivax*, the differences in haemoglobin parameters between those treated with artemether/lumefantrine and those treated with other drugs were compared using the non-parametric Dunn's multiple comparison test. Correlations between parasitaemia and haematological parameters were evaluated using either Pearson's or Spearman's correlation, depending on distribution.

Parasitaemia parameters evaluated included the log_{10} transformed pre-treatment peak parasitaemia (PP_{Pre}), peak parasitaemia (PP; the highest parasitaemia within 24 hours of treatment), and the pre-treatment total parasite burden (TPB_{Pre}). The TPB_{Pre} was determined using the area under curve (AUC) of the non-transformed 18S qPCR data from day 4 until the time of treatment. For *P. falciparum,* the TPB_{Pre} incorporated an adjustment to account for sequestered parasites, assumed to be approximately 25% of the total parasite burden at any given timepoint [52]. Linear regression analysis was used to evaluate the association between TPB_{Pre} or PP_{Pre} and the haematology parameters, adjusting for day of treatment and/or drug treatment. The parasite multiplication rates (PMR) and parasite reduction rates (PRR) were calculated as previously described [53,54]. Parasite recrudescence was defined as a parasitaemia increase by >1000 parasites/mL occurring more than 2 days post-treatment.

For each participant, the total erythrocyte loss was calculated by subtracting the red cell count (RCC) at the day of haemoglobin nadir from the baseline RCC. The malaria-attributable loss was then calculated by subtracting the estimated phlebotomy-related erythrocyte loss from the total erythrocyte loss. For this calculation, the individual total blood volume was calculated using Nadler's method [55], with the phlebotomy-related erythrocyte loss estimated by multiplying the participant's baseline RCC ($x10^{12}$/L) by the estimated total phlebotomy blood volume from inoculation until the median day of haemoglobin nadir (total 0.19 L/ total blood volume). To calculate the loss of parasitised RBCs as a proportion of the malaria-attributable erythrocyte loss, the TPB_{Pre} (parasites per mL) was divided by the malaria-attributable erythrocyte loss **(see supplementary information for calculations**). This calculation assumes that every parasitised erythrocyte is singly infected [56], and therefore

the TPB_{Pre} is equal to the number of parasitized erythrocytes lost. The use of TPB_{Pre} also assumes that parasite replication does not continue after treatment.

Results

Participant's demographics

The median ages of participants inoculated with Pf3D7 (n=254), PfK13 (n=15) and *P. vivax* (n=46) were 24 (IQR 22-28), 23 (IQR 21-27) and 24 (IQR 21-31) years, respectively. The majority of participants in all three groups were male (73% in Pf3D7, 60% in PfK13 and 65% in *P. vivax*).

Parasitaemia

The overall median PP_{Pre} for participants inoculated with *P. falciparum* was 10,277 (IQR 3,566-27,815) parasites/mL (**Table 1**). This was higher in participants inoculated with PfK13 (71,427 [IQR 33,236-180,218] parasites/mL) compared to Pf3D7 (9,008 [IQR 3,341-21,798] parasites/mL; p<0.0001), possibly due to the PfK13 participants being treated later (day 9) than those inoculated with Pf3D7 (day 7 or 8). The median PP in the *P. falciparum* group was 20,218 (IQR 8,350-55,570) parasites/mL, which was again higher in the PfK13 group (132,160 [IQR 69,160-309,057 parasites/mL]) compared to the 3D7 group (18,240 [IQR 7,901-49,995] parasites/ml; p<0.0001). For participants inoculated with *P. vivax* the median PP_{Pre} was 34,840 (IQR 13,302 – 77,064) parasites/mL, and the PP 53,696 (IQR 15,934-102,635) parasites/mL, with both these values being significantly higher than those inoculated with *P. falciparum* (p<0.001 for both comparisons). Recrudescence occurred in 96/269 (36%) participants in the *P. falciparum* group (15/15 [100%] in PfK13 and 84/254 [33%] in Pf 3D7), and 7/46 (15%) in the *P. vivax* group.

Table 1. Summary of haematology and parasite data

	Pf all (n=269)	Pf 3D7 (n=254)	Pf K13 (n=15)	*P. vivax* (n=46)	P-value (3D7 vs K13)	P-value (Pf all vs *P. vivax*)
Age, Years (Median, IQR)	24 (22-28)	24 (22-28)	23 (21-27)	24 (21-31)	0.38	0.87
Male Sex, n (%)	197 (73)	188 (74)	9 (60)	30 (65)	0.23	0.26
PP_{Pre}, Parasites/mL (Median, IQR)	10,277 (3,566-27,815)	9,008 (3,341-21,798)	71,427 (33,327 – 180,218)	34,840 (13,302-77,064)	**<0.0001**	**<0.0001**
PP, Parasites/mL (Median, IQR)	20,218 (8,350-55,570)	18,240 (7,901-49,995)	132,160 (69,160-309,057)	53,696 (15,934-102,635)	**<0.0001**	**0.0006**
TPB_{Pre}, Parasites/mL (Median, IQR)	30,223 [a] (14,320-90,970)	28,366 [a] (12,637-75,571)	410,274 [a] (166,853-1,106,184)	31,345 (12264-67,516)	**<0.0001**	0.38
PRR (Median, IQR)	0.058 (0.045-0.081)	0.060 (0.045-0.082)	0.051 (0.045-0.055)	0.055 (0.048-0.068)	**0.040**	0.37
Baseline Hb, g/L (Median, IQR)	149 (139-156)	149 (141-156)	145 (136-153)	147 (139-158)	0.27	0.74
Day of Treatment Hb, g/L (Mean, SD)	139 (12.09)	140 (11.81)	136 (16.11)	140 (12.61)	0.22	0.57
Nadir Hb, g/L (Median, IQR)	132 (123-140)	132 (123-140)	127 (110-135)	129 (121-140)	**0.024**	0.22
Hb Fractional Fall, % (Median, IQR)	10.6 (7.9-13.8)	10.3 (7.8-13.3)	14.8 (11.8-15.9)	11.7 (8.9-14.5)	**0.001**	0.07
Day Post Treatment of Hb Nadir (Median, IQR)	12 (5-21)	12 (5-21)	15 (7-22)	8 (7-15)	0.66	0.11
Day Post Treatment of return to baseline Hb (Median, IQR)	28 [b] (22-37)	28 [b] (22-37)	[c]	20 (18-25)	[c]	**<0.0001**
% Contribution of pre-treatment Hb drop to total drop in Hb (Median, IQR)	55 (23-78)	55 (24-78)	40 (11-76)	45 (27-56)	0.45	**0.020**
Baseline reticulocyte count, 10^9/L (Median, IQR)	55 [d] (44-68)	54 [d] (43-68)	58 (49-76)	58 (46-74)	0.33	0.34
Maximum post-treatment reticulocyte count, 10^9/L (Median, IQR)	68 [d] (54-90)	66 [d] (53-89)	84 (66-100)	72 [e] (58-89)	**0.048**	0.29
Reticulocyte difference, 10^9/L (Median, IQR)	12 [f] (3-28)	11 [g] (1-27)	20 (10-33)	13 [e] (4-23)	**0.049**	0.99

n-number of participants; IQR- interquartile range; PP- peak parasitaemia; PP_{Pre}- pre-treatment peak parasitaemia; TPB_{Pre}- pre-treatment total parasite burden, determined using the AUC of the 18S qPCR data from day 4 until the time of treatment; PRR- parasite reduction ratio; a- the TPB_{Pre} for the *P. falciparum* groups has been adjusted by a factor of 25% to account for sequestered parasites [52]; b- Pf all n=267 and Pf3D7 n=252 due to 2 participants not reaching haemoglobin baseline; c- none of the PfK13 participants' haemoglobin returned to baseline; d- Pf all n=254 and Pf3D7 n=239; e- n=44; f- n=246; g- n=231; Mann-Whitney test used unless otherwise stated.

Fall in haemoglobin following inoculation with *P. falciparum* and *P. vivax*

In all participants inoculated with *P. falciparum*, the median haemoglobin fell from a baseline of 149 (IQR 139-156) g/L to a nadir of 132 (IQR 123-140) g/L, representing a median fall of 17 g/L and a fractional fall of 10.6% (IQR 7.9-13.8%) (**Table 1**). The fall in haemoglobin prior to antimalarial treatment accounted for a median 55% (IQR 23 – 78%) of the total fall. The haemoglobin nadir occurred a median of 12 (IQR 5-21) days post treatment and returned to baseline a median of 28 (IQR 22-37) days post treatment.

There was no significant difference between the median baseline haemoglobin between participants inoculated with Pf3D7 (149 g/L [IQR 141-156]) and those inoculated with PfK13 (145 g/L [IQR 136-153]; p=0.27). In participants with PfK13, the haemoglobin fell by a median 18 (IQR 12-23) g/L compared to 17 (IQR 11 – 20) g/L in the Pf3D7 group, representing a median fractional fall of 14.8% (IQR 11.8 - 15.9%), compared to 10.3% (IQR 7.8 – 13.3%) in the 3D7 group (p=0.001) (**Figure 1**). The day of haemoglobin nadir occurred a median of 15 (IQR 7 –

22) days post treatment in those inoculated with PfK13 compared 12 (IQR 5 – 21) days in those inoculated with Pf3D7 (p=0.66). In participants inoculated with the Pf3D7, the haemoglobin returned to baseline a median 28 (IQR 22-37) days post-treatment, while in the PfK13 group, the haemoglobin did not recover to baseline levels prior to the end of study in any of the 15 participants (**Table 1**).

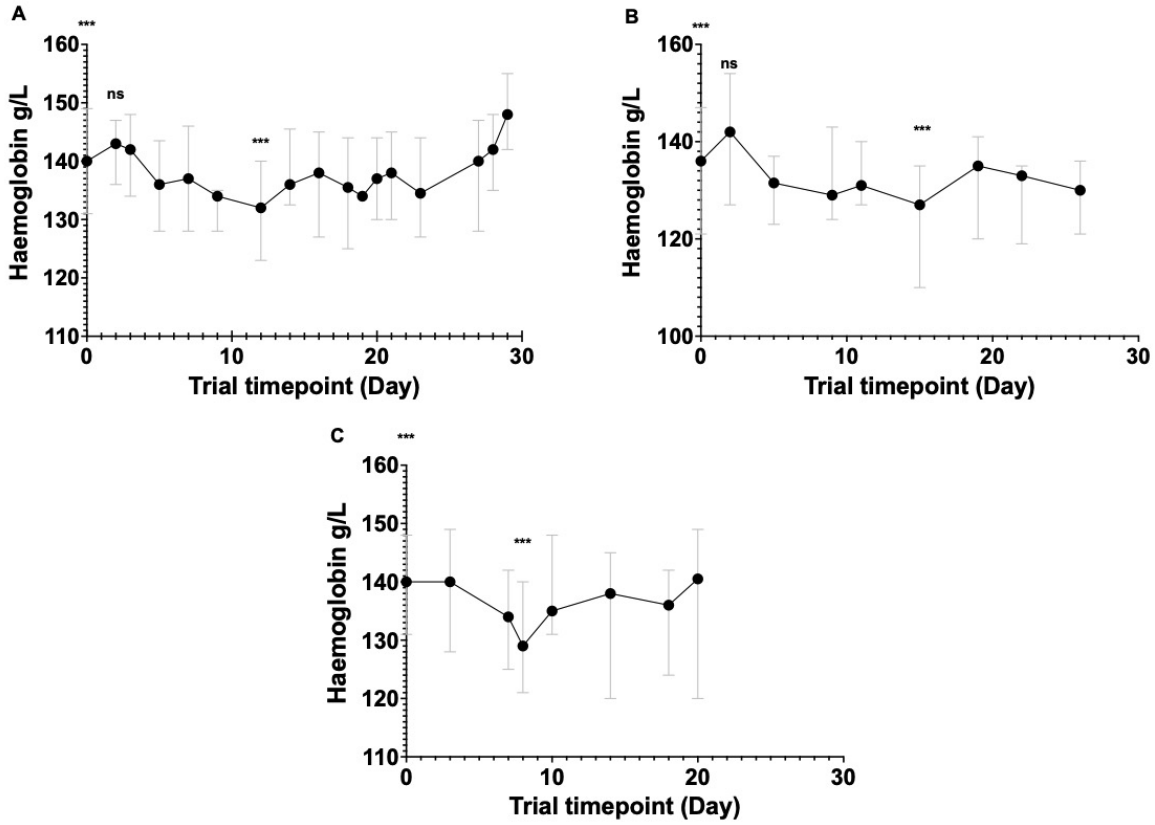

Figure 1. Changes in haemoglobin post-treatment over time in participants inoculated with Pf3D7 (A), PfK13 (B) and *P. vivax* (C).

Data points and error bars represent median and interquartile range, respectively. Values were compared to the values at baseline using the Wilcoxon matched-paired sign-rank test. *** represent a p-value <0.001 and represent the difference between baseline haemoglobin and day of treatment/day of haemoglobin nadir. NS represents a non-significant value.

In participants inoculated with *P. vivax*, the haemoglobin fell from a median of 147 (IQR 139—158) g/L to 129 (IQR 121-140) g/L, with a total median fall of 18 (IQR 14-21) g/L and a median fractional fall of 11.7% (IQR 8.9-14.5), which compared to 10.6% (IQR 7.9-13.8) in the *P. falciparum group* (p=0.07). The median fall in haemoglobin prior to treatment contributed to 45% (IQR 27-56) of the total fall. The haemoglobin nadir occurred a median of 8 (IQR 7-15) days following treatment and returned to baseline levels a median of 20 (IQR 18-25) days following treatment.

Factors influencing fall in haemoglobin

Recrudescence

In participants inoculated with *P. falciparum*, 99 (37%) participants who recrudesced had a fractional fall in haemoglobin of 12.0% (IQR 8.1 - 15.2; n = 99), compared to 10.2% (IQR 7.7-12.8; n=170) in those who did not (p=0.006). In the *P. vivax* group, the median haemoglobin fractional fall was similar in those who recrudesced (11.8% [IQR 8.7-16.4]; n=7) compared to those who did not (11.7% [IQR 8.8-14.5; n=39]; p=0.95).

Age, sex and day of treatment

In the *P. falciparum* participants, there was no significant difference observed in the haemoglobin nadir or fractional fall in haemoglobin between age groups (**supplementary table 4**). In participants inoculated with *P. vivax*, a trend was observed towards a greater fractional fall of haemoglobin in the older age group (median 13.6% [IQR 10.8-14.6; n=21] *vs* 10.5% [IQR 8.5-14.0; n=25]; p=0.09) (**supplementary table 5**), although this was not statistically significant.

Sex had no effect on the fractional fall in haemoglobin across all three species. Females had a lower median baseline haemoglobin than males (134 g/L [IQR 127-138; n=88] vs 153 g/L [IQR 147-158; n=227]; N=315; p<0.0001) as well as a lower haemoglobin nadir (117 g/L [IQR 111-123; n=88] vs 136 g/L [IQR 130-142; n=227]; N=315; p<0.0001).

In participants inoculated with Pf3D7, the day of treatment did not have any significant impact on the haematology parameters (**supplementary table 6**). However, for participants inoculated with *P. vivax*, where day of treatment was more variable, the median haemoglobin nadir was lower in participants treated on Day 10-14 (129 g/L [120-137]; n=36), compared to those treated on Day 8 (140 g/L [133-144]; n=10; p=0.049). The median fractional fall in haemoglobin was also greater in those treated at Day 10-14 (12.4% [IQR 9.7-14.6]), compared to those treated on Day 8 (8.8% [IQR 7.2-11.7]; p=0.030) (**supplementary table 7**). All participants in the PfK13 were treated on Day 9.

Drugs

There were sixteen single or combination drugs assessed in the Pf3D7 participants (**table 2**). However, substantial heterogeneity existed among these studies, precluding statistical analysis of drug effect on the fractional fall in haemoglobin. In particular, treatment was administered on day 7 in 10 studies, and on day 8 in 10 studies. The end of study (EOS) also varied, from Day 36 in the ZY-19489 study to Day 50 in the M5717 study. Recrudescence occurred more frequently in the ACT-451840 (7/8 participants) and SJ733 (6/16 participants) studies compared to the ferroquine study where no individuals recrudesced. Although statistical analysis was not performed, fractional falls in haemoglobin were numerically lowest

in those treated with griseofulvin (8.5% [IQR 6.8-9.3; n=3]), and highest in those treated with MMV048 (11.4% [IQR 8.4-14.2; n=13) (**figure 2**).

Table 2. Haemoglobin parameters in participants inoculated with Pf 3D7 who did not recrudesce, according to drug treatment

Pf3D7 (n=168) Drugs (n)	Nadir, g/L (median, IQR)	Fractional Fall (%, median, IQR)	Day post treatment of Hb Nadir (median, IQR)
ACT-451840 (1)	136	9.33	9
DSM265 (12)	135 (130-141)	10.9 (10.4-12.9)	21 (14-23)
DSM265 + Artefenomel (7)	115 (114-139)	10.6 (6.1-11.6)	21 (20-28)
Ferroquine (8)	129 (118-133)	11.3 (9.3-12.8)	20 (18-21)
Griseofulvin (3)	140 (136-146)	8.5 (6.8-9.3)	11 (4-20)
Cipargamin (4)	139 (133-144)	10.0 (8.0-13.0)	20 (12-23)
M5717 (20)	136 (132-142)	10.6 (8.5-12.9)	10 (6-14)
MMV048 (13)	138 (131-143)	11.4 (8.4-14.2)	9 (8-12)
Mefloquine (22)	127 (120-139)	9.1 (7.9-14.7)	4 (0-9)
Artefenomel (16)	125 (119-139)	8.6 (7.5-9.7)	8 (1-21)
Artefenomel + Piperaquine (21)	132 (127-140)	9.1 (7.0-13.2)	14 (0-27)
Piperaquine (23)	129 (120-140)	10.8 (6.3-13.0)	19 (0-23)
Piperaquine + Primaquine (2)	127-138	4.2-13.0	7-20
SJ733 (10)	136 (130-140)	9.8 (8.3-11.6)	12 (10-20)
ZY-19849 (6)	128 (122-137)	10.9 (5.5-11.6)	7 (3-8)

n- number of participants; b- no IQR as all 4 returned to baseline on this day; IQR- interquartile range

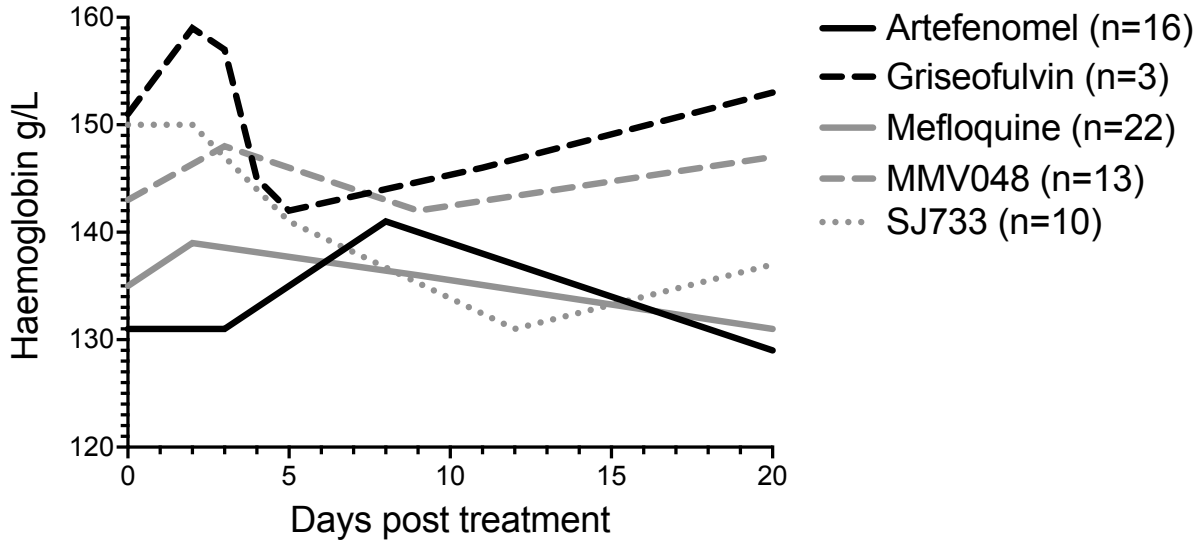

Figure 2. Median haemoglobin in participants inoculated with Pf3D7 who did not recrudesce, according to drug treatment

Participants inoculated with *P. vivax* were treated with either chloroquine (n=24), artefenomel (n=8) or artemether/lumefantrine (n=14), with no significant differences in the haemoglobin parameters observed between treatment groups (**supplementary table 8**). The PfK13 participants were all administered artesunate as the investigational medical product.

There was no correlation between speed of parasite killing (PRR) and fractional fall of haemoglobin in the overall *P. falciparum* and *P. vivax* groups (**supplementary table 9**). There was however a strong inverse correlation between PRR (r= -0.57, p=0.026) and the fractional fall in haemoglobin in the PfK13 group (**supplementary table 10**).

Effect of parasitaemia

In those inoculated with Pf3D7 and who did not experience recrudesce (n=170), there were no correlations between any of the parasitaemia variables and haemoglobin nadir or

fractional fall in haemoglobin, even after adjusting for drugs (**table 3**). There were however strong correlations between PP_{Pre} (r=0.66, p=0.007) and PMR (r=0.61, p=0.016) and the fractional fall in haemoglobin in the PfK13 group, although all (15/15) PfK13 participants recrudesced (**supplementary table 10**).

Table 3. Correlations between parasite parameters and haemoglobin variables in *P. falciparum* and *P. vivax* in those who did not recrudesce

Correlation	Pf3D7 (n=170)			*P. vivax* (n=39)		
	r-value	Unadjusted P-value [a]	Adjusted P-value [b]	r-value	Unadjusted P-value [a]	Adjusted P-value [c]
PP_{Pre} and Haemoglobin nadir	0.06	0.44	0.82	**-0.47**	**0.002**	**0.001**
PP_{Pre} and Fractional Fall of Haemoglobin	-0.05	0.49	0.43	**0.32**	**0.045**	**0.041**
TPB_{Pre} and Haemoglobin nadir	0.07	0.39	0.99	**-0.57**	**0.0001**	**0.002**
TPB_{Pre} and Fractional Fall of Haemoglobin	-0.01	0.89	0.78	**0.39**	**0.013**	**0.040**

n- number of participants; TPB_{Pre}- pre-treatment total parasite burden; PP_{Pre}- Pre-treatment peak parasitaemia; a- Pearson correlation- PP_{Pre} and TBP are log_{10} transformed; b-adjusted for drugs by including drug as an explanatory variable in a linear regression model; b-adjusted for drugs and by day of treatment by including drug and day of treatment as explanatory variables in a linear regression model

In participants inoculated with *P. vivax* and who did not experience recrudescence (n=39), the PP_{Pre} correlated with the fractional fall in haemoglobin (r=0.32, p=0.045), with this correlation remaining significant when adjusted for drugs (p=0.041). The TPB_{Pre} correlated with the haemoglobin nadir (r=-0.57, p=0.0001) and fractional fall in haemoglobin (r=0.39, p=0.013), with both correlations remaining significant after adjustment for drug (p=0.0001 and p=0.040, respectively) (**table 3**).

Malaria-attributable erythrocyte loss and the contribution of parasitised erythrocytes to the malaria-attributable erythrocyte loss

In participants inoculated with *P. falciparum*, the median total erythrocyte loss from inoculation until the day of haemoglobin nadir was 0.5 (IQR 0.3 – 0.7) x 10^{12} erythrocytes/L, with this loss being greater in those inoculated with PfK13 (median 0.7 [IQR 0.6 – 0.8] x 10^{12}/L) compared to Pf3D7 (median 0.5 [IQR 0.3 – 0.6] x 10^{12}/L; p=0.0005) (**Table 4**). The erythrocyte loss in those inoculated with *P. vivax* (median 0.5 [IQR 0.4 – 0.6] x 10^{12} erythrocytes/L) was similar to those inoculated with Pf3D7.

Table 4. Loss of infected erythrocytes as a percentage of total erythrocyte loss, in participants inoculated with Pf3D7, PfK13 and *P. vivax*

	Pf all (n=267) Median (IQR)	Pf 3D7 (n=254) Median (IQR)	Pf K13 (n=15) Median (IQR)	*P. vivax* (n=46) Median (IQR)	P-value (3D7 vs K13) [d]	P-value (Pf all vs *P. vivax*) [d]
Baseline RCC (x10^{12}/L)	5.0 (4.7-5.8)	5.0 (4.7-5.8)	5.1 (4.6-5.2)	5.0 (4.7-5.2)	0.58	0.57
Nadir RCC (x10^{12}/L)	4.5 (4.2-4.8)	4.5 (4.2-4.8)	4.4 (3.8-4.6)	4.4 (4.0-4.8)	0.07	0.31
Total RCC Loss (x10^{12}/L)	0.5 (0.3-0.7)	0.5 (0.3-0.6)	0.7 (0.6-0.8)	0.5 (0.4-0.6)	**0.0005**	0.57
Malaria-attributable RCC loss (x10^{12}/L) [a]	0.21 (0.04-0.36)	0.20 (0.04-0.33)	0.34 (0.12-0.50)	0.21 (0.04-0.33)	0.06	0.87
Malaria-attributable RCC loss/total RCC loss (%) [b]	42 (13-51)	40 (13-55)	49 (20-63)	42 (13-55)	0.29	0.46
Loss of pRBC/total malaria-attributable RBC loss (%) [c]	0.015 (0.006-0.060)	0.014 (0.005-0.039)	0.128 (0.068-0.616)	0.022 (0.008-0.082)	**<0.0001**	0.19

n-number of participants; IQR- interquartile range; a. calculated by subtracting the estimated phlebotomy-related RBC loss from the total RBC loss. For this calculation, the phlebotomy-related RBC loss was estimated by multiplying the participant's baseline RCC (x10^{12}/L) by the estimated total phlebotomy blood volume (0.19L) from day of inoculation to the median day of haemoglobin nadir.

b. The percentage contribution of the malaria-attributable erythrocytes losses from the total erythrocyte losses was determined by dividing the malaria-attributable losses by the erythrocyte losses and multiplying by 100 to generate a percentage.

c. The loss of pRBCs was equal to the TPB_{Pre}, which was determined using the AUC of the 18S qPCR data from day 4 until the time of treatment. For *P. falciparum*, the TPB_{Pre} incorporated an adjustment to account for parasite sequestration, assumed to be an assumed approximately 25% at any given timepoint. This calculation assumed that each erythrocyte was singly infected, and that parasite replication did not continue following treatment

d- Mann-Whitney test of significance which were not adjusted for multiple comparisons.

The malaria-attributable erythrocyte loss was estimated to be a median 0.21 (IQR 0.04 – 0.36) x 10^{12}/L in participants inoculated with *P. falciparum*, or ~42% of the total erythrocyte loss. Again, this loss was greater in the PfK13 group (median 0.34 [IQR 0.12 – 0.50] x 10^{12}/L, or 49% of the total erythrocyte loss) compared to Pf3D7 group (median 0.20 [IQR 0.04 – 0.33] x 10^{12}/L, or 40% of the total erythrocyte loss). In the *P. vivax* group, the median malaria-attributable erythrocyte loss was 0.21 (IQR 0.4 – 0.33) x 10^{12}/L, similar to the erythrocyte loss in the Pf3D7 group (**Table 4**). Applying these percentages to the fractional fall in haemoglobin, the malaria-attributable fractional fall in haemoglobin was 4.1% (IQR 3.1-5.3) in those inoculated with Pf3D7 group, 7.2% (IQR 5.8-7.8) in those inoculated with PfK13, and 4.9% (IQR 3.7-6.1) in those inoculated with *P. vivax*.

In participants inoculated with *P. falciparum*, parasitised cells accounted for an estimated 0.015% (IQR 0.006 – 0.060%) of the malaria-attributable erythrocyte loss. This proportion was

significantly higher in the PfK13 group (median 0.128 [IQR 0.068 – 0.616] %) compared to the Pf3D7 group (0.014 [IQR 0.005 – 0.039] %, p<0.0001). In those inoculated with *P. vivax*, parasitised cells accounted for an estimated 0.022 (IQR 0.008 – 0.082) % of the malaria-attributable erythrocyte loss. The loss of parasitised cells as a percentage of the total malaria-attributable erythrocyte loss was associated with the TPB_{Pre} in participants both species (*P. falciparum* r=0.83, p<0.001; *P. vivax* r=0.63, p<0.001) and both *P. falciparum* inocula (Pf3D7 r=0.81, p<0.001; PfK13 r=0.56, p=0.035).

Reticulocyte response

In those inoculated with *P. falciparum*, the reticulocyte count increased from a median of 55 (IQR 44-68) x 10^9/L at baseline to 68 (IQR 54-90) x 10^9/L at EOS, representing a median 22% increase (p=0.0001) (**Table 1, Figure 3**). A similar increase was seen among those inoculated with *P. vivax*. There was a significant correlation between the reticulocyte response and total erythrocyte loss in the *P. falciparum* group (r=0.13, p=0.04; n=240) but not in the in the *P. vivax* group (r=0.08, p=0.60; n=44). In the *P. falciparum* group, there was a correlation between age and the reticulocyte response (r=0.15, p=0.019; n=240), although this was not observed in the *P. vivax* group.

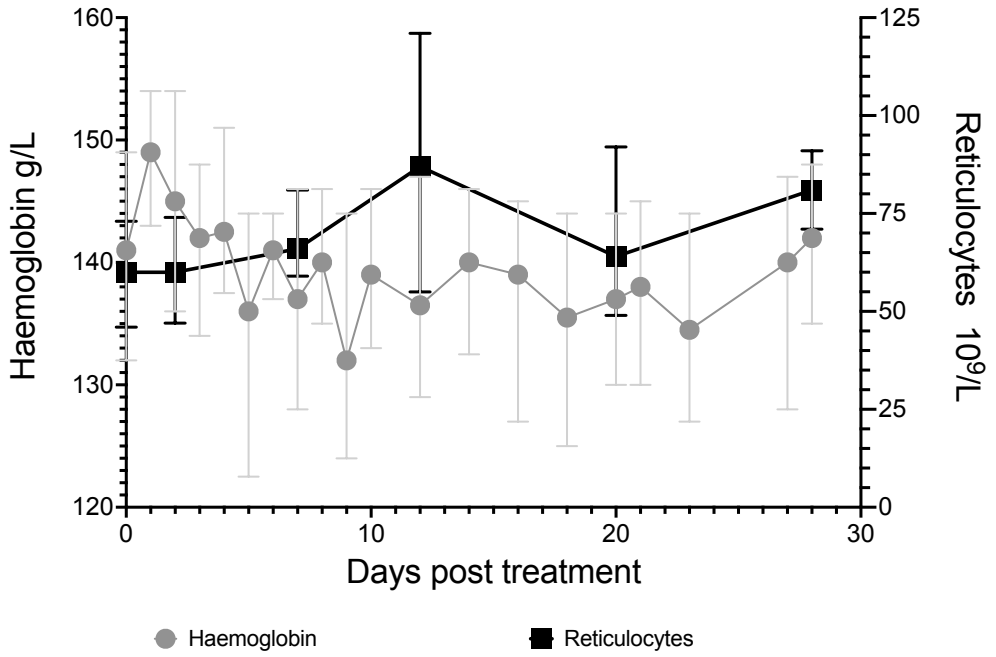

Figure 3. Median haemoglobin and reticulocyte count over time in participants inoculated with Pf3D7 (n=254).

Error bars represent interquartile range.

Discussion

In this study we describe the haematological response to early experimental *P. falciparum* and *P. vivax* infection. We show that in both *P. falciparum* and *P. vivax*, experimental infection results in an ~11% fractional fall in haemoglobin, approximately half of which occurs prior to treatment. The haemoglobin nadir occurred ~12 days after treatment in participants inoculated with *P. falciparum* and 8 days in participants inoculated with *P. vivax*, returning to normal by 28 days in *P. falciparum* and 20 days in *P. vivax*.

Volunteer infection studies are associated with frequent blood sampling, and we estimated that phlebotomy-related losses accounted for ~60% of the total fractional fall in haemoglobin. Nonetheless, the malaria-attributable loss from early experimental malaria, with parasitaemias only just reaching the limit of detection by microscopy, still accounted for a

fractional fall in haemoglobin of ~4% in Pf3D7, ~7% in PfK13 and ~5% in *P. vivax*, after accounting for phlebotomy related losses. It should be noted that in experimental malaria the duration of parasitaemia prior to treatment is only a few days, in contrast to endemic regions where low-level asymptomatic parasitaemia may persist in partially immune individuals for prolonged periods, thus the haemoglobin loss may be expected to be substantially greater. This is supported by recent studies from endemic regions, which have demonstrated a significant burden of anaemia associated with submicroscopic parasitaemia [10,11].

In participants inoculated with *P. falciparum*, the fractional fall in haemoglobin was greater in those inoculated with an artemisinin-resistant PfK13 strain, compared to those inoculated with the artemisinin-sensitive Pf3D7 strain. Contributing factors that may have accounted for this greater fractional fall include a later day of treatment and higher parasitaemias in participants inoculated with PfK13 compared to Pf3D7, as well as the slower parasite reduction ratio, and the recrudescence that occurred in all of the PfK13 participants following treatment with artesunate. The day of haemoglobin nadir also occurred later in the PfK13 participants, and no participant recovered their haemoglobin prior to the end of study, again likely reflecting delayed parasite clearance and recrudescent parasitaemia. In the PfK13 participants the parasite reduction ratio was significantly and inversely correlated with the fall in haemoglobin, with slower parasite clearance associated with greater haemoglobin fall. Although this study was not designed to directly compare haemoglobin losses in participants inoculated with PfK13 vs Pf3D7, our results are consistent with data from clinical studies reporting a greater incidence of anaemia in patients with drug-resistant *P. falciparum* [58,59] and those given drugs with slower parasite clearance effects [60–63]. Thus, while our study did not allow direct comparison of the effect of different drugs on haemoglobin loss, the

impact of parasite clearance on rates of anaemia should be considered in clinical trials evaluating antimalarials.

Consistent with the greater fall in haemoglobin in participants inoculated with PfK13, all of whom recrudesced, within the Pf3D7 group those who recrudesced also experienced a significantly greater fall in haemoglobin. These data are consistent with previous studies which have also demonstrated higher rates of anaemia associated with parasite recrudescence [13,57].

It is also possible that the greater fractional fall in haemoglobin observed in participants inoculated with PfK13 may have been due in part to the fact that these participants were treated with artesunate. Post-artesunate anaemia is well described, and results from delayed clearance of once-infected erythrocytes [68]. However, it is unlikely that this would be a significant contributor to erythrocyte loss at such low parasitaemias, and no participant inoculated with PfK13 had elevated markers of haemolysis [32].

In participants inoculated with *P. vivax*, we found that delaying day of treatment was associated with a greater fall in haemoglobin. This is also consistent with clinical studies reporting correlations between the time since symptom onset and degree of anaemia [69], and highlights the importance of early initiation of treatment.

In participants inoculated with *P. vivax*, we observed a correlation between parasitaemia (whether measured as peak parasitaemia, or pre-treatment total parasite burden) and the

fractional fall in haemoglobin. This is as expected, and consistent with clinical data demonstrating a correlation between parasitaemia and anaemia [70–72]. Unexpectedly, this association was not observed in the much larger group of participants inoculated with Pf3D7. It is possible the heterogeneity of the Pf3D7 studies (with different treatment days and different drugs evaluated), together with the low parasitaemias, may have obscured any association between parasitaemia and fractional fall in haemoglobin.

In this study we attempted to quantify the loss of parasitised cells as a proportion of the overall malaria-attributable erythrocyte loss. Similar analyses have been conducted previously, including in clinical malaria in endemic regions [13], and in neurosyphilis patients receiving malariotherapy [12] [14]. In the former study, Price et al [13] evaluated the haematological response to *P. falciparum* malaria in over 4000 children and adults in Thailand during 1990 – 1995, and estimated that parasitised cells accounted for 7.9% of the total malaria-attributable loss. A similar estimate was obtained by Jakeman et al [12], who used data from neurosyphilis patients undergoing malariotherapy to estimate that *P. falciparum* parasitised cells accounted for ~10.5% of erythrocytes lost. In neurosyphilis patients inoculated with *P. vivax*, Collins et al [14] estimated that only 2.9% of the reduction in haemoglobin was due to destruction of parasitised erythrocytes. In the current study involving very low parasitaemias, our analyses demonstrated that parasitised cells accounted for only 0.015% of erythrocytes lost in volunteers inoculated with Pf3D7, and 0.022% in those inoculated with *P. vivax*. This suggests that in submicroscopic infections, the relative contribution of the loss of unparasitised cells to malarial anaemia is likely much greater than that seen in higher parasitaemia infections.

The mechanisms mediating the loss of unparasitised cells at such low parasitaemias remain incompletely understood. In malaria volunteer infection studies, despite the low parasite counts, participants still experience a significant inflammatory response, with elevated levels of IFN-γ and IL-6 [68–70] potentially contributing to inhibition of erythropoiesis [21,71]. Additional contributors to the loss of unparasitised cells in acute clinical malaria include haemolysis, decreased red blood cell deformability, antibody and complement binding to erythrocytes, loss of complement regulatory proteins on the surface of unparasitised erythrocytes [72] and increase in splenic size [73] with associated splenic clearance of uninfected erythrocytes [74]. However, the role of these processes in low-parasitaemia infections such as volunteer infection studies has been more difficult to define [17].

Our study had a number of limitations. First, there was substantial heterogeneity between and within the individual malaria volunteer infection studies, including the drug used, the day of treatment, and the study duration, making it difficult to account for factors associated with haemoglobin loss. Second, in the *P. vivax* infection studies, our calculation of total parasite burden prior to antimalarial treatment did not account for parasite sequestration. Recent studies have suggested that in chronic *P. vivax* infection, a very high proportion of parasitized erythrocytes are sequestered in the spleen [55], and we have also shown that splenic accumulation may occur even in early *P. vivax* infection [73]. It is possible that this may in part explain the low contribution of peripheral parasitized cells to the malaria-attributable erythrocyte loss, in this study and in others [75]. Finally, our study was conducted in malaria-naïve healthy adults, and our data may not be applicable to children, or to adults in malaria-endemic regions where immunity maybe present.

In summary, we have demonstrated that a small but statistically significant fall in haemoglobin occurs in experimental malaria infection, despite parasitaemias that are only just at the level of microscopic detection. Our data add to studies from endemic regions reporting a significant burden of anaemia from asymptomatic and submicroscopic infections, highlighting the importance of treating these groups to reduce the overall burden of anaemia. Finally, our data on the expected haemoglobin loss in malaria volunteer infection studies can be used as a baseline against which to compare the haemoglobin losses that may occur when new antimalarial drugs are evaluated in this model.

Abbreviations

Abbreviation	Definition
ANOVA	Analysis of variance
AUC	Area under the curve
EOS	End of study
HB-FF	Fractional fall in haemoglobin
HREC	Human research ethics committee
IBSM	Induced blood-stage malaria
IFN	Interferon
IQR	Interquartile range
MMV	Medicines Malaria Venture
Pf	*Plasmodium falciparum*
Pf3D7	3D7 strain of *P. falciparum*
PfK13	K13 artemisinin-resistant strain of *P. falciparum*
PMR	Parasite multiplication rate
PP	Peak parasitaemia
PP_{Pre}	Pre-treatment peak parasitaemia
PRR	Parasite reduction ratio
pRBC	Parasitised red blood cell
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red blood cell
RCC	Red cell count
RNA	Ribonucleic acid
SD	Standard deviation
TPB_{Pre}	Pre-treatment total parasite burden
uRBC	Unparasitised red blood cell
VIS	Volunteer infection studies

Declarations

Ethics approval

Ethical approval for all studies was given by the QIMR Berghofer Human Research Ethics Committee (HREC)

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SDW, LM, JSM and BEB contributed to the design of the study. SDW, JW, LM, JSM and BEB participated in the implementation of the study and/or analysis of results. SDW, ML, SC,

JJM, JSM and BEB participated in writing the manuscript. All authors read and approved the final manuscript.

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Supplementary Appendix- Haemoglobin analysis paper

Supplementary Table 1. List of clinical trials

Clinical Trial ID	Drug/ Dose	Year	Inoculum	Day of Treatment	No of Subjects	Reference
ACTRN12612000323820	Mefloquine	2012	Pf 3D7	7 or 8	22	[1]
ACTRN12612000814875	Artefenomel	2012	Pf 3D7	7 or 8	24	[2]
ACTRN12612001096842	Artemether/lumefantrine	2012	*P. vivax*	14	2	[3]
ACTRN12613000533796	DSM265	2013	Pf 3D7	8	9	[4]
ACTRN12613000669796	Artemether/lumefantrine	2013	Pf 3D7	8	2	[5]
ACTRN12613000565741	Piperaquine	2013	Pf 3D7	7 or 8	24	[6]
ACTRN12613000698774	Griseofulvin	2013	Pf 3D7	8	5	[7]
ACTRN12613001008718	Artemether/lumefantrine	2013	*P. vivax*	14	6	[8]
ACTRN12613001040752	Ferroquine	2013	Pf 3D7	8	8	[9]
ACTRN12614000781640	ACT-451840	2014	Pf 3D7	7	8	[10]
ACTRN12614000930684	Artemether/lumefantrine	2014	*P. vivax*	8	2	[11]
NCT02281344	MMV390048	2014	Pf 3D7	7	6	[12]
NCT02389348	Artefenomel + DSM265	2015	Pf 3D7	7	13	[13]
NCT02431637	Piperaquine	2015	Pf 3D7	7	6	[14]
NCT02543086	Cipargamin + Piperaquine	2015	Pf 3D7	7	8	[15]
NCT02431650	Piperaquine + Artefenomel or Primaquine	2015	Pf 3D7	7	11	[14]
NCT02573857	DSM265 (Pf) and Artefenomel (*P. vivax*)	2015	Pf3D7/ *P. vivax*	7 or 10	15	[16,17]
ACTRN12616000174482	Chloroquine	2016	*P. vivax*	8 or 10	24	[11]
NCT02867059	SJ733	2016	Pf 3D7	8	16	[18]
NCT02783833 (Part B)	MMV390048	2016	Pf 3D7	8	15	[19]
ACTRN12617000244303	Artesunate	2017	Pf K13	9	2	[20]
NCT03261401	M5717	2017	Pf 3D7	8	22	[21]
ACTRN12617001394336	Artesunate	2017	Pf 3D7/Pf K13	8 or 9	22	[20]
ACTRN12617001502325	Artemether/lumefantrine	2017	*P. vivax*	10 or 11	4	[22]
NCT03542149	Artfenomel + Piperaquine	2018	Pf 3D7	8	24	[23]
ACTRN12619001215112	ZY-19489	2019	Pf 3D7	8	15	[24]

Calculations

i. Total RCC Loss ($x10^{12}$ cells/L) =

Baseline RCC – RCC at haemoglobin nadir

The baseline RCC is the RCC on the day of inoculation. The RCC at haemoglobin nadir, which was calculated as the lowest haemoglobin from the day of first antimalarial treatment onwards.

ii. Individual total blood volume in litres (using Nadler's method)

Male= $[0.3669*(\text{height in metres})^3] + [0.03219*(\text{weight in kg})] + 0.6041$

Female= $[0.3561*(\text{height in metres})^3] + [0.03308*(\text{weight in kg})] + 0.1833$

iii. Phlebotomy losses (x10^{12} cells/L) =

*Baseline RCC * (0.19/total individual blood volume)*

The estimated total phlebotomy losses from inoculation to the median day of haemoglobin nadir was 0.19 L, which is divided by the individual total blood to give a proportion of the total blood volume. The volume is then multiplied against the baseline RCC to give the phlebotomy erythrocyte losses.

iv. Malaria attributable losses (x10^{12} cells/L) =

Total RCC Loss – Phlebotomy Loss

v. Malaria attributable losses (cells/L) =

Malaria attributable losses x 10^{12}

vi. Malaria attributable losses (cells/μL) =

$$\frac{\textit{Malaria attributable losses (cells/L)}}{10^6}$$

Division by 10^6 is the conversion factor from cells/L to cells/μL

vii. TPB_{PreSeq}/mL [for *P. falciparum* species only] =

*TPB_{Pre} [calculated via AUC] *1.33*

For *P. falciparum* species only, the TPB_{Pre} was adjusted for sequestered parasites, which is assumed to 25% of the TPB at any one time, therefore the TPB_{Pre} is multiplied by 1.33.

viii. TPB_{PreSeq}/μL or TPB_{Pre} for *P. vivax* =

TPB_{PreSeq} or TPB_{Pre} [P. vivax]/ 1000

Conversion of the TPB_{PreSeq}/μL or TPB_{Pre} from parasites/mL to parasites/μL, by dividing by 1000. This ensures both the malaria-attributable RBC losses and TPB_{PreSeq} or TPB_{Pre} are in cells/parasites per μL.

ix. Loss of pRBC/total malaria-attributable RBC loss (%)

$$\frac{TPB_{PreSeq}\,[P.\ falciparum]\ or\ TPB_{Pre}\,[P.\ vivax]}{Malaria\ attributable\ losses\ (cells/\mu L)} \times 100$$

Supplementary Table 2. **Effect of recrudescence on haemoglobin parameters in all participants inoculated with *P. falciparum***

Pf (n=269)	Recrudescence (n=99)	No recrudescence (n=170)	P-value [a]
Baseline Hb, g/L (Median, IQR)	150 (141-157)	148 (138-155)	0.47
Nadir Hb, g/L (Median, IQR)	130 (123-141)	133 (123-140)	0.39
Hb Fractional Fall, % (Median, IQR)	12.0 (8.1-15.2)	10.2 (7.7-12.8)	**0.006**
Day post treatment of Hb Nadir, Day (Median, IQR)	14 (6-20)	11 (4-21)	0.74
Day post treatment of return to baseline Hb, Day (Median, IQR)	28 (22-36)	29 (21-37)	0.68

n-number of participants; SD- standard deviation; IQR- interquartile range; a- Mann-Whitney test used

Supplementary Table 3. Effect of recrudescence on haemoglobin parameters in participants inoculated with *P. vivax*

P. vivax (n=46)	Recrudescence (n=7)	No recrudescence (n=39)	P-value [a]
Baseline Hb, g/L (Median, IQR)	147 (136-152)	136 (126-167)	0.65
Nadir Hb, g/L (Median, IQR)	139 (125-141)	129 (119-140)	0.21
Hb Fractional Fall, % (Median, IQR)	11.8 (8.7-16.4)	11.7 (8.8-14.5)	0.95
Day post treatment of Hb Nadir, Day (Median, IQR)	15 (8-15)	8 (7-16)	0.38
Day post treatment of return to baseline Hb, Day (Median, IQR)	21 (18-33)	20 (18-25)	0.57

n-number of participants; SD- standard deviation; IQR- interquartile range; a- Mann-Whitney test used

Supplementary Table 4. Effect of age on the haemoglobin parameters in all participants inoculated with *P. falciparum*

Pf 3D7 (n=254)	Ages			
	18-24 (n=143)	**25-34 (n=102)**	**35+ (n=24)**	**P-value [a]**
Nadir Hb, g/L (Median, IQR)	133 (125-141)	130 (120-139)	134 (127-139)	0.09
Hb Fractional Fall, % (Median, IQR)	10.2 (7.9-13.3)	11.0 (8.2-14.4)	10.4 (6.0-12.8)	0.15
Day post treatment of Hb Nadir, Day (Median, IQR)	14 (5-21)	10 (5-20)	14 (7-20)	0.16
Day post treatment of Hb Return, Day (median, IQR)	28 (22-36)	28 (22-36)	33 (27-43)	0.30+

n-number of participants; IQR- interquartile range; a- Kruskal-Wallis test used

Supplementary Table 5. Effect of age on the haemoglobin parameters in participants inoculated with *P. vivax*

P. vivax (n=46)	Ages		
	18-24 (n=25)	**25+ (n=21)**	**P-value [a]**
Nadir Hb, g/L (Median, IQR)	135 (125-141)	124 (120-135)	0.07
Hb Fractional Fall, % (Median, IQR)	10.5 (8.5-14.0)	13.6 (10.8-14.6)	0.09
Day post treatment of Hb Nadir, Day (Median, IQR)	8 (7-15)	8 (5-16)	0.56
Day post treatment of Hb Return, Day (median, IQR)	20 (18-21)	19 (16-27)	0.43

n-number of participants; IQR- interquartile range; a- Mann-Whitney test used

Supplementary Table 6. Effect of Day of Treatment on the haemoglobin parameters in participants inoculated with Pf3D7

Pf 3D7 (n=254)	Day of Treatment		
	7 (n=74)	**8 (n=180)**	**P-value [a]**
PP_{Pre}, Parasites/mL (Median, IQR)	7,329 (3,375-14,982)	10,243 (3,275-28,546)	0.05
TPB_{Pre}, Parasites/mL (Median, IQR)	13,505 (4,261-26,364)	44,877 (18,157-97,669)	**<0.0001**
Nadir Hb, g/L (Median, IQR)	130 (120-141)	133 (125-140)	0.49
Hb Fractional Fall, % (Median, IQR)	9.7 (7.2-12.8)	10.6 (8.0-13.8)	0.12
Day post treatment of Hb Nadir, Day (Median, IQR)	19 (6-21)	10 (5-20)	0.052
Day post treatment of Hb Return, Day (median, IQR)	28 (22-35)	29 (22-38)	0.15

n-number of participants; IQR- interquartile range; PP_{Pre}- Peak parasitaemia pre-treatment; TPB_{Pre}- Pre-treatment total parasite burden which has been adjusted by a factor of 25% for sequestered parasites; a-TPBPre Mann-Whitney test used

Supplementary Table 7. Effect of Day of Treatment on the haemoglobin parameters in participants inoculated with *P. vivax*

***P. vivax* (n=46)**	Day of Treatment		
	8 (n=10)	**10-14 (n=36)**	**P-value [a]**
PP_{Pre}, Parasites/mL (Median, IQR)	6,480 (3,952-8,949)	45,268 (30,437-82,905)	**0.003**
TPB_{Pre}, Parasites/mL (Median, IQR)	8,333 (4,690-10,895)	45,557 (26,732-87,598)	**<0.0001**
Hb Fractional Fall, % (Median, IQR)	8.8 (7.2-11.7)	12.4 (9.7-14.6)	**0.030**
Day post treatment of Hb Nadir, Day (Median, IQR)	14 (7-20)	8 (5-15)	0.09
Day post treatment of Hb Return, Day (median, IQR)	20 (19-21)	19 (18-27)	0.34

n-number of participants; IQR- interquartile range; PP_{Pre}- Peak parasitaemia pre-treatment; TPB_{Pre}- Pre-treatment total parasite burden; a- Mann-Whitney test used

Supplementary table 8. Effect of drugs on the haemoglobin parameters in participants inoculated with *P. vivax*

***P. vivax* (n=46)**	Drug Classes			
	Chloroquine (n=24)	**Artefenomel (n=8)**	**Artemether/lumefantrine (n=14)**	**P-value [a]**
Nadir Hb, g/L (Median, IQR)	129 (114-140)	137 (128-140)	129 (121-139)	0.44
Hb Fractional Fall, % (Median, IQR)	11.3 (8.8-14.2)	11.3 (9.6-14.4)	13.8 (8.9-14.7)	0.91
Day post treatment of Hb Nadir, Day (Median, IQR)	7 (6-18)	11 (8-15)	8 (3-14)	0.56
Day post treatment of Hb Return, Day (median, IQR)	20 (18-21)	19 (18-27)	20 (14-28)	0.96

n-number of participants; IQR- interquartile range; Dunn's test used

Supplementary Table 9. Spearman's correlations between parasite parameters and haemoglobin variables in all participants inoculated with *P. falciparum* and *P. vivax* who did not recrudesce

Correlation	Pf All(n=170)		*P. vivax* (n=39)	
	r-value	P-value	r-value	P-value
PP_{Pre} and Haemoglobin nadir	0.07	0.37	**-0.51**	**0.0008**
PP_{Pre} and Fractional Fall of Haemoglobin	-0.04	0.61	**0.38**	**0.018**
TPB_{Pre} and Haemoglobin nadir	0.07	0.35	**-0.60**	**0.0001**
TPB_{Pre} and Fractional Fall of Haemoglobin	0.01	0.93	**0.44**	**0.005**
PMR and Haemoglobin nadir	0.01 [a]	0.94	0.05 [b]	0.81
PMR and Fractional Fall of Haemoglobin	-0.05 [a]	0.53	-0.27 [b]	0.17
PRR and Haemoglobin nadir	0.05 [c]	0.59	0.20 [d]	0.35
PRR and Fractional Fall of Haemoglobin	0.01 [c]	0.87	-0.23 [d]	0.26

n-number of participants; TPB_{Pre}- pre-treatment total parasite burden; PP_{Pre}- Pre-treatment peak parasitaemia; Spearman's correlation used; PMR- parasite multiplication rate; PRR- parasite reduction ratio; a- n=155; b- n=27; c- n=142; d- n=25.

Supplementary Table 10. Spearman's correlations between parasite parameters and haemoglobin variables in participants inoculated with PfK13

Correlation	Pf K13 (n=15)*	
	r-value	P-value
PP_{Pre} and Haemoglobin nadir	-0.46	0.09
PP_{Pre} and Fractional Fall of Haemoglobin	**0.66**	**0.007**
TPB_{Pre} and Haemoglobin nadir	-0.37	0.18
TPB_{Pre} and Fractional Fall of Haemoglobin	0.50	0.06
PMR and Haemoglobin nadir	-0.49	0.04
PMR and Fractional Fall of Haemoglobin	**0.61**	**0.016**
PRR and Haemoglobin nadir	**0.72**	**0.002**
PRR and Fractional Fall of Haemoglobin	**-0.57**	**0.026**

n-number of participants; TPB_{Pre}- pre-treatment total parasite burden; PP_{Pre}- Pre-treatment peak parasitaemia; PMR- parasite multiplication rate; PRR- parasite reduction ratio; Spearman's correlation used

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4.5 Significance of this paper

The key findings from this paper are as follows:

- **Malaria attributable losses**. The fractional fall in haemoglobin in IBSM VIS is between 10 and 15%, depending on the species that was inoculated. After factoring in the losses through phlebotomy, the fractional fall in haemoglobin that were attributable to the malaria were approximately 4% in *P. falciparum* 3D7, 7% in *P. falciparum* K13 and 5% in *P. vivax.*
- **Day of haemoglobin nadir and return to baseline.** In those inoculated with *P. falciparum,* the median day of haemoglobin nadir occurred at day 12 post-treatment, with a return to haemoglobin baseline occurring at day 28 post-treatment. In those inoculated with *P. vivax* the median day of haemoglobin nadir was day 8 post-treatment, returning to baseline on day 20 post-treatment.

- **The contribution of parasitised erythrocytes to the malaria attributable losses.** In previous studies looking at *P. falciparum* infections by Price *et al* [63] and Jakeman *et al* [75], the estimated contribution of parasitised erythrocytes was 7.9% and 10.5% respectively. A similar study looking at the contribution in those infected with *P. vivax* was 2.9% [154]. In this study the contributions were considerably smaller, with the parasitised erythrocytes contributing 0.015% in those inoculated with *P. falciparum* and 0.022% in those inoculated with *P. vivax*. The mechanisms remain poorly understood but are an area for further investigation.
- **Factors affecting the fractional fall in haemoglobin**. Those inoculated with *P. falciparum* who experienced recrudescence had a higher fractional fall in haemoglobin compared to those who did not. In those inoculated with *P. vivax*, the use of a later treatment schedule (day 14 vs day 8) was associated with a higher fractional fall in haemoglobin, which is linked to those individuals having higher parasite burdens.
- **Reticulocyte response**. The reticulocyte response was correlated with the erythrocyte losses in those inoculated with *P. falciparum*, unlike those inoculated with *P. vivax*. This is likely linked to *P. vivax* having a predilection for younger erythrocytes especially reticulocytes, when compared to *P. falciparum*.

This study provides a detailed analysis of the haemoglobin trends that occur in malaria VIS. This data will provide a baseline against which haemoglobin losses in future malaria VIS can be evaluated, informing the assessment of any drug-specific effect on haemoglobin. Of note is the low contribution of parasitised erythrocytes to the overall malaria-attributable losses observed. Erythrocyte loss in malaria is multi-factorial, however dyserythropoiesis especially the metabolism of iron, could be a significant mechanism. The next chapter investigates the markers of iron metabolism in early malaria as a possible mechanism for the development and progression of malaria-associated erythrocyte losses and anaemia.

Chapter 5. Iron metabolism in malaria volunteer infection studies using the induced blood-stage model

5.1. Declaration

This study was designed by Bridget Barber, Fiona Amante, Jeremy Gower, James McCarthy and me. The sample preparation and ELISA processing was conducted by Jeremy Gower and me. The data analysis was performed by me with advice and assistance from Louise Marquart and Bridget Barber.

5.2. Introduction

Anaemia was estimated to affect up to 800 million women and children globally in 2011, with the highest prevalence in children aged 6-59 months and pregnant women, aged 15 to 49 [109]. According to regional estimations in 2011, the greatest burden of anaemia was in Africa and South-East Asia. Iron deficiency is the leading cause of anaemia globally, accounting for up to 60% of cases of anaemia [105]. It affects up to 2 billion people worldwide, of which a quarter develop anaemia [106]. The key finding by Muriuki *et al* [118], was that any intervention that reduces the risk of malaria would halve the prevalence of iron deficiency, which is the first time reported in the literature of the causation of iron deficiency by malaria. The mechanism which is proposed is through the upregulation of hepcidin via inflammatory and non-inflammatory pathways, subsequently reducing the absorption of iron across the small bowel [118].

The relationship between iron deficiency and malaria is complex and the debate on whether to give supplemental iron in malaria endemic settings is ongoing. Previous studies by Sazawal *et al* [121] and Murray *et al* [120] have suggested that giving iron supplementation in malaria endemic regions increases the risk of malaria and also increased mortality. However the latest Cochrane review by Neuberger *et al* [106], suggested that iron supplementation in malaria endemic regions does not increase the risk of malaria in the context of effective malaria-control programmes, via readily available malaria prevention interventions and treatment services [106].

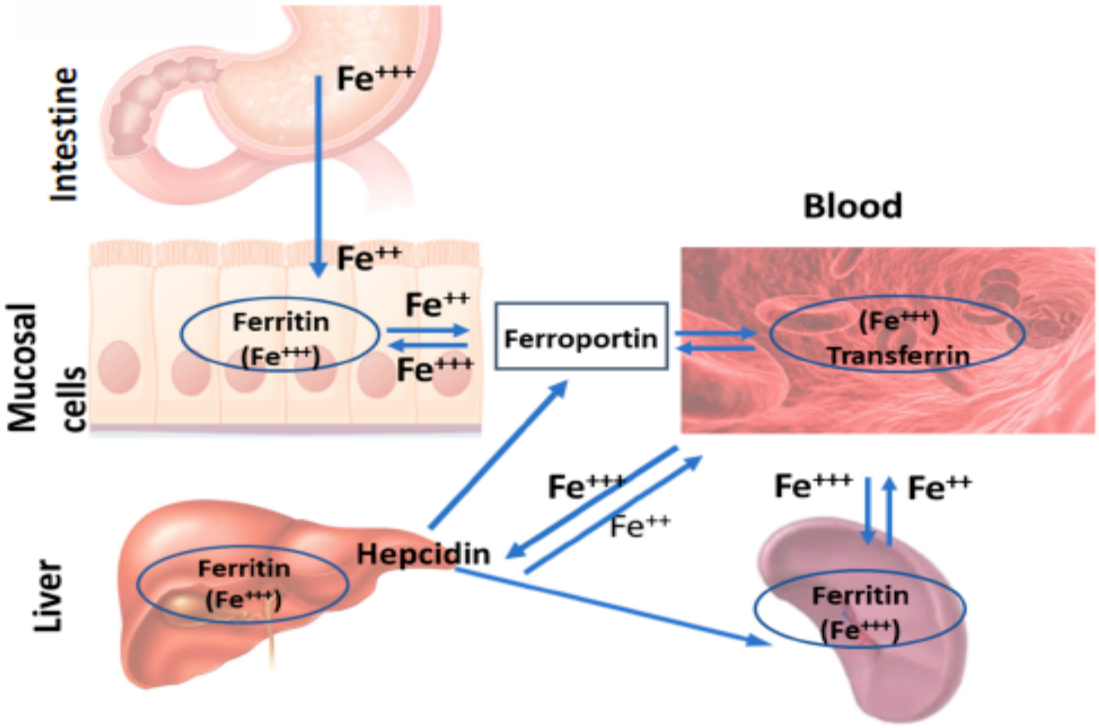

Figure 5.1. Iron transportation and storage. Fe++ is the ferrous ion of iron. Fe+++ is the ferric ion of iron. Iron is absorbed by the enterocytes and transported by the plasma protein transferrin. Iron is intracellularly stored in ferritin. As well as iron storage, the liver produces hepcidin which controls the movement of iron through the degradation of ferroportin. Adapted from Yiannikourides *et al* [155].

In normal iron metabolism, iron is absorbed via enterocytes in the small bowel (**figure 5.1**). The control of iron across the enterocytes is through the hepcidin mediated control of ferroportin [92,94,96,155]. Iron is then transported in the peripheral circulation via transferrin, the iron transport plasma transport which transports iron in the ferric state [155], which under normal physiological parameters is only 30% saturated. This is an important buffering function, which allows uptake of toxic non-transferrin bound iron [156]. All nucleated cells express plasma membrane transferrin receptors, which directly correlate with cellular iron requirements [155]. Ferritin is a large protein, whose primary role is intracellular iron storage, which can hold up to 4500 atoms of iron [94], and when the cell becomes saturated with iron-laden the ferritin aggregates with lysosomes resulting in haemosiderin [94,157]. Haemosiderin is insoluble and predominantly found in macrophages, and is also controlled by hepcidin [155]. There is some intracellular release of ferritin, and this is correlated with the total intracellular iron stores [94,155]. Ferritin is also an acute phase

protein and is released in acute inflammation, which can lead to false interpretations of the total iron stores. Soluble transferrin receptor (sTfR) is regarded by Suominen *et al* [112] to be a more accurate reflection of iron status, as it is not elevated in acute inflammation. Suominen *et al* [112] also evaluated the TfR-log ferritin index and determined it was even better in determining those the iron-deficient states prior to the development of iron-deficiency anaemia.

The major controller of body iron metabolism is the hormone hepcidin, which is produced predominantly by hepatocytes [92]. Hepcidin acts by binding to and inducing the degradation of ferroportin, a transmembrane iron transporter, therefore inhibiting cellular iron efflux [92,94,96]. As a consequence, hepcidin controls systemic iron by inhibiting iron export from macrophages, enterocytes, hepatocytes and other body cells and induces a hypoferraemia [94]. Hepcidin is upregulated by Interleukin-6 (IL-6) via JAK/STAT3 signalling in response to inflammation and this response is observed in acute infection as well as in chronic diseases such as chronic renal disease and inflammatory conditions such as rheumatoid arthritis [92,94,158].

Hepcidin action is also regulated by the recently discovered hormone, erythroferrone (ERFE) [102–104]. ERFE is produced by erythroblasts in response to erythropoietin (EPO) [104]. Hypoxia, oxidative stress and subsequently anaemia, cause EPO to be produced by the kidney [104]. This increase in EPO causes ERFE to be secreted into the systemic circulation and it acts on hepatocytes to decrease the production of hepcidin [104]. Latour *et al* [130], noted that in their murine malaria model using *P. berghei*, ERFE mRNA expression was concomitantly raised with elevated levels of EPO post infection. Hepcidin levels were elevated in both wild-type and ERFE deficient mice up to day 7 after infection, however hepcidin was supressed below baseline in the wild-type mice only with no significant blunting of the hepcidin suppression in the ERFE deficient mice [130]. Presently, ERFE has not been studied in human malaria infection.

Alterations in iron metabolism may also contribute to the elevations in liver transaminases observed in IBSM VIS. As mentioned in chapter two, these elevations were initially presumed to be drug induced liver injury, however studies have hypothesised systematic inflammation via TNF could be an explanation [134]. However, the relationship of iron has not investigated in

the context of these elevations in the liver transaminases. It is understood from chronic liver disease, that the increase in intrahepatic iron can cause local inflammation and tissue necrosis [141,142]. With an acute rise in hepcidin, free iron is retained in hepatocytes and in the reticulo-endothelial system [94,132]. The IBSM VIS participants may be more likely to be iron-replete when compared to individuals from a malaria endemic region, which could explain why raised liver transaminases are more common in IBSM VIS compared to clinical malaria.

The further evaluation and characterisation of iron homeostasis in malaria will provide insights into the pathophysiological mechanisms and regulators of malarial anaemia, with the aim of informing the development of treatments for malarial anaemia. The primary objective of this study was to determine longitudinal changes in EPO, ERFE, hepcidin and iron metabolism markers (ferritin, soluble transferrin receptor [sTfR] and the $\log_{10}$ ferritin/ sTfR index) in VIS subjects inoculated with *P. falciparum* and *P. vivax.*

The secondary objectives of this study were to:

1. Correlate iron metabolism markers with haemoglobin and parasitaemia

2. Correlate the effect of iron and hepcidin on parasite multiplication rates

3. Correlate the effect of iron status with post-treatment elevations in liver function tests

5.3. Methods

5.3.1. Ethics

Ethics approval was granted by the QIMR Berghofer Human Ethics Committee (HREC) on 10 October 2019 (**appendix 3**). The samples utilised were from participants involved in previous QIMR IBSM VIS Phase 1 studies, and all had provided written consent for their retained samples to be used in future research. All IBSM VIS studies were conducted in accordance with the Declaration of Helsinki and the International Committee of Harmonisation Good Clinical Practice guidelines, with ethical approval being granted by the QIMR Berghofer HREC.

5.3.2. Haematology, liver function, iron, and parasite data

Phlebotomy was carried at specified timepoints throughout the trials. The haematology tests included a full blood count, which included a haemoglobin, mean corpuscular volume (MCV) and a reticulocyte count at selected timepoints. At certain timepoints, liver function tests were taken, which included alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin. Some of the earlier studies did not have iron studies and C-reactive protein (CRP) conducted, whereas the newer studies had these two tests conducted during the trial. Where the ferritin and CRP levels were not conducted, aliquots from those participants were sent for ferritin and CRP testing at Sullivan and Nicolaides (SNP) Laboratories. The ferritin was then adjusted for CRP, as ferritin is an acute phase protein and liable to be elevated in pro-inflammatory conditions. The adjustment would give a more accurate picture of the iron status. The adjustment calculation was based on the biomarkers reflecting inflammation and nutritional determinants of anaemia (BRINDA project) [159–161]. The calculation utilisng the BRINDA adjustment was:

1. If CRP < 5mg/mL then $Ferritin_{Unadj} = Ferritin_{Adj}$
2. If CRP>5mg/mL then $Ferritin_{Unadj} \times 0.67 = Ferritin_{Adj}$

The log ferritin/sTfR index was calculated by dividing the log_{10}-transformed adjusted ferritin level by the concurrent sTfR. In the literature, the index is calculated by sTfR/ log_{10} ferritin [112], however for ease of interpretation the index was performed inversely; therefore a low log ferritin/sTfR index represents a low-level of iron status. The ferritin/AST ratio, used to estimate the intrahepatic iron concentration, was calculated as per Cippa *et al* [143].

The baseline haemoglobin was defined as the haemoglobin on the day of inoculation or day -1. The haemoglobin nadir was the lowest haemoglobin concentration following treatment, and this data was taken from the haemoglobin analysis. The fractional fall in haemoglobin was the difference between the baseline and haemoglobin nadir expressed as a percentage of the baseline haemoglobin. The reticulocyte difference, which was the difference between baseline and EOS, which was also taken from the haemoglobin analysis.

Parasitaemia was monitored by quantitative PCR (qPCR) targeting the species specific 18S rRNA gene as previously described [152,153]. The peak parasitaemia was the highest parasitaemia within 24 hours of treatment. The pre-treatment total parasite burden (TPB) was determined using the area under curve (AUC) of the non-transformed 18S qPCR data from day 4 until the time of treatment. For those inoculated with *P. falciparum,* the TPB incorporated an adjustment to account for sequestered parasites, assumed to be approximately 25% of the total parasite burden at any given timepoint [162]. The PMR and PRR were calculated as previously described [152,153]. Parasite recrudescence was defined as a parasitaemia increase by >1000 parasites/mL occurring more than 2 days post-treatment. This definition for parasite recrudescence was used, as we had frequent 18S qPCR data which was more sensitive at the submicroscopic levels of infection in determining infection especially in view of low-level circulating gametocytes that could remain post-treatment.

5.3.3. Statistical analysis

A statistical analysis plan was agreed prior to the start of this pilot study between Bridget Barber, Louise Marquart and I. Samples were taken from five IBSM trials. The following endpoints were based on the objective:

Primary endpoints

- Longitudinal changes in EPO, ERFE, hepcidin, ferritin, sTfR and the $\log_{10}$ ferritin/sTfR index in those inoculated with *P. falciparum* and *P. vivax* between day 0 and day 30 (EOS).

Secondary

- Correlations between the hormones (EPO, ERFE and hepcidin) and markers of iron status (adjusted ferritin, sTfR and $\log_{10}$ ferritin/ sTfR index) with haemoglobin and total parasite burden in both *P. falciparum* and *P. vivax*
- Correlations between baseline (day 0) markers of iron status and the parasite multiplication rates in both *P. falciparum* and *P. vivax*
- Correlations between baseline (day 0) markers of iron status and the post-treatment (day 15) liver transaminases in both *P. falciparum* and *P. vivax*

- Longitudinal changes in the ferritin/AST ratio those inoculated with *P. falciparum* and *P. vivax* between day 0 and day 30 (EOS).
- Correlations pre-treatment (day 8 in *P. falciparum* and day 10 in *P. vivax*) and post-treatment ferritin/AST ratio and the post-treatment (day 15) ALT in both *P. falciparum* and *P. vivax*

Data were analysed using Stata V17.0 and GraphPad Prism V9.1.0. For normally distributed continuous variables, the mean and standard deviation (SD) were reported and differences between groups were compared using the Student's t-test or analysis of variance (ANOVA) test. A repeated measures ANOVA was used on normally distributed variables and $\log_{10}$ transformed variables. The Geisser and Greenhouse correction using a cut-off of $p<0.05$ was used to determine sphericity. With up to six pairwise comparisons per repeated ANOVA, the Bonferroni correction was used to adjust the p-values, with α=0.05/6 [the number of pairwise comparisons], leading to an adjusted p-value of 0.008 being used to determine significance. Non-normally distributed continuous variables were summarised using the median and interquartile range (IQR); where two groups were compared the Wilcoxon rank-sum test was used to test for significance and differences between more than two groups were compared using the Friedmann's test, adjusted using the Bonferroni correction as above. Where there were more than 2 groups for example when comparing the effect of drug on post-treatment elevations in liver function tests, a Dunn's multiple comparison test was used. The artemisinin drug class was considered the reference group, as artemisinins are the gold standard of treatment in both uncomplicated and complicated falciparum malaria [87]. Spearman's correlation was used to investigate the correlation of the non-parametric iron-axis and the haematology, liver, and parasite data.

5.3.4. Malaria volunteer infection studies

Stored frozen plasma samples were retrieved from malaria volunteer infection studies conducted from 2015 to 2020. In these studies, participants were intravenously inoculated with a low dose of either *P. falciparum* or *P. vivax* infected erythrocytes at day 0 and treated with an antimalarial agent either day 7 or 8 in *P. falciparum* and day 8-14 in *P. vivax*. Bloods were usually taken at an eligibility visit (day -3 to -1), day of inoculation, day of antimalarial

administration and at days 14, 15, 18, 19, 20 and day of rescue. There was variability in the timing of the bloods, which was dependent on the study and cohort used. The studies utilised are highlighted below in table 5.1:

Study	*ANZCTR Number*	*Drug Candidate*	*Inoculation Agent*	*No. of Subjects*
QP15C20	NCT02867059	*SJ733*	*P. falciparum* 3D7	8
QP16C04	NCT02783833 (Part B)	MMV390048	*P. falciparum* 3D7	5
QP17C11	ACTRN12617001394336	Artesunate	*P. falciparum* 3D7	4
QP17C11	ACTRN12617001394336	Artesunate	*P. falciparum* K13	10
QP17C14	ACTRN12617001502325	Artemether/ lumefantrine	*P. vivax* HMPBS02	4
QP18C07	ACTRN12619001215112	ZY-19489	*P. falciparum* 3D7	14
P3491	ACTRN12619001079134	Artemether/ lumefantrine	*P. falciparum* 3D7-MBE008	2

Table 5.1. Summary table of QIMR Berghofer studies used. ANZCTR- Australian New Zealand Clinical Trials Registry

The sample size for those inoculated with *P. falciparum* 3D7 was 33, with 10 inoculated with *P. falciparum* K13 and 4 with *P. vivax*. Ferritin and CRP were performed at Sullivan Nicholades Pathology, while ERFE, EPO, Hepcidin and sTfR were performed at QIMR Berghofer by ELISA. The ELISA assays were commercial assays from Intrinsic Lifesciences (ERFE and Hepcidin), BioLegend (Human EPO) and BioVendor (sTfR). All ELISAs were performed as per manufacturer's instructions.

5.4. Results

5.4.1. Demographics

The median age of volunteers was 26 years [IQR 22-23; n=43] for those inoculated with *P. falciparum* and 34 [IQR 28-42; n=4] for those inoculated with *P. vivax*. All four participants who were inoculated with *P. vivax* group were male, whilst 70% (30/43) were male in those inoculated with *P. falciparum*. Of those inoculated with *P. falciparum*, 76% (25/33) in the Pf3D7 subgroup were male compared to 50% (5/10) in the PfK13 subgroup; this difference was not statistically significant (p=0.12).

	Pf all (n=43)	Pf 3D7 (n=33)	Pf K13 (n=10)	Pv (n=4)	P-value (3D7 vs K13)	P-value (Pf all vs Pv)
Age, Years (Median, IQR)	26 (22-33)	27 (23-34)	23 (21-27)	34 (28-41)	0.19	0.13
Male Sex, n (%)	30 (70)	25 (76)	5 (50)	4 (100)	0.12	0.20
Peak Parasitaemia at Day of Treatment, Parasites/mL (Median, IQR)	20,411 (7,535-74,773)	14,256 (6,825-31,399)	92,063 (57,454-184,891)	29,928 (17,751-49,982)	**0.003**	0.68
Total Parasite Burden, Parasites (Median, IQR)	65,213 (27,013-297,055)	47,121 (24,560-138,303)	530,260 (189,004-1,736,391)	37,370 (26,115-94,637)	**<0.0001**	0.34
Parasite multiplication rate slope, (Median, IQR)	0.69 (0.64-0.74)	0.70 (0.64-0.75)	0.68 (0.66-0.71)	0.54 (0.50-0.60)	0.58	**0.005**
Parasite reduction ratio (median, IQR)	0.050* (0.045-0.063)	0.050** (0.044-0.072)	0.051 (0.045-0.053)	N/A	0.48	N/A

Table 5.2. Summary table of the demographics and parasite parameters. n- sample size; IQR- inter-quartile range; Pf- *P. falciparum* all; Pf3D7- *P. falciparum* 3D7 group; PfK13- *P. falciparum* K13 group; Pv- *P. vivax* group. Wilcoxon rank-sum test used to test significance between groups. * n=38, **n=28

The median peak parasitaemia on the day of treatment prior to receiving any drug treatment was significantly different between those inoculated with Pf3D7 (14,256 parasites per mL [IQR 6,825 -31,999]) and PfK13 (92,063 parasites per mL [IQR 57,454-184,891]; p=0.003). The median peak parasitaemia was similar in those inoculated with *P. falciparum* and *P. vivax* (**table 5.2**). The median total parasite burden was also significantly larger in those inoculated with PfK13 (530,260 parasites [IQR 189,004- 1,736,391]) when compared to Pf3D7 (65,213 parasites [IQR 27,013-297,055]; p<0.0001). The parasite burden in the *P. vivax* participants was smaller but was not statistically significant when compared to the *P. falciparum* participants (**table 5.2**). Those inoculated with *P. falciparum* had a greater median PMR when compared to those with *P. vivax* (0.69 [IQR 0.64-0.74] vs 0.54 [IQR 0.50-0.60]; p=0.005). The PRR was similarly the same between those inoculated with both *P. falciparum* strains. There was no *P. vivax* PRR data available.

5.4.2. Changes in the markers of iron metabolism in *P. falciparum*

Based on Suominen *et al* [112] and mentioned previously, the log ferritin/sTfR index is considered the most sensitive marker of iron status. Overall, participants became iron deplete over the study with the mean log ferritin/sTfR index being significantly lower at the EOS (0.83

[95%CI: 0.76-0.90]) when compared to baseline (1.08 [95%CI: 1.02-1.15]; p<0.0001) (**figure 5.2**). There was no significant change between the mean baseline EPO and the mean day of treatment EPO, however there was a significant increase from day 15 onwards (5.73 [95% CI: 5.04-6.52] mIU/mL to 7.21 [95%CI: 6.35-8.20] mIU/mL; p=0.013). There was no correlation between baseline log ferritin/sTfR index and the change in EPO. Following inoculation, hepcidin rose slightly from a median of 21.60 ng/mL (95%CI: 18.67-25.00) at baseline to 25.36 ng/mL [95%CI: 21.92-29.34] on the day of treatment, although this was not statistically significant (p=0.13). Following antimalarial treatment, the median hepcidin level fell to a median of 16.59 mg/mL [14.34-19.19], which was significantly lower than the baseline level (p=0.012). There were no differences in the markers of iron metabolism between Pf3D7 and PfK13.

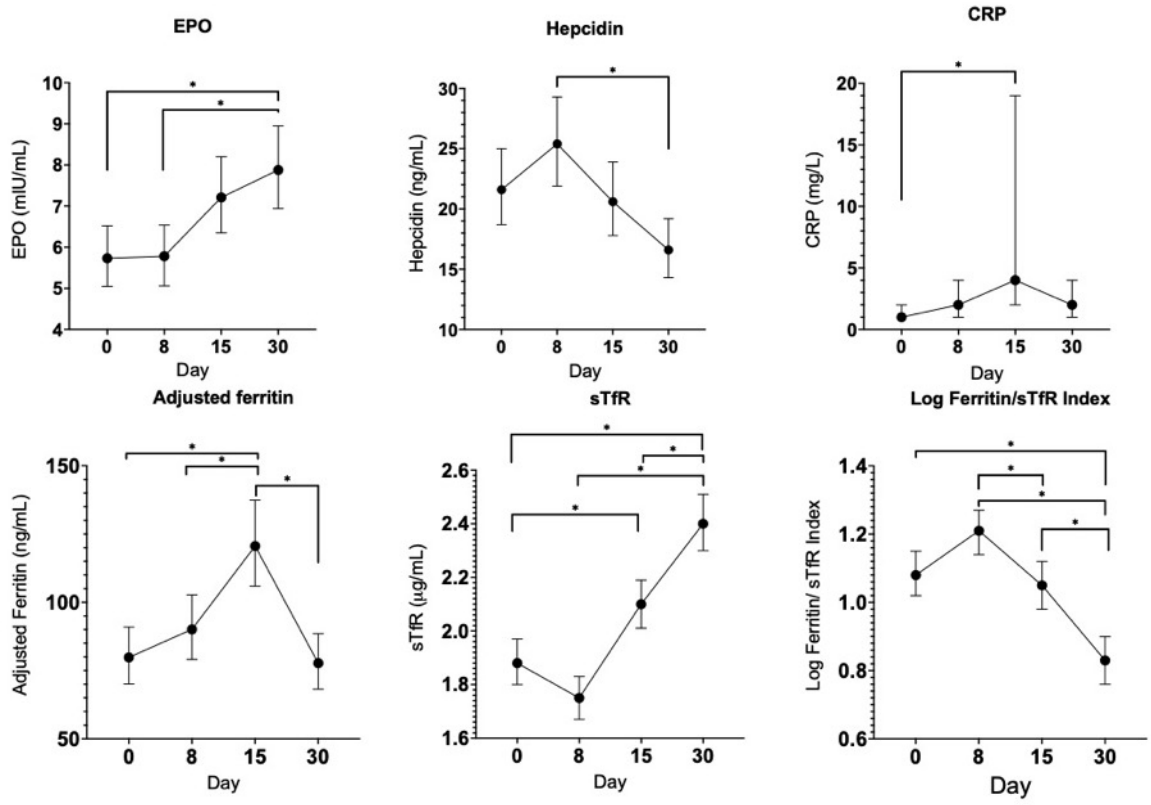

Figure 5.2. Longitudinal changes in the markers of iron metabolism in *P. falciparum*. The iron-axis over the duration of the study. A repeated measures ANOVA was performed on Log_{10} transformed data, with the dots represented the back-transformed means and lines the back-transformed 95% confidence intervals. The p-values have been adjusted using Bonferroni's correction. For CRP the dots represent the median and lines represent the interquartile range,

with the Friedman's test adjusted using Bonferroni's correction, used to identify the any significant difference between variables. * Represents a p-value <0.008

5.4.3. Changes in the markers of iron metabolism in *P. vivax*

Similarly, to the *P. falciparum* participants, the mean log ferritin/sTfR index was lower at EOS (0.76 [95%CI: 0.66-0.86]) than at baseline (1.05 [95%CI: 0.95-1.16]; p=0.001), indicating participants lost iron as the study progressed (**figure 5.3**). There was a gradual increase in the mean EPO, with a difference between baseline (2.49 mIU/mL [95%CI: 1.54-4.40]) and the EOS EPO levels (4.14 mIU/mL [95%CI: 2.55-6.71]; p=0.13). As expected, there was a significant rise in hepcidin from baseline to the day of treatment in keeping with the rise in CRP, with a mean hepcidin level at baseline of 13.77 ng/mL [95%CI: 8.83-21.50]) increasing to 116.27 ng/mL [95%CI: 74.50-181.48]; p<0.0001. This was followed a significant decrease in hepcidin from the day of treatment to EOS (116.27 ng/mL [95%CI: 74.50-181.48] vs 7.68 ng/mL [95%CI: 4.92-11.99]; p<0.0001).

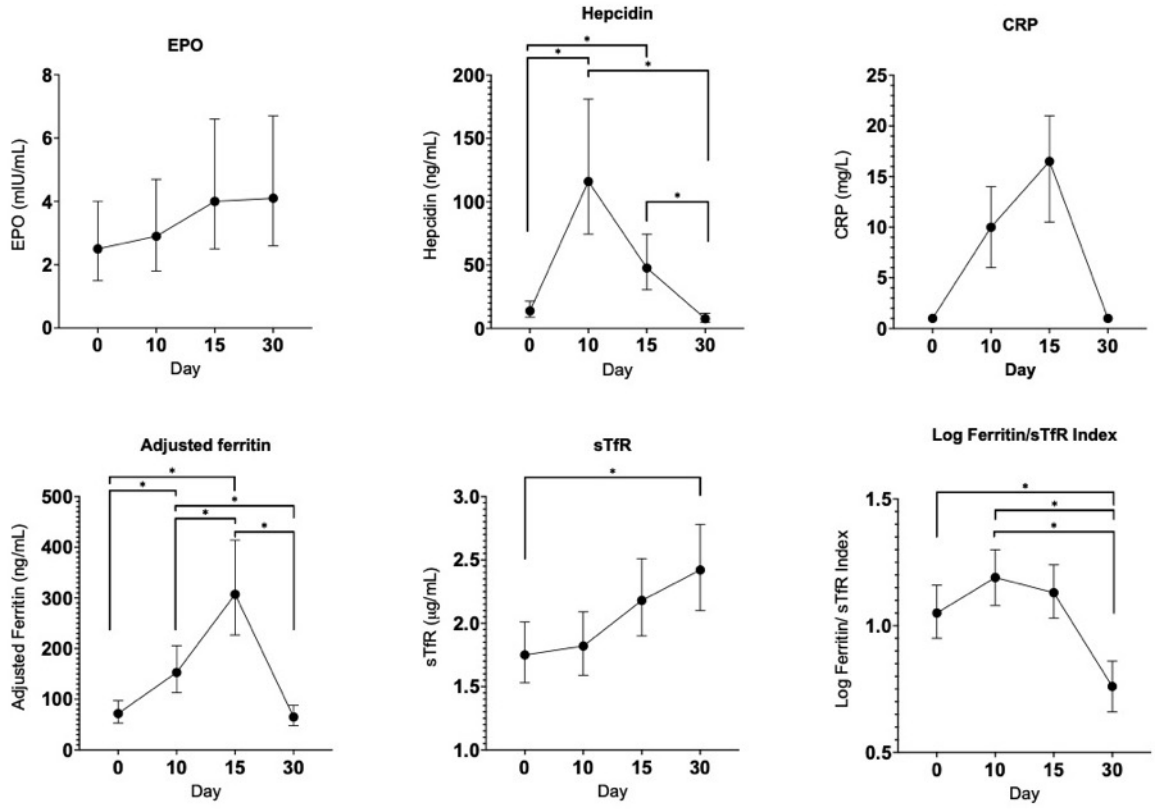

Figure 5.3. Longitudinal changes in the markers of iron metabolism in *P. vivax*. The iron-axis over the duration of the study. A repeated measures ANOVA was performed on Log_{10} transformed data, with the dots represented the back-transformed means and lines the back-transformed 95% confidence intervals. The p-values have been adjusted using Bonferroni's correction. For CRP the dots represent the median and lines represent the interquartile range, with the Friedman's test adjusted using Bonferroni's correction, used to identify the any significant difference between variables. * Represents a p-value <0.008

5.4.4. Effect of age, recrudescence and sex on the markers of iron metabolism in *P. falciparum*

Age was not analysed as a categorical variable i.e. age groups, as physiologically there were no distinct groups. When age was analysed as a continuous variable, there were no significant correlations between age and the haematology/parasite parameters.

Females had a lower median baseline log ferritin/sTfR index than males (0.74 [IQR 0.67-1.41] vs 0.97 [IQR 0.81-1.26]; p= 0.27) (**figure 5.4**). There were no other differences observed in the markers of iron metabolism between the two sexes. There were no significant differences

between the changes in levels of the markers of iron metabolism by sex (from baseline to pre-treatment and pre-treatment to EOS), when using the Wilcoxon rank-sum test.

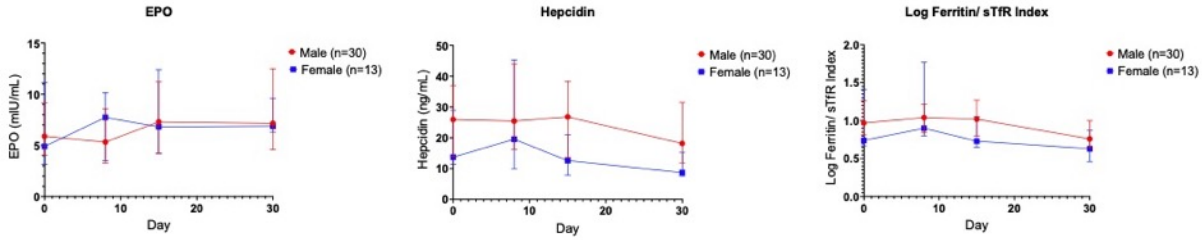

Figure 5.4. Effect of sex on the markers of iron metabolism in those inoculated with *P. falciparum*. These graphs show the key components of the iron-axis over the duration of the studies in those inoculated with *P. falciparum* by sex. The dots represent the median and the lines represent the inter-quartile range.

Figure 5.5 illustrates the markers of iron metabolism in those inoculated with *P. falciparum* who either experienced recrudescence or did not. There was no significant difference in the log ferritin/sTfR index between those who experienced recrudescence and those who did not (no recrudescence- -0.16 [IQR -0.33 – 0.02] vs -0.22 [IQR-0.32- -0.05] in the recrudescence group; p=0.61). There were no observable significant differences between the other markers of iron metabolism by recrudescence status. There were no significant differences between changes in levels of the markers of iron metabolism (from baseline to pre-treatment and pre-treatment to EOS), when using the Wilcoxon rank-sum test, by recrudescence status.

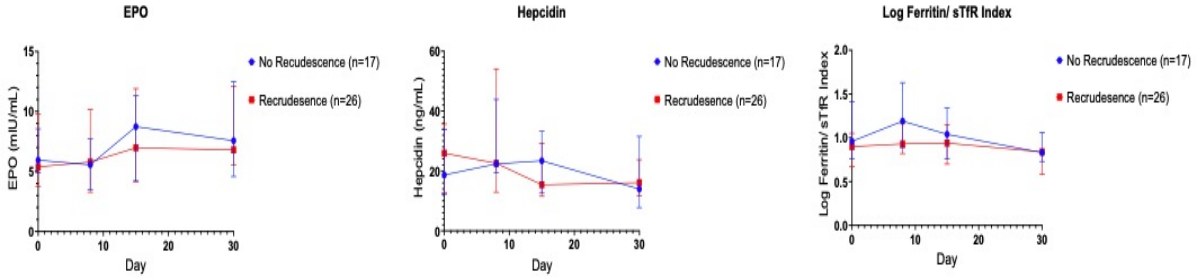

Figure 5.5. Effect of recrudescence on the iron axis in those inoculated with *P. falciparum*. These graphs show the key components of the iron-axis over the duration of the studies in those inoculated with *P. falciparum* by recrudescence status. The dots represent the median and the lines represent the inter-quartile range.

5.4.5. Haematology data

The median baseline haemoglobin was similar between those inoculated with either *P. falciparum* or *P. vivax*, 147 g/L (IQR 137-154) vs 146 g/L (IQR 140-153); p=0.93; however, it was significantly lower in the PfK13 participants (136 g/L [IQR 132-145]), when compared to the Pf3D7 participants (150 g/L [IQR 143-154]; p=0.006). The difference in baseline haemoglobin is likely to have been explained by the increased number of female participants inoculated with PfK13, 5/10 (50%) vs 8/33 (24%), as females have an average 10 g/L lower haemoglobin concentrations when compared to males [163]. There was also a significant difference in the median EOS haemoglobin concentration between those inoculated with PfK13 (130 g/L [IQR 127-135]) and those with Pf3D7 (141 g/L [133-146]; p=0.009). Similarly, the median nadir haemoglobin concentration was lower in the PfK13 participants (121 g/L [118-127]) compared to 131 g/L (IQR 121-138) in the Pf3D7 participants (p=0.022) (**table 5.3**). The median EOS and nadir haemoglobin concentrations were similar between those inoculated with either *P. falciparum* or *P. vivax* (**table 5.3**). There was a large difference, albeit not significant, in the fractional fall between the *P. falciparum* and *P. vivax* participants (10.9% [IQR 7.1 – 14.9] vs 13.7% [IQR 10.0-14.0]; p=0.77)

There were no significant differences observed in the fractional fall in haemoglobin when analysed by sex or recrudescence and correlation between age and the fractional fall in haemoglobin.

	Pf all (n=43)	Pf 3D7 (n=33)	Pf K13 (n=10)	Pv (n=4)	P-value (3D7 vs K13)	P-value (Pf all vs Pv)
Baseline Hb, g/L (Median, IQR)	147 (137-154)	150 (143-154)	136 (132-145)	146 (140-153)	**0.006**	0.93
EOS Hb, g/L (Median, IQR)	139 (132-145)	141 (133-146)	130 (127-135)	132 (125-137)	**0.009**	0.21
Nadir Hb, g/L (Median, IQR)	131 (121-138)	133 (127-141)	121 (118-127)	129 (125-132)	**0.022**	0.91
Frac Fall % (Median, IQR)	10.9 (7.1-14.9)	10.9 (7.1-14.7)	11.7 (6.3-15.8)	13.7 (10.0-14.0)	0.87	0.77
Baseline reticulocyte count, x10^9/L (Median, IQR)	70 (56-82)	72 (62-87)	55 (49-65)	49 (46-72)	**0.029**	0.21
EOS reticulocyte count, x10^9/L (Median, IQR)	84 (66-100)	80 (68-91)	87 (65-107)	72 (66-96)	0.75	0.81
Change between baseline and EOS reticulocyte counts, x10^9/L (Median, IQR)	14 (3-20)	10 (2-18_	19 (14-28)	15 (2-24)	0.034	0.95

Table 5.3. Summary table of haematology data. n- sample size; IQR- inter-quartile range; Pf- *P. falciparum* all; Pf3D7- *P. falciparum* 3D7 group; PfK13- *P. falciparum* K13 group; Pv- *P. vivax* group. Wilcoxon rank-sum test used to test significance between groups.

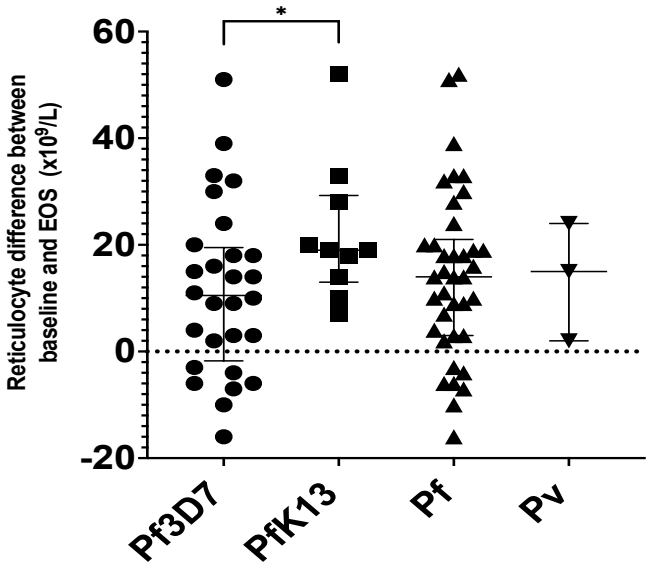

Figure 5.6. Reticulocyte difference between baseline and EOS. The difference in reticulocytes from baseline to EOS were compared between species and both *P. falciparum* sub-groups. Each point represents the individual reticulocyte difference per participants. The lines represent the median and interquartile range. The Wilcoxon rank-sum test was used to compare *P. falciparum* and *P. vivax* and the Pf3D7 and PfK13. * Represents a p-value of <0.05.

The median corpuscular volume (MCV) remained constant for those inoculated in both species (**figure 5.7**).

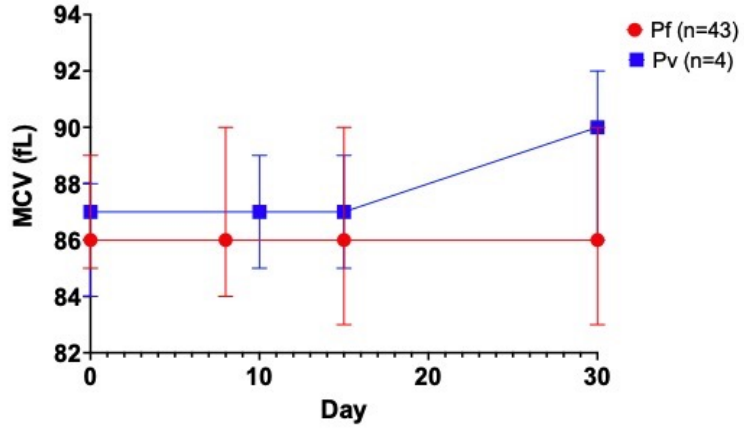

Figure 5.7. Longitudinal change in the mean corpuscular volume (MCV) in those inoculated with *P. falciparum* and *P. vivax*. The dots represent the median and the lines represent the inter-quartile range.

5.4.6. The effect of the markers of iron metabolism on the parasite and haematology parameters in those inoculated with *P. falciparum*

There were no correlations between baseline iron status (log ferritin/sTfR index) and any of the parasitaemia parameters evaluated including peak parasitaemia (r=0.13, p=0.41), total parasite burden (r=0.16, p=0.30), and PMR (r=-0.21, p=0.17). There was also no correlation between the baseline iron status and the fractional fall in haemoglobin (r=-0.02, p=0.92). Baseline iron status was however correlated with the change in the reticulocyte count, with participants who were more iron replete having a greater reticulocyte response (r=0.39, p=0.015) (**figure 5.8**). There was no correlation between change in EPO and baseline iron status using the log ferritin/sTfR index. The baseline MCV was correlated with peak parasitaemia in those inoculated with *P. falciparum* (r=0.37, p=0.015) as well as with total parasite burden (r=0.36, p=0.018) (**figure 5.9**) but not PMR.

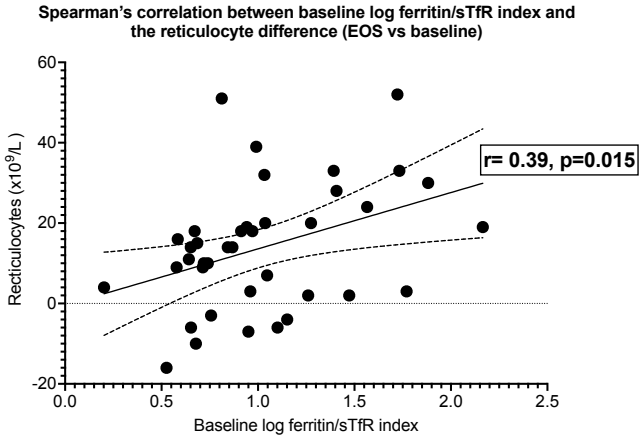

Figure 5.8. Correlation between baseline iron status and the reticulocyte difference. Spearman's correlation between the baseline log ferritin/sTfR index and the difference between baseline and EOS reticulocyte counts. The straight line represents the Spearman's correlation, with the dotted lines representing the 95% confidence interval.

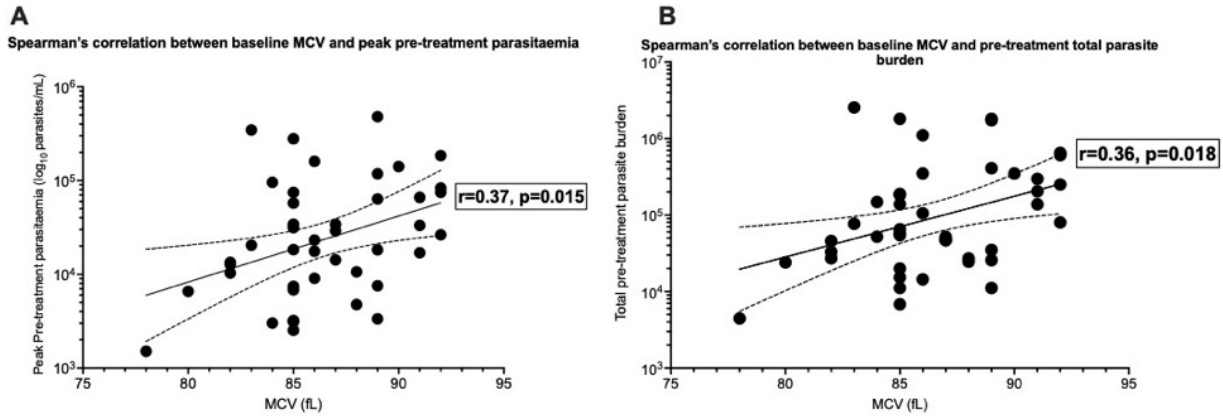

Figure 5.9. Correlations between mean corpuscular volume (MCV) and parasite parameters. (A) Spearman's correlation between MCV and peak pre-treatment parasitaemia. (B) Spearman's correlation between MCV and pre-treatment total parasite burden. The straight line represents the Spearman's correlation, with the dotted lines representing the 95% confidence interval.

5.4.7. Longitudinal changes in liver function tests

Figure 5.10 below graphically illustrates the liver function tests in both *P. falciparum* and *P. vivax*, including both *P. falciparum* strains. In both species and both *P. falciparum* strains, there are significant increases in the mean ALT and AST from day 0 to day 15. Following the rise in ALT there were observed significant declines in the ALT from day 15 to the EOS in all

groups except those inoculated with *P. vivax*. The decline in AST from the day to treatment to EOS was significant in all groups. Bilirubin remained consistent in all groups.

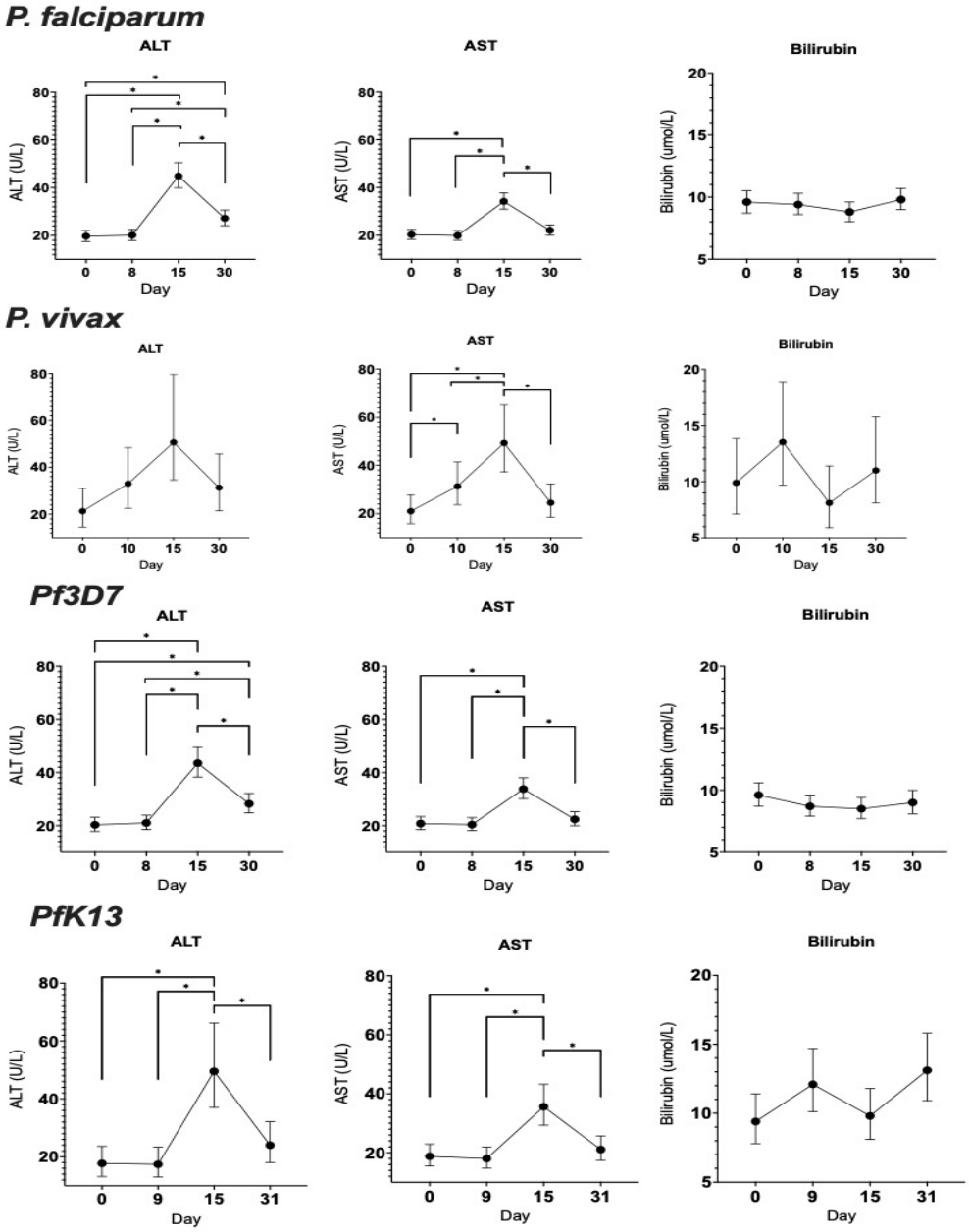

Figure 5.10. Longitudinal changes in liver function tests. A repeated measures ANOVA was performed on Log_{10} transformed data, with the dots represented the back-transformed means and lines the back-transformed 95% confidence intervals. The p-values have been adjusted using Bonferroni's correction. * Represents a p-value <0.008

5.4.8. Changes in baseline to post-treatment ALT by age, sex, drug and recrudescence status in those inoculated with *P. falciparum*

Figure 5.11 graphically presents the median change in ALT from baseline to post-treatment (day 15) by age, sex, recrudescence status and drug treatment. There was no effect of sex, age, or recrudescence status on changes in the liver transaminases. When comparing the different drug classes, there was a difference observed between the median change in ALT from baseline to day 15 between the artemisinin group (27 U/L [IQR 13-104]; n=16) and ZY-19849 (4 U/L [IQR 0-10]; n=14; p=0.005), however there was no difference observed between the other two drug groups and the reference artemisinin group.

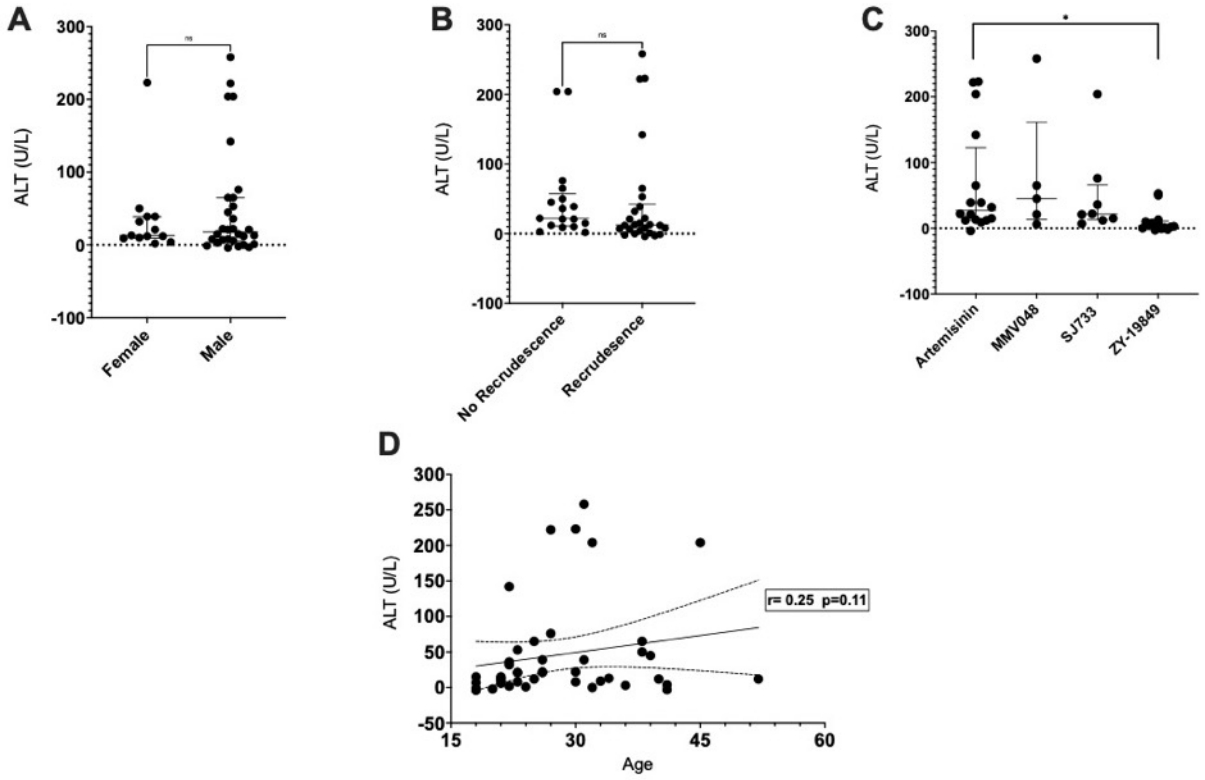

Figure 5.11. Changes in ALT (baseline to post-treatment) by age, sex, drug and recrudescence status. The difference in the change of ALT (baseline to the day of treatment) by sex (A), recrudescence status (B) and drug (C). The lines represent the median and interquartile range. The Wilcoxon rank-sum test was used to compare the difference by group. ns represents no significance was observed, * represents a p-value <0.05 and ** represents a p-value <0.005. (D) Spearman's correlation between age and change in ALT (baseline to post-treatment).

5.4.9. Interactions between the markers of iron metabolism and the liver transaminases in those inoculated with *P. falciparum*

There were strong significant correlations between the baseline log ferritin/sTfR index and the post-treatment ALT and AST [day 15] (ALT- r= 0.54, p<0.001; AST- r=0.31, p=0.047). The correlations were even stronger when the post-treatment log ferritin/sTfR index was correlated with liver transaminases (ALT- 0.61, p<0.001; AST- 0.54, p<0.001) (**figure 5.12**).

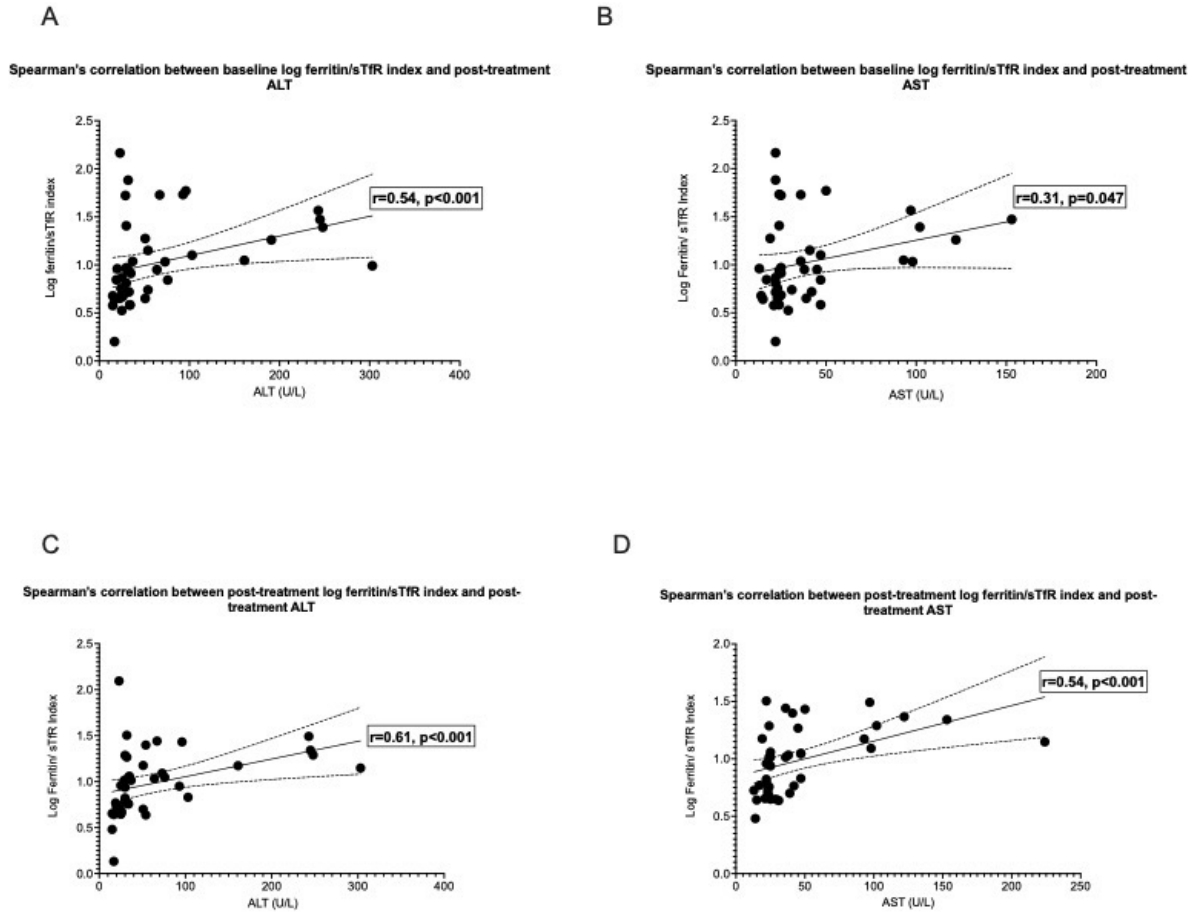

Figure 5.12. Correlations between markers of iron metabolism and the liver transaminases in those inoculated with *P. falciparum*. (A) Spearmans's correlation between baseline log ferritin/sTfR index and post-treatment ALT. (B) Spearman's correlation between the baseline log ferritin/sTfR index and post-treatment AST. (C) Spearman's correlation between post-treatment log ferritin/sTfR index and post-treatment ALT. (D) Spearman's correlation between post-treatment log ferritin/sTfR index and AST. The straight line represents the Spearman's correlation, with the dotted lines representing the 95% confidence interval.

5.4.10. Intrahepatic iron concentrations

When comparing the ferritin/AST ratio as a crude marker of intrahepatic iron concentration[143] in both species of malaria, there was a small increase on the day of the treatment from baseline in those inoculated with either *P. falciparum* and *P. vivax,* although this was not statistically significant in either group. However, in those inoculated with *P. vivax* the ferritin/AST ratio increased from baseline to day 15 (3.42 ng/U [95%CI: 2.36-4.94] vs 6.25 ng/U [4.32-9.04]; p=0.028), unlike those inoculated with *P. falciparum*, where the ratio dropped after the day of treatment. Figure 5.13 shows the longitudinal changes in the ferritin/AST ratio between those inoculated with *P. falciparum* and *P. vivax*.

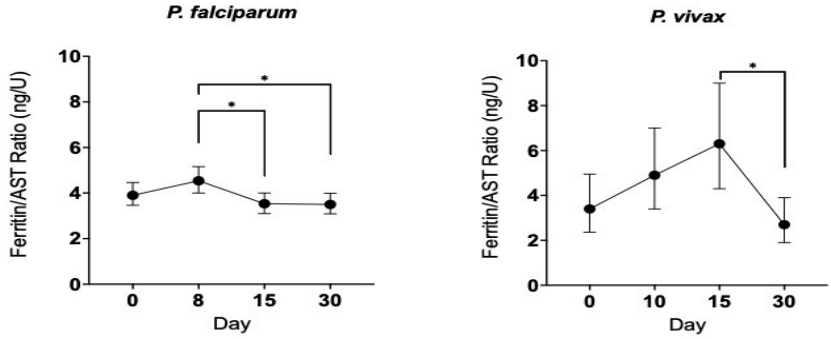

Figure 5.13. The ferritin/AST ratios by species and sub-groups over the course of the studies.

A repeated measures ANOVA was performed on Log_{10} transformed data, with the dots representing the back-transformed means and lines the back-transformed 95% confidence intervals. The p-values have been adjusted using Bonferroni's correction.

* Represents a p-value <0.008

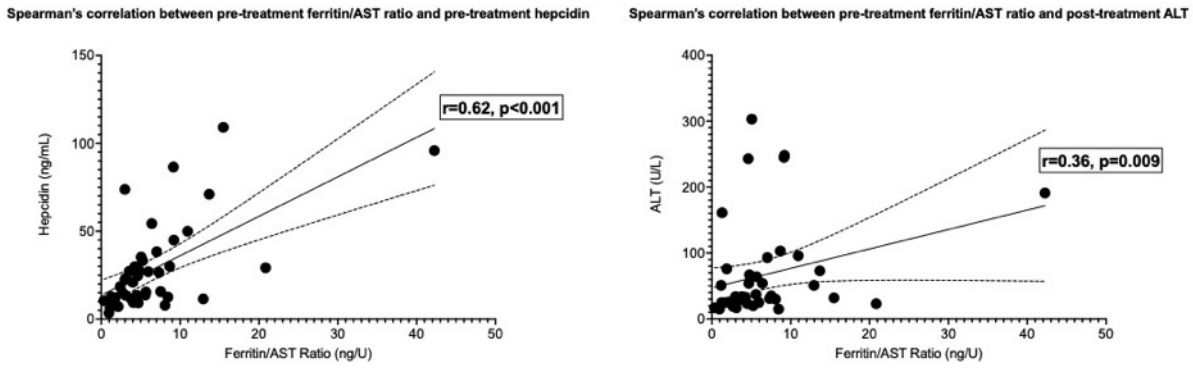

Figure 5.14. Spearman's correlation between pre-treatment hepcidin and post-treatment ALT in those inoculated with *P. falciparum*. Spearman's correlations between pre-treatment ferritin/AST ratio and pre-treatment hepcidin/ALT. The straight line represents the Spearman's correlation, with the dotted lines representing the 95% confidence interval.

There was a strong correlation between the pre-treatment ferritin/AST ratio and pre-treatment hepcidin (r=0.62, p<0.001), suggesting the retention of iron within the liver is controlled by hepcidin. There was also a significant correlation between the pre-treatment ferritin/AST ratio and the post-treatment ALT (r=0.36, p=0.009) (**figure 5.14**). There was however no correlation between the pre-treatment ferritin/AST ratio and AST.

5.5. Discussion

Dyserythropoiesis is an important mechanism in the development of malaria-associated anaemia [13]. Plasmodia require iron for their growth and development, which they acquire through the digestion of intra-erythrocytic haemoglobin [71,72]. While the co-existence of malaria and iron deficiency is well-known, the recent study by Muriuki *et al* [118] has provided the first data to suggest causation of iron deficiency by malaria. Current literature suggests that iron deficiency appears to be protective in the development of malaria, especially severe malaria [117,119,164], although there are conflicting reports that iron supplementation in endemic settings may increase mortality and morbidity associated with malaria [106,120,121]. This exploratory study aimed to evaluate markers of iron metabolism in early malaria infection using longitudinal data from 43 participants inoculated with *P. falciparum* and 4 participants

inoculated with *P. vivax* and associations between haematology, parasite and liver biochemical parameters.

EPO is known to be elevated in malaria infection, which would be in keeping with an increase in oxidative stress and haemolysis [165,166]. In our study, EPO was elevated in participants inoculated with *P. falciparum* and *P. vivax*, although the response in EPO was larger in those inoculated with *P. falciparum*. The increase in EPO in both species occurred between the day of treatment (day 8 in Pf3D7, day 9 in PfK13 and day 10 in *P. vivax*) and post-treatment (day 15). There was a significant increase between baseline and EOS EPO in those inoculated with *P. falciparum*. This increase could be explained by the increase in oxidative stress and haemolysis post drug administration. In the PfK13 participants, the increase occurred between day 15 and the EOS, although this rise was not significant. The later rise observed in the PfK13 participants is likely related to recrudescence, as those inoculated with PfK13 had parasite recrudescence between day 11 to day 26 [56].

In our study the increase in hepcidin between baseline and day of treatment was associated with a corresponding increase in CRP. This is consistent with previous studies demonstrating that the early increase in hepcidin in malaria is related to an increase in IL-6 [94,167]. Also, in this study hepcidin appeared to have a biphasic response to malaria, rising following inoculation before falling to below baseline levels by the end of study. These findings are consistent with a study by De Mast *et al* [168], which demonstrated normalisation of hepcidin following treatment in natural infection. The rise in hepcidin in our study also corresponded with an elevation in the ferritin/AST ratio, a marker of intrahepatic iron concentration, with a significant, strong correlation observed between the pre-treatment ferritin/AST ratio and pre-treatment hepcidin in the *P. falciparum* participants. These findings support current thinking that in infection hepcidin upregulation causes the retention of iron within the liver [94]. This study also supports other findings that following treatment, hepcidin is downregulated in order to release stored iron to facilitate erythropoiesis [94]. This is demonstrated by the significant reductions in the ferritin/AST ratios observed in those inoculated with *P. falciparum*, *P. vivax* and PfK13 following treatment, mirroring the reductions observed in hepcidin in these groups.

Ferritin is an acute phase protein that increases in the acute phase of many inflammatory conditions including infection, followed by a slow decline [169,170]. As this can affect the utility of ferritin as a marker of iron status, we corrected the ferritin for inflammation using adjustments previously published from the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia project (BRINDA) [159–161]. The BRINDA project was a multi-agency internal collaboration with an aim to refine estimates of nutritional status in the context of anaemia [159–161]. One of the outcomes was adjusting ferritin for inflammation, using CRP and α-1-glycoprotein [159–161]. In our study, the peak in the adjusted ferritin was observed at day 15 in all groups, with significant rises from baseline to day 15 seen in all groups. These observations in the adjusted ferritin likely represent the utility of ferritin as an acute phase protein, as they correlate with CRP, which was also significantly elevated at day 15. Castberg *et al* [171] noted that ferritin remained significantly elevated in children with a positive RDT for *P. falciparum* in Ghana (n=52) 4 weeks post-treatment with no evidence of recrudescence or re-infection. However, our findings differ from those of Castberg *et al* [171], as the adjusted ferritin returned to near baseline levels. The mean parasitaemia in those children was 40,326 parasites/μL [171], which is 3-log greater than observed in the IBSM VIS participants. The mean CRP was 93 mg/L on the day of presentation in Castberg *et al* [171], which is significantly higher than observed in this study (median day 15 was CRP 4 mg/L in those inoculated with *P. falciparum*). In the IBSM *P. falciparum* participants, there were strong correlations between the change in CRP from baseline to day 15 and the change in hepcidin levels from baseline to pre-treatments, as well as pre-treatment to EOS, in keeping with the literature that the rise in hepcidin is linked to IL-6 [92,94,158]. The differences in parasitaemia and inflammatory response likely explain why the adjusted ferritin is lower in the IBSM participants. Kabore *et al* [172], noted in children that circulating pro-inflammatory cytokines such as IL-6/IL-10 were increased in asymptomatic and clinical malaria in children and was related to parasite densities. The findings also support Castberg *et al* 's suggestion [171], that the optimum time to give iron supplementation would be 7 days after the treatment of infection, when the hepcidin levels decrease.

Our findings also support not using ferritin alone as a marker of total iron stores in malaria [112,115,116]. Based on Suominen *et al* [112], the log ferritin/sTfR index ratio has been used as the marker of total iron stores. The ratio was inversed in this study for ease of interpretation, with

a low log ferritin/sTfR index representing low iron stores and vice versa; when the ratios were converted from Suominen *et al* [112], a log ferritin/sTfR value of <0.49 represents iron-deficient erythropoiesis and <0.37 representing iron-deficiency anaemia. The observed trend in the two species was an overall decrease in the index ratio from baseline until EOS. This observation would suggest that individuals lose iron during low-level malaria infection, especially post-treatment. This supports the findings of Muriuki *et al* [118], that malaria causes a functional iron-deficiency and ultimately iron-deficiency anaemia. Parasite bound iron is retained with the RES until reprocessed, as parasites are scavenged in the spleen by macrophages or remains within live parasites in sanctuary sites such as the spleen as identified by Kho et al [78]. This then induces a functional rather than true iron deficiency as iron is not lost from the body. This study also observed a significant correlation between the reticulocyte difference in the *P. falciparum* participants, from baseline to EOS, and baseline log ferritin/sTfR index, suggesting a poor reticulocyte response in those with lower iron stores [173].

Overall, age, sex and recrudescence had no observed effect on the iron-axis. In the IBSM VIS, the median age of the *P. falciparum* participants was 26, whereas in natural infection individuals most susceptible to malaria-associated anaemia are children [13,123,174]. The effect of age should therefore be interpreted with caution. It was surprising that there was no effect on the iron-axis markers secondary to parasite recrudescence. This may be related to lower parasitaemias observed in the IBSM participants when compared to the natural infection.

The next aim of this study was to evaluate the impact that changes in the iron-axis had on haematology parameters. The median fractional fall was highest in those inoculated with *P. vivax* (13.7%) when compared to those inoculated with *P. falciparum* (10.9%) which may be sample size related. The difference between those inoculated with PfK13 and Pf3D7 is likely explained by the significant difference in the total parasite burden between the two groups (**table 5.2**).

There were no correlations between the total parasite burden and the changes in the markers of iron metabolism in those inoculated with *P. falciparum*. Also, in the *P. falciparum* participants, there was no observable correlation between baseline log ferritin/sTfR index and

the parasite parameters including peak parasitaemia, TPB and PMR. These findings suggest that in malaria VIS there is no relationship between baseline iron status and either the parasite growth rate or subsequently higher parasite burdens. The only finding that iron-deficiency could be protective was a moderate correlation between baseline MCV and peak parasitaemia and total parasite burden, which supports the *in vitro* findings of Clark *et al* [127]. The MCV was observed to increase which is likely linked to the increased production of reticulocytes in response to the erythrocyte destruction. Reticulocytes are 8% larger than more mature erythrocytes, therefore without the red cell distribution width, it is difficult to ascertain a true reflection on the MCV of the circulating erythrocytes longitudinally.

There was a significant inverse relationship between the EOS log ferritin/sTfR index and the PMR in *P. falciparum*, although there were no correlations with either peak parasitaemia or total parasite burden, suggesting those individuals with higher parasite growth rates are more iron deplete at the EOS.

Elevation in the liver transaminases have been previously reported [133,134,140], and we sought to evaluate whether the hepcidin-mediated movement of intrahepatic iron could be contributory. Both the post-treatment ALT and AST were strongly correlated with the baseline log ferritin/sTfR index, suggesting low iron stores protect against developing higher elevations in liver transaminases at day 15. The correlations were similarly strong between the day 15 log ferritin/sTfR index and day 15 liver transaminases, further demonstrating the relationship between lower iron stores and lower liver enzymes. Pre-treatment hepcidin levels and ferritin/AST (a marker of intrahepatic iron) were also correlated with day 15 ALT. This suggests that movement and retention of iron into the liver in the acute phase of the infection could be causing a temporary iron overload with associated inflammation. This is consistent with the known association between intra-hepatic iron and local inflammation and tissue necrosis in chronic liver diseases [141,142].

The limitations of this exploratory study include the variability in the sampling time for post-treatment and EOS. In those inoculated with Pf3D7, the post-treatment day ranged from day 11 to 17, with the EOS day ranging from day 24 to 59. Secondly, the baseline iron status of the individuals participating in the IBSM VIS are higher than individuals in malaria-endemic regions, which may account for the frequency of raised liver enzymes in VIS compared to

natural infection. Individuals with anaemia would not be eligible to participate in the IBSM VIS although two individuals who had iron deficiency if defined as a log-ferritin/sTfR index of <0.49. This limits the understanding of what the impact of iron-deficiency anaemia is on the parasite and biochemistry markers. Lastly, this study included only 4 *P. vivax* participants, limiting our ability to evaluate associations in this group.

The ERFE assays across the 5 runs did not produce a full set of longitudinal data for any individual and as such have not been used in the analyses. The assays used were from a commercial provider. The preferred medium to test was serum, although advice was sought which stated that frozen plasma would be acceptable. Possible hypotheses for the failure of the assays include degradation of the hormone during freeze-thaw cycles, inhibition of the assay using plasma and/or poor sensitivity of the assay [175,176]. ERFE has not been described previously in human malaria and was one of the novel aspects of this study. Due to limited samples and sample volumes, further repeat runs were not possible. Further investigation of ERFE in the context of malaria and the development of malaria-associated anaemia, is likely to provide key insights into the role this hormone plays in the pathophysiology of dyserythropoiesis. This study could be repeated during future IBSM VIS, using fresh serum that has not undergone any freeze/thaw cycles instead of plasma. This with paired longitudinal data would be useful in determining the role ERFE plays in the iron-axis during early malaria.

In summary, this exploratory study has characterised the iron-axis in early experimental malaria infection. We have demonstrated experimental malaria infection leads to a functional iron deficiency which is consistent with the clinical findings of Muriuki *et al* [118]*.* Reduced baseline iron status leads to a reduced reticulocyte and EPO response, suggesting of an effect of iron status on bone marrow function. Lastly, increased iron stores at baseline were associated with increased post-treatment liver transaminases; providing reassurance that these changes are of minimal clinical significance and not often suggestive of drug-induced liver injury.

Chapter 6. Discussion

Malaria-associated anaemia is a significant cause of mortality and morbidity in malaria endemic countries [60,61,99,174,177–179]. There are several different mechanisms that are recognised to contribute to malaria-associated anaemia including erythrocyte haemolysis [180], splenic clearance of uRBC [63,75,181], dyserythropoiesis secondary to direct effects of the parasite plus indirect effects of hemozoin and cytokines on the bone marrow [71,72,182,183], as well as iron deficiency [69,118,131,132,167]. Severe anaemia (<50 g/L) in the context of *P. falciparum* infection increases mortality five-fold [61]. Iron deficiency is very common in malaria-endemic regions, with malaria-associated anaemia now being increasingly recognised as a contributor to this disease burden, especially in asymptomatic and low-level infection [118].

The aim of this thesis was to characterise anaemia in early malaria using VIS, through the development of a new IBSM MCB, a pooled analysis of historic haematology and parasite data and finally through investigation of iron metabolism in the IBSM VIS. The IBSM VIS are routinely used in antimalarial development and provide a unique opportunity to investigate the haematological response in low-level infection, using repeated measures of blood counts and parasite data via 18S qPCR quantification. The research questions I wanted to answer through this research were as follows:

1) Can human malaria volunteer infection studies (VIS) be used as a model for malaria-associated anaemia in early malaria?
2) What is the development and progression of malaria-associated anaemia in healthy malaria-naïve individuals in early infection?
3) Is iron metabolism altered in early malaria in VIS subjects?

6.1. Aim 1- Conduct and IBSM malaria VIS utilising a newly developed malaria cell bank, 3D7-MBE-008

The two participants who were inoculated with 3D7-MBE-008 had detectable parasitaemia on day 4 and subsequently reached threshold parasitaemia (>10,000 parasites/mL) on day 8, which mirrors recent clinical trials using the 3D7-V2 MCB. Both participants, reached peak

parasitaemia two hours post the administration of artemether-lumefantrine. The PMR_{48} for both participants was also like the existing 3D7-V2 MCB [55]. The parasite killing as determined by the PRR, using artemether-lumefantrine was also similar to 3D7-V2 MCB [55]. The majority of adverse events (91.4%) were mild or moderate and were attributable to early malaria (71.4%) [55]. There were three severe AES, including lymphopenia in each participant and raised ALT (4.8x ULN) in one participant, which have all been previously reported in other studies [133,134,184]. These similarities demonstrate the new bioreactor-expanded 3D7-MBE-008 MCB has an equivalent safety, parasite growth and parasite clearance profile compared to the existing 3D7-V2 MCB.

This study has added to the existing bank of IBSM VIS safety data. These studies remain a safe and effective tool in investigating early malaria and associated erythrocyte loss. The biomanufacture process of malaria cell banks has the potential to increase the supply of a documented safe *P. falciparum* cell bank which could be used in malaria-endemic settings.

6.2. Aim 2- Utilise data from previous IBSM VIS conducted at QIMR Berghofer, including the above-mentioned MBE-008 IBSM VIS, to investigate the haematological response to malaria

The pooled analysis investigated the development and progression of anaemia in low-level malaria infection in malaria-naïve participants. Data from 315 participants who participated in 26 IBSM trials over a ten-year period at QIMR Berghofer were utilised. Of note, the analysis not only investigated the anaemia in participants inoculated with *P. falciparum* but also with the K13 artemisinin-resistant strain of *P. falciparum* and *P. vivax*. We also characterised the contribution of parasitised erythrocytes to the overall contribution to the total erythrocyte losses in the context of early and low-level infection.

In a study by Price *et al* [63] the day of haemoglobin of 4000 individuals infected with P. falciparum is reported to 7 days post the commencement of treatment, however the individual haematocrit levels were only taken weekly from the day of treatment. Therefore, the true day of the haemoglobin nadir could have occurred either side of day 7. This study demonstrated the median haemoglobin nadir in those inoculated with *P. falciparum* and did not experience parasite recrudescence occurred on day 11 (IQR 4-21), although the 95%

confidence intervals were large. The day of haemoglobin nadir has been reported to occur between day 3 and 7 in individuals infected with *P. vivax* by Leslie *et al* [185], and this study has observed the median haemoglobin nadir occurred on day 8 (IQR 7-16; n=39) in those inoculated with *P. vivax* and did not experience parasite recrudescence. The differences between Leslie *et al* [185] and this study could be explained by the sample size (767 vs 39), as the individuals in Leslie *et al* [185] had haematocrit levels taken daily for 4 days and then weekly. The haemoglobin nadir in those infected with *P. vivax* appears to be occur earlier than observed in those infected with *P. falciparum*.

The day of return to baseline haemoglobin is reported by Price *et al* [63] to occur by day 42, which occurs 13 days later than observed in the IBSM VIS participants inoculated with *P. falciparum.* These differences may be explained by the differences in the cohorts investigated by Price *et al* [63]and this study, which were displaced persons living on the Thai-Myanmar border compared to healthy malaria-naïve individuals who had undertaken an intensive health screen prior to enrolment on the IBSM VIS. In Price *et al* [63], it would be anticipated a significant population would have significant medical co-morbidities or be nutritionally less well off when compared to the IBSM volunteers, which could account for the differences observed in the return to haemoglobin baseline.

In Price *et al* the contribution of parasitised erythrocytes to the total erythrocyte losses was estimated to be 7.9%, with similar findings in neurosyphilis patients who were treated with malariotherapy [75], which was estimated to be 10.5%. However, this study has observed the contribution of parasitised erythrocytes to be <0.1%. These findings are similar when comparing existing literature in those infected with *P. vivax*, 2.9% [74] compared to <0.1%. The contributions of parasitised erythrocytes in low-level infections have not previously been reported. The findings in this study were in the context of healthy malaria-naïve individuals, with future research exploring the contribution of parasitised erythrocytes in individuals living in malaria endemic regions. Future IBSM studies could also be used to investigate potential mechanisms for this observed difference in contributions such as iron deficiency/replacement and the effect of the pro-inflammatory cytokines.

This analysis was unable to directly compare the progression of anaemia between the different drugs used. This was because of the heterogeneity within and between studies including day of treatment, drug doses and study duration, leading to variable recrudescence rates. As such, only descriptive analyses were undertaken, although there are some differences in the haematology parameters. Drug effect on the progression of anaemia is important, with phenomenon such as Blackwater fever and post-artesunate delayed haemolysis, secondary to quinine and artesunate respectively, causing significant anaemia that can sometime require transfusion [84,90].

6.3. Aim 3- Utilise data from previous IBSM VIS to evaluate iron metabolism in participants inoculated with *P. falciparum* and *P. vivax*

The aim of this study was to evaluate the longitudinal measures of markers of the iron-axis in early malaria, including measures of EPO, ERFE, hepcidin and iron status markers such as ferritin, sTfR and the $\log_{10}$ ferritin/sTfR index, with the latter being the preferred measure of iron stores. ERFE has not been previously commented upon in human malaria, although it has been described in a murine model [130]. Hepcidin and ferritin have been previously investigated in VIS [118,132] and clinical infection, although the $\log_{10}$ ferritin/sTfR has not. Hepcidin mediated iron control is increasingly being recognised as an important mechanism of malaria-associated anaemia, especially in low level infection [118,131,167]. The association of the iron-axis has not previously been described in the context of elevations in liver transaminase in malaria either.

This study aimed to characterise the iron-axis in early malaria through repeated longitudinal measurements of EPO, ERFE, hepcidin and iron status markers (ferritin, sTfR and $\log_{10}$ ferritin/sTfR) with paired haematology, biochemistry and 18S qPCR parasite data. Unfortunately, the ERFE assays did not yield sufficient results, with the majority under the LLOD. The likely reasons for the failure of these commercial assays include the use of frozen plasma instead of serum (despite manufacturer assurances), multiple freeze-thaw cycles and poor assay sensitivity [176].

EPO is known to be elevated in malaria, which is secondary to increases in oxidative stress and hypoxia from haemolysis of erythrocytes [104]. It was observed in this study the EPO levels

were significantly increased from the day of treatment onwards in both *P. falciparum* and *P. vivax*, which is related to the parasite killing. Hepcidin was observed to be biphasic in nature in *P. falciparum* and *P. vivax*, which was consistent with the literature [131,132], except for PfK13 where there was a gradual decline in the mean hepcidin through the four timepoints. The rise in hepcidin was correlated with the rise in CRP and independent of EPO, which is in keeping with the established literature [94]. There was a decrease in hepcidin in all groups, with all experiencing a mean EOS hepcidin lower than the baseline, which also corresponds the body's response to free iron for utilisation in erythropoiesis [94]. The changes between baseline and EOS sTfR and $\log_{10}$ ferritin/sTfR support the findings of Muriuki *et al* [118], that malaria is a cause of iron-deficiency. Age, sex, and recrudescence status had no effect on the iron-axis in either species.

There were no correlations between the iron-metabolism markers and the haematology parameters. There were no correlations between the baseline $\log_{10}$ ferritin/sTfR index and the fractional fall in haemoglobin as well as the change in hepcidin from baseline to the day of treatment with the fractional fall in haemoglobin in those inoculated with *P. falciparum*.

There were no correlations between the parasite markers and changes in the iron-axis in those inoculated *with P. falciparum*. Especially, there was no correlation between the parasite multiplication rate and the baseline $\log_{10}$ ferritin/sTfR index. These findings would suggest those with higher iron stores do not develop higher levels of parasite burden and faster parasite growth rates in those inoculated with *P. falciparum* in the VIS. The study observed that those with a lower baseline MCV had lower parasitaemias and total parasite burdens in *P. falciparum*, supporting Clark *et al* [127], that microcytic erythrocytes can evade the parasite invasion. The findings for this study would support giving iron 7-days after treatment [127], which appears to show the hepcidin returning to near baseline levels in both *P. falciparum* and *P. vivax.*

Finally, there appears to be evidence that shows the movement of hepcidin-mediated iron into the liver maybe a cause of elevated liver transaminases. There were strong correlations with baseline and day 15 $\log_{10}$ ferritin/sTfR levels and day 15 ALT and AST. The correlations between pre-treatment ferritin/AST ratio, which can be used as a crude estimate of

intrahepatic iron, and day 15 ALT were also strong in *P. falciparum*. There is evidence from chronic liver diseases such as haemochromatosis and non-alcoholic fatty liver disease, that the retention of iron mediated by hepcidin can cause hepatic inflammation and necrosis [141,142].

6.4. Limitations

The IBSM VIS have several limitations. The IBSM VIS are used in the development of novel therapeutics especially the pharmacokinetic and pharmacodynamics of the investigational drugs. As such there is variability within drugs and drug doses within and between cohorts of studies [186,187], which leads to significant heterogeneity when analysing these studies together. In the pooled haemoglobin analysis, there is variability in the day of treatment, day 7 or 8 in *P. falciparum* and day 7,8,10,11 and 14 in *P. vivax*. Through refinement of the process, in the more recent studies, there is now consistency of the day of treatment and when participants reach parasite threshold.

The parasitaemias and parasite burdens encountered in the IBSM VIS are significantly less than observed in natural infection. In the haemoglobin pooled analysis, the parasitaemias in the IBSM VIS were 4-fold smaller than observed in natural infection, with the median TPB in *P. falciparum* being 30 parasites/μL, compared to 158,000 parasites/μL in uncomplicated malaria and 440,000 parasites/μL in severe malaria [188]. As such, these differences are likely to account for the differences observed in the pRBC:uRBC ratios seen in the IBSM VIS participants and degree of erythrocyte loss.

Lastly, in the IBSM VIS, the participants can have large volumes of blood taken during the trials. For every rolling 30 days the maximum permitted blood volume that can be withdrawn is 400 mLs. The phlebotomy is particularly intense in the confinement period, as intensive PK/PD and matched 18S qPCR samples are taken. For every 1 mL of blood taken it is anticipated the haemoglobin level will drop by 0.07 g/L [189], with the haemoglobin dropping by a maximum of 28 g/L if the full 400 mLs is taken. The phlebotomy losses were considered in the pooled analysis, however, should be considered in all further malaria-associated anaemia work.

6.5. Further work

The disappointing results from the ERFE assays should not deter further investigation of this potentially important hormone in the control of hepcidin. As knowledge progresses on this key hormone, other commercially available assays are likely to be developed which could be considered. Also, the use of fresh serum for current trials would also be another method of overcoming the issues with the detection of the hormone.

Lastly, to further investigate the movement of iron in to the liver and the role it plays in raised liver transaminases would be to use non-invasive methods of measuring hepatic iron such as magnetic resonance imaging (MRI) and elastography [190]. The use of MRI during IBSM VIS has been conducted previously and could be used successfully to investigate iron at baseline, treatment and EOS [191].

6.6. Summary of key findings

IBSM VIS are a safe and powerful tool in the drug development pipeline and has been further demonstrated with the pilot study investigating the safety and infectivity of the new *P. falciparum* 3D7-MBE-008 MCB. They are also a useful model to investigate malaria associated erythrocyte losses.

The haematological response in early malaria can be characterised using the IBSM VIS, demonstrating significant malaria-attributable erythrocyte losses in both *P. falciparum* and *P. vivax*, despite the parasitaemias being 3-log smaller than observed in natural infection, the malaria-attributable fractional fall in haemoglobin was approximately 4% in those inoculated with *P. falciparum*. The percentage loss of pRBC of the total malaria-attributable erythrocyte losses was 0.1% in PfK13 vs 7.9% in natural infection [63].

There appears to be no evidence from this study to suggest those with lower baseline iron from developing higher parasite growth rates and parasite biomass. Individuals are more iron deplete at the EOS, when compared to commencing the studies. Hepcidin mediated movement of iron may be a mechanism for elevations in liver transaminases observed in *P. falciparum* IBSM VIS participants. Evidence suggests the movement of iron into the liver at

the day of treatment is significantly correlated with post-treatment elevations in the liver transaminases.

6.7 Lessons learnt

I have learnt several key lessons through this clinical fellowship which will be used to enhance my future professional and academic practice. Below are some of the key lessons:

- *Be prepared to remain flexible in academia.* This more so when on the first day of my fellowship at QIMR, I was told the CTU had been sold and some of the planned trials had been paused or cancelled. It was also true when the COVID-19 pandemic hit and paused the clinical trials, and therefore hastily re-dedicating effort on new projects.
- *Be prepared for failures*. This was true in the context of the failed ERFE assays, which was the novel aspect of my pilot study in the iron metabolism in early malaria. Also, the lack of *P. vivax* samples for the iron metabolism was really disappointing.
- *Be organised*. A likely explanation for the failure of the ERFE assays is the repeated free-thaw cycles those samples had undergone. Going forward it makes sense to break down initial samples into smaller aliquots, space depending.
- *Enjoy the research*. I enjoyed learning from experienced clinicians and research nurses conducting these volunteer trials. Early phase clinical trials design and implementation were areas of medicine and academia that I had never done before and I had a near vertical but enjoyable and rewardable learning curve. The basic skills I have learnt conducting early phase trials have prepared me to continue similar work in the future.

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Appendices

Appendix 1. Approved sponsor laboratory values

		SNP Normal Ranges		Acceptable Inclusion Range		Gender acceptable inclusion range	
		Male	Female	Low	High	Male	Female
Biochemistry							
Sodium	mmol/L	135-145	135-145	130	150		
Potassium	mmol/L	3.5-5.5	3.5-5.5	3.0	5.5		
Chloride	mmol/L	95-110	95-110	85	120		
Calcium (Corr)	mmol/L	18 years: 2.20-2.65	18 years: 2.20-2.65	2.05	2.75		
		19-55 years: 2.10-2.60	19-55 years: 2.10-2.60	2.05	2.67		
Urea	mmol/L	18-29 years: 3.0-7.5	18-29 years: 2.5-6.5	N/A	1.75xULN	N/A – 13.1	N/A – 11.4
		30-49 years: 3.0-8.0	30-49 years: 2.5-7.0	N/A	1.75xULN	N/A – 14.0	N/A – 12.3
		50-55 years: 3.5-8.5	50-55 years: 3.0-8.0	N/A	1.75xULN	N/A – 14.9	N/A – 14.0
Urate	mmol/L	0.20-0.50	0.15-0.40	N/A	1.75xULN	N/A – 0.88	N/A – 0.70
Creatinine	umol/L	60-110	45-85	N/A		N/A – 111	N/A – 86
Creatine kinase	U/L	45-250	30-150	N/A	>5.0xULN	>1250	>750
eGFR	mL/min/ 1.7 3m2	≥60	≥60	≥85	N/A		
Glucose Fasted	mmol/L	3.6-6.0	3.6-6.0	N/A	6		
Total Protein	g/L	18-49 years: 66-83	18-49 years: 64-81	55	95		
		50-55 years: 63-80	50-55 years: 63-80	55	95		
Albumin	g/L	18-50 years: 35-48	18-50 years: 33-46	30	55		
		50-55 years: 32-44	50-55 years: 32-44	25	55		
Total Bilirubin	umol/L	4-20	3-15	N/A	1.xULN	N/A – 21	N/A – 16
Direct Bilirubin	umol/L	0-7	0-7	N/A	1.5xULN	N/A – 11	N/A – 11
ALP	U/L	18-19 years: 60-200	18 years: 45-120	N/A	1.5xULN	N/A – 300	N/A – 180
		20-55 years: 35-110	19-49 years: 20-105	N/A	1.5xULN	N/A – 165	N/A – 158
			50-55 years: 30-115	N/A	1.5xULN		N/A – 173
AST	U/L	10-40	10-35	N/A	1.0xULN	N/A – 40	N/A – 35

ALT	U/L	5-40	5-30	N/A	1.0xULN	N/A – 40	N/A – 30
GGT	U/L	5-50	5-35	N/A	1.5xULN	N/A – 75	N/A – 53
Cholesterol	mmol/L	3.9-5.5	3.9-5.5	N/A	6.5		
HDL Cholesterol	mmol/L	0.9-1.5	1.1-1.9	0.8	N/A		
LDL Cholesterol	mmol/L	0-4	0-4	N/A	5.0		
Haematology							
Hb	g/L	135-175	115-165			135-180	115-170
Plats	$x10^9$/L	150-400	150-400	145	450		
WCC	$x10^9$/L	3.5-10.0	3.5-12.0	3.5	12.0		
Neuts	$x10^9$/L	1.5-6.5	1.5-8.0	1.5	8.0		
Lymphs	$x10^9$/L	1.0-4.0	1.0-4.0	1.0	4.0		
Monos	$x10^9$/L	0.0-0.9	0.0-0.9	N/A	1.1		
Eos	$x10^9$/L	0.0-0.6	0.0-0.6	N/A	0.6		
Baso	$x10^9$/L	0.0-0.15	0.0-0.15	N/A	0.3		
Urine							
Protein (dipstick)		N/A	N/A	N/A	1+		
Ketones (dipstick)		N/A	N/A	N/A	<3+		
Red Blood Cells (MCS)		<10	<20			<20	<20*
White Blood Cells (MCS)		<10	<10	N/A	<10		
Casts (MCS)		Not seen	Not seen	N/A	<2/high power field		

* Results >20 are NCS if female subject is menstruating.

Appendix 2. Ethics for pilot banks studies

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A	Barber	B	A/Prof
B	McCarthy	J	Prof

Dear A/Prof Barber,

HREC Reference Number: **P3487**
Project Title: **3D7-GL A pilot study to characterise the in vivo safety and infectivity of a *Plasmodium falciparum* 3D7-GL isolate in healthy subjects**

Thank you for submitting the above research project for single ethical review. This project was considered by the QIMR Berghofer-HREC at its meeting held on 12 July 2019.

I am pleased to advise you that at this meeting the QIMR Berghofer-HREC granted ethical approval of this research project. The nominated participating sites for this project are;

- Q-Pharm Pty Ltd
- Herston Imaging Research Facility (HIRF)

This letter constitutes ethical approval only. The project cannot commence until separate research governance authorisation has been obtained from relevant participating research sites.

The study meets the requirements of the *NHMRC National Statement* and the documents listed below are approved:

Document	Version Submitted
E-Form P:3487	10 July 2019
• *2019-07-10- P3487_3D7 GL Pilot Study_Version 2.0_01Jul19_Fully signed.pdf* • *2019-07-01- P3487_3D7-GL_ IBSM PICF_Version 2.0_28June19_Final.pdf* • *2019-07-01- 3D7_IB_GL_Version 1.0_210619_Final.pdf* • *2019-07-01- P3487_PET Scan Preparation Sheet_Version2.0_28Jun19_Final.pdf* • *2019-07-01- P3487_3D7-GL_Generic Advertising Material.pdf* • *2019-07-01- CV_Earlleen Gunning_2019.pdf* • *2019-07-01- Q-Pharm Internet Public Use Policy_11OCT2012.pdf* • *2019-07-01- Q-Pharm Code of Conduct V1.1_10JAN2018.pdf* • *2019-06-11- P3487_Radiation Assessment Report_11Jun19.pdf* • *2019-06-11- P3487_MRI Safety Questionnaire_Version 1.0_04Jun19.pdf* • *2019-06-11- Beck Depression Inventory.pdf* • *2019-06-11- QIMRB Clinical Trials CoC 2018.pdf*	

300 Herston Road, Herston Qld 4006 Australia | QIMR Berghofer Locked Bag 2000, Royal Brisbane Hospital, Qld 4029
T +61 (7) 3362 0222 F +61 (7) 3362 0111 W www.qimrberghofer.edu.au
ABN 31 411 813 344

- *2019-06-11- P3487_3D7-GL_Participant Trial Card_V1.0_7Jun2019.pdf*
- *2019-06-11- P3487_3D7-GL_Participant Diary_v1_7Jun19.pdf*
- *2019-06-11- P3487_3D7-GL_GP Letter_v1.0_7June2019.pdf*
- *2019-06-11- P3487_3D7-GL_Generic Participant Card_v1.0_7Jun2019.pdf*
- *2019-06-11- P3487_3D7-GL Participant Stickers.pdf*
- *2019-06-11- Riamet_PI_2012.pdf*
- *2019-06-11- Primacin PI_2017.pdf*
- *2019-06-11- Malarone PI_2016.pdf*
- *2019-06-11- FDG_PI.pdf*
- *2019-06-11- CMI_Primacin_2017.pdf*
- *2019-06-11- CMI_Riamet_2012.pdf*
- *2019-06-11- CMI_Malarone_2013.pdf*
- *2019-06-11- Artesunate IV PI.PDF*
- *2019-06-11- CV_Stephen Woolley_2019.pdf*
- *2019-06-11- CV_Stephen Rose_2018.pdf*
- *2019-06-11- CV_Paul Thomas_2018.pdf*
- *2019-06-11- CV_John Woodford_2018.pdf*
- *2019-06-11- CV_James McCarthy_2019.pdf*
- *2019-06-11- CV_Bridget Barber_2019.pdf*

Approval of this project from QIMR Berghofer-HREC is valid from 12 July 2019 to 12 July 2022 subject to the following conditions being met:

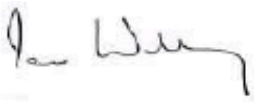
- This QIMR Berghofer-HREC approval is subject to governance approval/s from all collaborating institutions.
- The Principal Investigator will submit an annual report to the QIMR Berghofer-HREC by the approval anniversary date.
- The Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Principal Investigator will notify the QIMR Berghofer-HREC of any event that requires a modification to the protocol, including site changes, or other project documents and submit any required amendments in accordance with the ToR provided by the HREC. These instructions can be found at http://www.qimrberghofer.edu.au/about-us/ethics-committees/qimr-berghofer-human-research-ethics-committee/
- The Principal Investigator will submit any necessary reports related to the safety of research participants in accordance with QIMR Berghofer-HREC policy and procedures. These instructions can be found at http://www.qimrberghofer.edu.au/about-us/ethics-committees/qimr-berghofer-human-research-ethics-committee/
- The Coordinating Principal Investigator will notify the QIMR Berghofer-HREC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation.

- The Coordinating Principal Investigator will notify the QIMR Berghofer-HREC of his or her inability to continue as Coordinating Principal Investigator including the name of and contact information for a replacement.

Should you wish to discuss this matter, please contact the HREC Secretariat via email at HREC.Secretariat@qimrberghofer.edu.au.

The QIMR Berghofer-HREC wishes you every success in your research.

Yours sincerely,



Ian Wilkey
QIMR-Berghofer HREC Chair (NHMRC HREC #EC00278)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*. The processes used by this HREC to review multi-centre research proposals have been certified by the National Health and Medical Research Council.

QIMR Berghofer
Medical Research Institute

17 July 2019

Phone: 07 3362 0117
Fax: 07 3362 0109
E-mail:
HREC.Secretariat@qimrberghofer.edu.au

Investigator(s)	Surname	Initials	Title
A	Barber	B	A/Prof
B	McCarthy	J	Prof

Dear A/Prof Barber,

HREC Reference Number: P3491
Project Title: **3D7-MBE008 A pilot study to characterise the in vivo safety and infectivity of an in vitro expanded *Plasmodium falciparum* 3D7-MBE008 master cell bank in healthy subjects**

Thank you for submitting the above research project for single ethical review. This project was considered by the QIMR Berghofer-HREC at its meeting held on 12 July 2019.

I am pleased to advise you that at this meeting the QIMR Berghofer-HREC granted ethical approval of this research project.

The study meets the requirements of the *NHMRC National Statement* and the documents listed below are approved:

Document	Version Submitted
E-Form P:3491	10 July 2019
• *2019-07-10- P3491_3D7 MBE008 Pilot Study_Version 2.0_01Jul19_Fully signed.pdf* • *2019-07-01- P3491_3D7-MBE008_ IBSM PICF_Version 2.0_28June19_Final.pdf* • *2019-07-01- MBE008_IB_Version1.0_030619_Final.pdf* • *2019-07-01- P3491_3D7-MBE008_Generic Advertising Material.pdf* • *2019-07-01- Q-Pharm Code of Conduct V1.1_10JAN2018.pdf* • *2019-07-01- Q-Pharm Internet Public Use Policy_11OCT2012.pdf* • *2019-07-01- CV_Earlleen Gunning_20* • *2019-06-11- Beck Depression Inventory.pdf* • *2019-06-11- Clinical Trials CoC 2018.pdf* • *2019-06-11- P3491_3D7-MBE008_Participant Trial Card_V1.0_7Jun2019.pdf* • *2019-06-11- P3491_3D7-MBE008_Participant Diary_v1_7Jun19.pdf* • *2019-06-11- P3491_3D7-MBE008_GP Letter_v1.0_7June2019.pdf* • *2019-06-11- P3491_3D7-MBE008_Generic Participant Card_v1.0_7Jun2019.pdf* • *2019-06-11- P3491_3D7-MBE008 Participant Stickers.pdf* • *2019-06-11- Riamet_PI_2012.pdf* • *2019-06-11- Primacin PI_2017.pdf* • *2019-06-11- Malarone PI_2016.pdf*	

- *2019-06-11- CMI_Riamet_2012.pdf*
- *2019-06-11- CMI_Primacin_2017.pdf*
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- *2019-06-11- Artesunate IV PI.PDF*
- *2019-06-11- CV_James McCarthy_2019.pdf*
- *2019-06-11- CV_Stephen Woolley_2019.pdf*
- *2019-06-11- CV_Bridget Barber_2019.pdf*

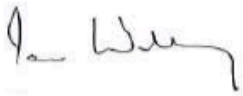
Approval of this project from QIMR Berghofer-HREC is valid from 12 July 2019 to 12 July 2022 subject to the following conditions being met:

- This QIMR Berghofer-HREC approval is subject to governance approval/s from all collaborating institutions.
- The Principal Investigator will submit an annual report to the QIMR Berghofer-HREC by the approval anniversary date.
- The Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Principal Investigator will notify the QIMR Berghofer-HREC of any event that requires a modification to the protocol, including site changes, or other project documents and submit any required amendments in accordance with the ToR provided by the HREC. These instructions can be found at http://www.qimrberghofer.edu.au/about-us/ethics-committees/qimr-berghofer-human-research-ethics-committee/
- The Principal Investigator will submit any necessary reports related to the safety of research participants in accordance with QIMR Berghofer-HREC policy and procedures. These instructions can be found at http://www.qimrberghofer.edu.au/about-us/ethics-committees/qimr-berghofer-human-research-ethics-committee/
- The Coordinating Principal Investigator will notify the QIMR Berghofer-HREC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation.
- The Coordinating Principal Investigator will notify the QIMR Berghofer-HREC of his or her inability to continue as Coordinating Principal Investigator including the name of and contact information for a replacement.

Should you wish to discuss this matter, please contact the HREC Secretariat via email at HREC.Secretariat@qimrberghofer.edu.au.

The QIMR Berghofer-HREC wishes you every success in your research.

Yours sincerely,



Ian Wilkey
QIMR-Berghofer HREC Chair (NHMRC HREC #EC00278)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007).* The processes used by this HREC to review multi-centre research proposals have been certified by the National Health and Medical Research Council.

Australian Red Cross
BLOOD SERVICE

7 August 2019

Associate Professor Bridget Barber
Head of Clinical Research
Clinical Tropical Medicine
QIMR Berghofer Medical Research Institute
300 Herston Road
Herston QLD 4006

Dear A/Prof. Barber,

Reference number: 2019#12
Project title: A pilot study to characterise the *in vivo* safety and infectivity of an *in vitro* expanded *Plasmodium falciparum* 3D7-MBE008 master cell bank in healthy subjects

Thank you for your submission to the Australian Red Cross Blood Service Ethics Committee. Your project, listed above, was considered by the Blood Service Ethics Committee at a meeting on 15 July 2019. I am pleased to advise that the Ethics Committee has granted ethical approval of this submission, for an initial period of three years, from 24 July 2019 to 24 July 2022.

Approval of this submission is subject to the following conditions being met:

- The Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Principal Investigator will notify the Blood Service Ethics Committee of any event that requires a modification to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the Ethics Committee.
- The Principal Investigator will report to the Blood Service Ethics Committee annually and notify the Ethics Committee when the project is completed.
- The Principal Investigator will notify the Blood Service Ethics Committee of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation.
- The Principal Investigator will arrange for the Blood Service Ethics Committee to receive a copy of any monitoring reports submitted to the Sponsor.

The approved documents include:

Document	Version	Date
P3491-3D7-MBE008 Protocol	2.0	1 July 2019
P3491-3D7-MBE008 PICF	3.0	1 August 2019
3D7-MBE008_IB	1.0	3 June 2019
P3491-3D7-MBE008 Code of Conduct	1.1	10 January 2018
P3491-3D7-MBE008 Generic Advertising Material	-	-

Australian Red Cross Blood Service
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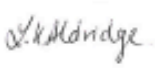
Alexandria | NSW | 2015
P: +61 2 9234 2368 | F: +61 2 9234 2411
Web: www.donateblood.com.au

P3491-3D7-MBE008 Generic Participant Card	1.0	7 June 2019
P3491-3D7-MBE008 Participant Trial Card	2.0	1 August 2019
P3491-3D7-MBE008 Participant Diary Card	2.0	1 August 2019
P3491-3D7-MBE008 GP letter	1.0	7 June 2019
P3491-3D7-MBE008 Participant Stickers	1.0	7 June 2019

Should you require any further information, please contact the Ethics Secretary on 02 9234 2368 or at ethics@redcrossblood.org.au.

Yours faithfully,



Dr Larissa Aldridge
Ethics Secretary
Research and Development
Australian Red Cross Blood Service

The Blood Service Ethics Committee is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*.

Australian Red Cross Blood Service
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Appendix 3 Ethics for iron metabolism study

QIMR Berghofer
Medical Research Institute

28 February 2020

Phone: 07 3362 0117
Fax: 07 3362 0109
E-mail:
HREC.Secretariat@qimrberghofer.edu.au

Investigator(s)	Surname	Initials	Title
A	Woolley	S	Dr

Dear Dr Woolley,

HREC Reference Number: **P3540**

Project Title: **Evaluating markers of iron metabolism during early Plasmodium infection: use of stored samples**

I am pleased to advise that an amendment to the above named research project was considered and approved by the QIMR Berghofer-HREC chairperson out of session on 27 February 2020.

The amendment and documents listed below are approved and meet the requirements of the *NHMRC National Statement*:

- *2020-02-27- P3540_Iron Studies Protocol_3.0_Final.docx*
- *2020-02-27- P3540_Iron Studies Protocol_3.0_tracked.docx*
- *2020-02-27- Iron studies Cover letter Amendment.docx*

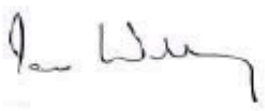
Provisos:
(1) This QIMR Berghofer-HREC approval is subject to governance approval/s from all collaborating institutions.
(2) The Principal Investigator must submit an annual report to the QIMR Berghofer-HREC by the approval anniversary date.

Please note that all requirements of the original ethical approval for this project still apply.

Should you wish to discuss this matter, please contact the HREC Secretariat at HREC.Secretariat@qimrberghofer.edu.au.

The QIMR-Berghofer HREC wishes you every continued success in your research.

Yours sincerely,



Ian Wilkey
QIMR-Berghofer HREC Chair (NHMRC HREC #EC00278)

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