Universidade de Lisboa
Faculdade de Medicina

In-vitro, Investigation of Interindividual Variability on *Plasmodium falciparum* Infection – Red Blood Cells Underlying Mechanisms

Yara de Sousa Lobo Almeida

Supervisor:
Prof. Doutor Thomas Hänscheid

Dissertation especially elaborated for obtaining the Master´s degree in Clinical Microbiology and Emerging Infectious Diseases

2024

Universidade de Lisboa
Faculdade de Medicina

In-vitro, Investigation of Interindividual Variability on *Plasmodium falciparum* Infection – Red Blood Cells Underlying Mechanisms

Yara de Sousa Lobo Almeida

Supervisor:
Prof. Doutor Thomas Hänscheid

Dissertation especially elaborated for obtaining the Master´s degree in Clinical Microbiology and Emerging Infectious Diseases

2024

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião 19 de janeiro de 2021.

ACKNOWLEDGMENTS

This journey towards completing my master's thesis has been lengthy and transformative, with my passion for research and its encompassing challenges continuously fuelling my drive. Research, to me, symbolizes resilience, engagement, dedication, critical thinking, and an unending quest for knowledge, often casting a positive light on each subject it touches. This endeavour has enriched my knowledge, sculpted me into a more rounded professional, and honed my critical outlook on a multitude of topics. Yet, these accomplishments would not have been realized without the invaluable individuals I've encountered along the way.

First and foremost, my heartfelt gratitude goes to Professor Thomas Hänscheid, the most exceptional supervisor one could wish for. His willingness to share his knowledge, the use of his computers when mine faltered, his guidance, dedication, and immense patience during my moments of doubt, remain deeply appreciated. His motivational "FORÇA" came when I needed it most, making me always feel welcomed and supported in the office, and never letting me "sink" while pushing me to think deeply. Above all, thank you for believing in my potential and understanding my limits.

I extend my acknowledgment to my team: Sara, Anita, and Tomás. Their support was crucial in rallying numerous volunteers and cultivating successful cultures. A heartfelt thanks to Sara, who transcended the role of a colleague to become a friend, involving me in her project, teaching me the ropes of laboratory work, especially *in-vitro falciparum* cultures. Her trust, our brainstorming sessions, the afternoon snacks, and, most importantly, her unwavering support and prompt assistance have been invaluable. I am truly grateful for her presence in this journey.

My gratitude also reaches out to all the volunteers who participated in this study, making this project feasible. Special thanks to Profª. Emília Valadas, Drª. Ana Miranda, and laboratory technician Ana Lúcia for their assistance in the study's second phase.

I am thankful for my colleagues from Hospital de Santa Cruz for their support and camaraderie during the laboratory work and their help with our haemoglobin standard curve.

Warm thanks to my 2019 MCDIE professors and classmates, and a special nod to the entire MRamirez team. I extend my gratitude to Nuno Leal and Melissa, for their companionship and for keeping me company during those long evenings and weekends in the lab. I must also express my gratitude to Yenine, for embarking on this master's journey alongside me.

My family deserves my deepest gratitude for their unwavering belief and pride in me. A special mention goes to my parents, whose support and motivation have always propelled me to pursue my dreams. And to Nuno, my deepest thanks for your constant presence, patience, support, friendship, and for always wanting the best for me, encouraging me to stay focused on my thesis.

I must also acknowledge the role of artificial intelligence, especially ChatGPT, in this thesis. As English is not my first language, ChatGPT was instrumental in correcting semantic or vocabulary errors and contributed to proofreading this work.

ABSTRACT

Background: Malaria, impacting 249 million globally in 2022, ranges from asymptomatic to severe cases, significantly affecting young children and pregnant women. Disease severity correlates with pathogen load, underscoring the importance of red blood cells (RBCs) factors like membrane properties, enzymatic activities and haemoglobin variations in influencing *Plasmodium* growth and malaria outcomes. Significant interindividual variability in *Plasmodium falciparum* (*Pf*) growth rates *in-vitro* and *in-vivo* underscores the complex dynamics of host-parasite interactions.

Methods: Malaria-naive volunteers from the Faculty of Medicine, University of Lisbon, were recruited for a three-phase study that included questionnaire assessments, blood sampling for parasite culture to establish growth patterns, and a series of haematological and biochemical analyses to identify associations with RBC traits among low (LG) and high growth (HG) groups.

Results: The study of 69 volunteers identified distinct growth patterns (low: LG, normal: NG, high: HG) with significant variability not accounted for by demographic or blood group factors. A trend towards ancestry from malaria-endemic regions in the LG group hinted at genetic factors. No significant effects of dietary habits, health status, or medication on growth rates were found. Notably, variations in haemoglobin levels and histories of borderline anaemia were observed between LG and HG despite no detected haemoglobinopathies. Preliminary results suggest potential RBC membrane alterations through Cell-Trace-Far-Red marker analysis.

Conclusion: The study highlights the variability in *Pf* growth rates caused by RBC factors, independent of immunological influences, demographics, or blood group, with borderline anaemia (history and blood results) playing a role. Future research should increase the LG and HG cohorts and expand on these findings through detailed exploration of RBC characteristics, possibly using advanced genomic and proteomic techniques, aiming to elucidate the mechanisms affecting malaria susceptibility and outcomes.

Key words: Malaria, *Plasmodium falciparum*, disease severity, parasite load, red blood cell**.**

RESUMO

Contexto: A malária continua a ser um desafio significativo para a saúde pública. O relatório mais recente destaca 249 milhões de casos e 608,000 mortes em 2022, o que marca uma inversão na tendência de queda observada de 2000 a 2015. A doença manifesta-se num espectro que vai desde a infeção assintomática, que contribui para a transmissão, até à malária não complicada, comum em adultos semi-imunes, e malária grave, que afeta principalmente crianças e mulheres grávidas.

A carga patogénica de um hospedeiro impacta significativamente na gravidade das doenças infeciosas, em que cargas mais elevadas resultam em doenças graves e morte. Esta associação é estabelecida em várias doenças, tais como a infeção por vírus da imunodeficiência humana, COVID-19 e sépsis, incluindo a malária. Por exemplo, marcadores elevados como o *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2) sinalizam um risco aumentado de doença grave. Além disso, a efetividade do artesunato no tratamento da malária grave resulta da rápida taxa de redução do parasita, visto que atua em múltiplas fases do ciclo de vida do mesmo. Destacando o princípio de que, uma maior carga parasitária correlaciona-se com doença mais grave e morte.

Fatores do hospedeiro, particularmente aqueles associados aos glóbulos vermelhos (GVs), influenciam o crescimento do *Plasmodium* e, consequentemente, o espectro da malária: (i) A membrana, crucial para funções fisiológicas, é relevante para a sobrevivência e interação com o parasita da malária. Alterações na membrana dos GVs têm sido demonstradas a mitigar a gravidade da doença. (ii) Os GVs abrigam um conjunto único de enzimas para o seu metabolismo energético. Alterações nessas enzimas podem afetar o crescimento do parasita no hospedeiro. (iii) Variações na hemoglobina, sendo o traço falciforme um exemplo primário, oferecem efeitos protetores contra a malária. Estes elementos sublinham a complexidade das interações hospedeiro-parasita na malária e destacam a importância das características dos GVs na determinação da gravidade da doença.

Estudos controlados de infeção humana por malária e experiências *in-vitro* demonstraram uma notável variabilidade interindividual nas taxas de crescimento do *Plasmodium falciparum* (*Pf*), destacando diferenças nas parasitémias entre indivíduos nunca expostos à malária e indivíduos imunes. Culturas *in-vitro* mostraram taxas de crescimento altamente variáveis, atribuídas a diferenças nos GVs do dador. Esta variabilidade também foi observada num estudo cujo objetivo era investigar os efeitos da ingestão de açúcar por voluntários no crescimento do parasita, no nosso laboratório. Tal variabilidade

interindividual levantou questões sobre os fatores do hospedeiro que podem causar estas diferenças, sugerindo que uma caracterização mais detalhada poderia fornecer informações importantes e potencialmente úteis para intervenções contra a malária.

Objetivos: Este estudo tem como objetivo determinar a variabilidade das taxas de crescimento do *Pf in-vitro*, utilizando GVs de voluntários sem exposição prévia à malária, categorizando-os em padrões de crescimento baixo ou alto, com base em fatores dos GVs do hospedeiro. Bem como com a colheita de dados dos voluntários para encontrar ligações potenciais com esses padrões e análise de parâmetros clínico-laboratoriais para identificar associações.

Métodos: O estudo recrutou voluntários adultos sem exposição prévia à malária, predominantemente da Faculdade de Medicina da Universidade de Lisboa (FMUL) e do Instituto de Medicina Molecular (iMM), todos tendo dado consentimento informado antes da participação. O estudo foi dividido em três fases distintas:

1. Avaliação inicial e realização das culturas: Foi solicitado aos participantes que preenchessem um questionário abrangente cobrindo dados demográficos, hábitos alimentares, exposição prévia à malária, estado de saúde atual e histórico médico, com um foco em qualquer histórico de anemia. Cada voluntário contribuiu com aproximadamente 14 mL de sangue, usado para testes de glicemia capilar, determinação dos níveis de hemoglobina e grupos sanguíneos AB0/Rh, e início de culturas com a estirpe laboratorial *Pf* 3D7. As culturas de *Pf* foram estabelecidas seguindo protocolos padrão para culturas de parasitas *in-vitro*, utilizando meio de cultura completo, com distintas suplementações. Durante um período de 96 horas, estas culturas foram monitorizadas e a parasitémia foi avaliada por microscopia ótica. O objetivo foi categorizar os voluntários em coortes de crescimento distintas—crescimento baixo (LG), crescimento normal (NG) e crescimento alto (HG)—com base nas parasitémias de cada voluntário.
2. Reprodução dos resultados e testes adicionais: Para confirmar os achados iniciais, um subconjunto de vinte e quatro voluntários forneceu amostras de sangue adicionais. Os voluntários foram convidados a fornecer sangue para um conjunto de testes hematológicos e bioquímicos de rotina, realizados num laboratório de referência credenciado, com o objetivo de determinar correlações com os padrões de crescimento do parasita observados.
3. Exploração do fenótipo de invasão: Na fase conclusiva do estudo, oito voluntários forneceram novas amostras de sangue para experiências adicionais.

Os GVs desses voluntários foram marcados com o corante Cell-Trace-Far-Red (CTFR) para investigar os fenótipos de invasão do *Pf*, com um foco específico na observação da variabilidade na marcação do CTFR entre os GVs dos voluntários.
O tratamento e análise de dados envolveu vários métodos estatísticos, incluindo os testes: exato de Fisher; t de Student; ANOVA unilateral e U de Mann-Whitney para comparar variáveis categóricas e numéricas, com resultados apresentados em frequências, médias, intervalos, desvios padrão e medianas conforme apropriado. Este estudo recebeu aprovação ética do Centro Académico de Medicina de Lisboa (Número de Aprovação Ética 496/18).

Resultados: Este estudo analisou culturas de *Pf* de 69 voluntários. O crescimento do parasita foi categorizado em grupos de crescimento LG, NG e HG, com diferenças significativas entre os grupos (d de Cohen = 5.4; $P<0.0001$). O grupo LG teve uma média de razão de crescimento 4.7, NG de 9.7 e HG de 16.0. A reprodutibilidade foi alta num subgrupo de 24 voluntários ($R^2 = 0.99$).
Na análise de fatores demográficos como idade, género e nacionalidade, não surgiram diferenças significativas entre os grupos de baixo e alto crescimento (LG e HG). No entanto, uma tendência notável foi a maior prevalência de indivíduos com ascendência de regiões endémicas de malária no grupo LG, sugerindo possíveis fatores genéticos que influenciam a resistência à malária, merecendo estudo adicional. Quanto à saúde e medicação, ambos grupos tinham membros com condições médicas, particularmente alergias ou asma, e usavam medicamentos como contracetivos e anti-histamínicos, não sendo encontrada correlação significativa com as taxas de crescimento do parasita. Os hábitos alimentares foram consistentes entre os voluntários, tendo todos consumido uma refeição rica em carbohidratos antes da colheita. Membros do grupo LG foram mais propensos a ter comido menos de uma hora antes da colheita. Não foram observadas diferenças impactantes nos níveis de glicose ou marcadores metabólicos e inflamatórios básicos entre os grupos.
Ao longo do estudo, nenhum participante relatou casos atuais de anemia, e o menor crescimento do parasita no grupo LG não foi devido a sintomas de anemia, como detalhado na Tabela 14. Cinco voluntários do grupo LG tinham históricos de anemia, identificados através de diagnóstico médico, durante doações de sangue ou exames de rotina, mas nenhum tinha o diagnóstico de anemia durante as participações no estudo. O grupo LG também relatou um histórico familiar superior de anemia do que os outros. Interessantemente, o grupo sanguíneo AB0 não mostrou implicações significativas nas taxas de crescimento do parasita.

No grupo HG, as variáveis hematológicas estavam dentro dos intervalos normais. Testes iniciais revelaram níveis de hemoglobina abaixo do limite inferior de referência para três voluntários LG na 1ª fase (n=69) e para quatro voluntários LG na fase 2 (n=24). O grupo LG também mostrou menor média de hemoglobina em comparação com HG, tanto na fase um (12.3 *vs* 14.8 g/dL, P=0.03) quanto na fase dois (12.7 *vs* 13.9 g/dL, P=0.04). Os níveis de reticulócitos e ferro tendiam a ser mais baixos no grupo LG, embora não significativamente, com reticulócitos (%) em 0.9 *vs* 1.2, P=0.07, e ferro (µg/dL) em 75.5 *vs* 103.1, P=0.07. Não foram detetadas variantes anormais de hemoglobina nos voluntários da fase 2. Uma análise preliminar (n=4 LG e n=4 HG) do CTFR mostrou uma tendência para maior média na intensidade de fluorescência mediana no grupo HG em comparação com o grupo LG.

Conclusão: Este estudo realça a significativa variabilidade nas taxas de crescimento do *Pf* entre os GVs dos voluntários, revelando um contraste acentuado entre GVs que suportam taxas de crescimento baixas *versus* altas em condições *in-vitro*, excluindo influências imunológicas e fatores séricos. Tal variabilidade não pôde ser explicada por variáveis demográficas ou pelo grupo sanguíneo, desafiando algumas observações de estudos anteriores. As principais diferenças entre os grupos de crescimento baixo (LG) e alto (HG) estavam ligadas a históricos pessoais e familiares de anemia, refletidos em níveis distintos de hemoglobina, contagens de reticulócitos e níveis de ferro, apesar da sobreposição substancial de valores. A ausência de hemoglobinopatias comuns adiciona complexidade à identificação da causa desta variabilidade. Achados preliminares indicam possíveis alterações na membrana dos GVs, sugeridos pela análise do marcador CTFR, potencialmente associados a níveis reduzidos de hemoglobina e históricos de anemia.

Pesquisas futuras devem focar na expansão dos coortes LG e HG para explorar de forma mais abrangente fatores relacionados à membrana, enzimas ou hemoglobina, utilizando possivelmente estudos genómicos e proteómicos. Esta abordagem visa desvendar os mecanismos intrínsecos subjacentes à variabilidade dos GVs em suportar o crescimento do *Pf*.

Palavras-chave: Malária, *Plasmodium falciparum*, doença severa, carga parasitária, glóbulo vermelho**.**

ABBREVIATIONS

2,3 DPG	2,3-diphosphoglycerate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATP2B4	ATPase Plasma Membrane Ca2+ Transporting 4
BC	Before Christ
BMI	Body mass index
BSC	Biosafety Cabinet
CCR5	CC chemokine receptor type 5
CHMI	Controlled human malaria infection
CHULN	Centro Hospitalar Universitário Lisboa Norte
CR1	Complement receptor 1
CRP	C reactive protein
CTFR	Cell Trace Far Red
CyRPA	Cysteine-rich protective antigen
DARC	Duffy antigen/chemokine receptor
EBA	Erythrocyte-binding antigen
EBL	Erythrocyte binding like
FBC	Full blood count
FMUL	Faculdade de Medicina da Universidade de Lisboa
G6PD	Glucose-6-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GP	Glycophorins
Hb	Haemoglobin
HbA1C	Glycated haemoglobin
HDLc	High density cholesterol
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HG	High growth
HIV	Human immunodeficiency virus
HRP2	histidine rich protein 2
IBSM	Infection Biology and Molecular Surveillance
ICAM-1	Intracellular adhesion molecule 1
iMM	Instituto de Medicina Molecular
IPST	Instituto Português do Sangue e da Transplantação
iRBC	Infected RBCs
LDH	Lactate dehydrogenase
LG	Low growth
LIP	Labile iron pool

McC	McCoy
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCM	Malaria complete parasite medium
MCV	Mean corpuscular volume
MFI	Median Fluorescent Intensity
MPS	Merozoite Surface Proteins
NG	Normal growth
NHE1	Sodium hydrogen antiporter 1
NPP	New permeation pathways
PBS	Phosphate Buffered Saline
Pf	*Plasmodium falciparum*
*Pf*AMA1	*Pf* apical membrane antigen 1
*Pf*EMP1	*Pf* erythrocyte membrane 1
*Pf*HRP2	*Pf* histidine rich protein 2
*Pf*Rh	*Pf* Reticulocyte binding protein homologues
*Pf*SPZ	*Pf* sporozoites
Pk	*Plasmodium knowlesi*
PK	Pyruvate kinase
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
pO_2	Partial pressure of oxygen
PRR	Parasite reduction rates
Pv	*Plasmodium vivax*
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
RCDW	Red Cell Distribution Width
Rh	Rhesus
RON	Rhoptry neck protein
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SARS	CoV2 – Severe acute respiratory syndrome 2
SD	Standard Deviation
SIa	Swain Langley
TNF	Tumour necrosis factor
uRBC	Uninfected red blood cell
VFR	Visiting friends and relatives
WHO	World Health Organization

TABLE OF CONTENTS

FIGURES – INDEX

TABLES - INDEX

1. INTRODUCTION

1.1. Malaria – an overview and history

Malaria, a parasitic disease caused by the *Plasmodium* genus, is transmitted through the bite of infected female *Anopheles* mosquitoes [1][2]. Historical accounts of malaria date back to ancient civilizations, with references found in ancient Chinese texts (2700 BC), Mesopotamian clay tablets (2000 BC), Egyptian papyri (1570 BC), and Hindu scriptures (6 BC) [3]. This disease is believed to have co-evolved with non-human primates and humans, spreading from tropical and temperate regions, especially Africa, to global extents [4]. Presently, malaria predominantly affects tropical countries, though historical records indicate its presence in Asia, North America, and Europe [5].
In Europe, malaria was rampant, particularly in marshy areas. For example, in Italy, where the term 'malaria' originates from 'mal'aria' (Italian for 'bad air'), the disease was prevalent, causing around 2 million infections and 15.000 to 20.000 deaths annually by the late 19th century [6]. In England, known as the 'marsh fever', it caused high mortality rates, particularly in the Kent and Essex marshlands from the 16th to the 19th century [7]. In Portugal, the disease gained prominence in the 19th century, coinciding with the expansion of rice cultivation. Historical surveys indicate that malaria was endemic in 15 out of 18 districts, with about 100.000 cases and 500 deaths annually [8].

Malaria has also impacted significant historical figures, including popes, kings, and military leaders [9]. It significantly affected soldiers during the World Wars, with over 1.5 million cases in World War I and varying case fatality rates [9]. Post-World War II, with health improvements and interventions like draining swamps (decreasing mosquito breeding grounds) [10] and the World Health Organization's (WHO) global malaria eradication program (1955-1969), malaria incidence declined in the northern hemisphere [11].

Nowadays, malaria's prevalence has been resurging, posing a significant public health challenge [12]. This resurgence is attributed to various factors: i) antimalarial drug resistance [13], ii) insecticide resistance [14], iii) climate change [15], iv) urbanization and migration [16], v) inadequate healthcare systems in endemic regions [17], vi) conflict and displacement [18], and vii) travel and migration [19]. Importantly, people from several countries, especially in Africa continue to be at risk of *falciparum* malaria, the most severe form [20].

1.2. Malaria – epidemiology

The incidence of malaria is highly influenced by environmental factors, including altitude, climate, and vegetation, as well as the effectiveness of control strategies [21]. This disease affects continents worldwide, with varying degrees of frequency and mortality.

The most recent World Malaria Report indicated an estimated 249 million cases of malaria and 608.000 associated deaths in 2022 [22]. There was significant progress from 2000 to 2015, with cases declining from 243 million to 231 million, reflecting a ~ 5,1% global reduction in malaria incidence [22][23]. However, this trend reversed between 2015 and 2022, as cases raised almost each year reflecting an ~ 7,2% increase in malaria incidence [24]. The majority of these cases and deaths are concentrated in Africa (as shown in Figure 1) [25].

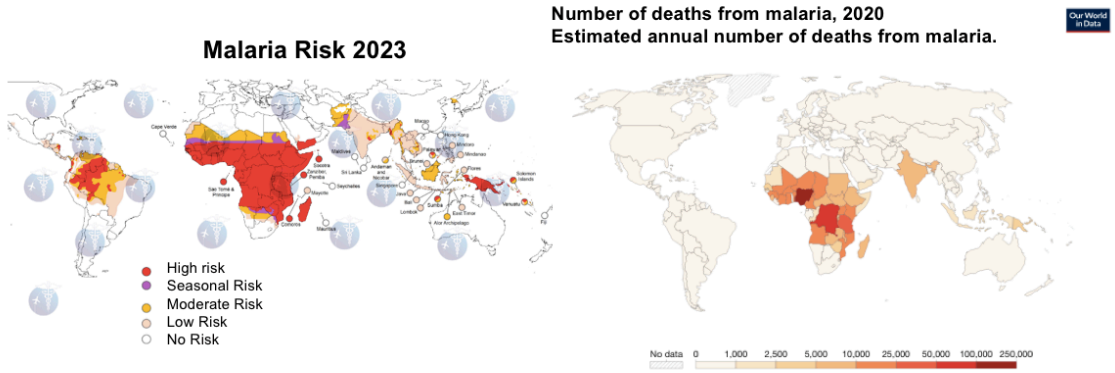


Figure 1 Global epidemiology of malaria
Adapted from [26][27].

Countries with underdeveloped healthcare systems, particularly in Africa, bear the highest burden of disease incidence and mortality [28][29]. In 2021, sub-Saharan Africa accounted for approximately 95% of all malaria cases. *Plasmodium falciparum* (*Pf*) is the most prevalent species in these regions, responsible for the majority of severe cases and fatalities [30]. Given its significant impact on public health, socio-economic development, and human suffering, malaria remains a critical global health priority [31].

1.3. Malaria – biology

Malaria in humans is caused by six *Plasmodium* species: *Plasmodium falciparum (Pf)*, *Plasmodium vivax* (*Pv*), *Plasmodium malariae* (*Pm*), *Plasmodium ovale curtisi* (*Po*), *Plasmodium ovale wallikeri* (*Po*), and *Plasmodium knowlesi* (*Pk*) [32]. Even though, the latter being recognized as a zoonotic infection, human-

mosquito-human transmission is biologically possible [33] [34] . A notable feature of *Pf* is its ability to infect red blood cells (RBCs) of all ages, resulting in higher pathogen burdens, in contrast to *Pv* for example, which mainly infects reticulocytes (approximately 1% of circulating RBCs). Thus, *Pf* often leads to more severe disease outcomes.

The life cycle of *Pf* involves two hosts: the vector, which is the female *Anopheles* mosquito, and the vertebrate host, humans [35]. The asexual cycle of the parasite occurs within the human host and is categorized into two principal phases: the pre-erythrocytic phase and the intra-erythrocytic phase, as depicted in Figure 2 [35][36]. This life cycle is critical to understanding the pathogenesis and transmission of malaria.

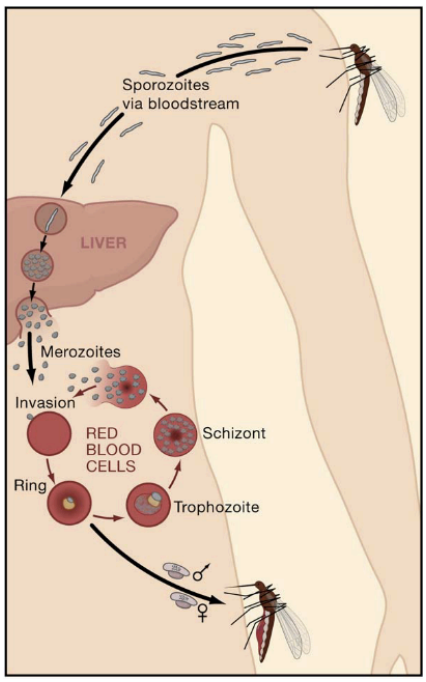


Figure 2 *Pf* life cycle

This diagram outlines the life cycle of *Pf* in the human host. It begins with the inoculation of sporozoites into human skin, their migration to the liver where they multiply within hepatocytes (pre-erythrocytic phase), and subsequent release into the bloodstream. Inside red blood cells (intra-erythrocytic phase), the parasites mature through several stages, leading to exponential infection growth. Some parasites develop into gametocytes, completing the cycle when taken up by mosquitoes during a blood meal. Image adapted from [37].

Pf infection exhibits distinct characteristics compared to other *Plasmodium* species, as represented in Table 1. *Pf*, *Pv* and *Po* share a similar replication cycle duration, and especially *Pv* and *Po* show tertian fever spikes every 48 hours due to the synchronous release of merozoites into the bloodstream, which infect new RBCs [38]. However, *Pf* distinguishes itself in two important ways: i) it infects RBCs

of all ages and ii) it releases a higher number of progeny [39]. Both result in a significant proportion of newly infected RBCs and lead to a higher parasite burden and thus, more severe disease [40]. Additionally, *Pf* parasites are known to sequester in blood capillaries, meaning the total biomass of *falciparum* malaria includes both circulating and sequestered parasites, contributing to high parasite burdens [41]. *Falciparum* malaria, capable of reaching high parasite loads, is associated with the high morbidity and mortality rates of the disease. In particular, *falciparum* hyperparasitaemia is often linked to poor prognosis [42]. Despite this, *falciparum* infections can present a wide range of clinical outcomes, from mild to life-threatening conditions [43].

In contrast, *Pv* and *Po* predominantly infect reticulocytes (about 1% of circulating RBCs), limiting their potential to achieve high parasite burdens [44]. *Pm*, on the other hand, has a 72-hour asexual cycle and releases fewer progeny, preferring older RBCs [45]. This preference, coupled with the faster removal of older RBCs from circulation, results in lower overall pathogen numbers [46]. *Pk* has the shortest asexual life cycle among these species, completing it in 24 hours and leading to daily fever spikes. This species also shows tropism for all types of RBCs [47].

Table 1 Characteristics of *Plasmodium* species infecting humans

Characteristic	*Plasmodium* species				
	Pf	*Pv*	*Pm*	*Po*	*Pk*
World distribution (%)	Global: 80- 90% (Africa)	50 – 80% (Asia)	Global: 0.5 – 3%	5 – 8% (Africa)	1 – 60% (Southeast Asia)
Replication cycle in hours (h)	48h (tertian)	48h (tertian)	74h (quartan)	48h (tertian)	24h (quotidian)
Progeny - number of merozoites released per replication cycle	16-32	12-24	6-12	6-14	Up to 16
Preferred RBC (age)	All	Young (reticulocytes)	Old	Young (reticulocytes)	All
Parasitaemia	Can be high, up to >50%	<2%	<2%	<2%	Can be high

Based on [37][48].

1.4. Malaria - disease and spectrum of presentation

Malaria typically presents with symptoms such as fever, shivering, headache, myalgia, arthralgia, and diarrhoea, potentially progressing to neurological symptoms and coma [49]. In endemic areas, the onset of clinical symptoms is often dependent on reaching a certain parasite density, known as the pyrogenic threshold (~100 parasites/μL) [49]. Notably, adults in African endemic areas often show no symptoms due to continuous low-level exposure and resulting low parasite burdens [50].

The incubation period for *Pf* malaria is about 10-15 days, with common symptoms like fever occurring at the end of the parasite's intra-erythrocytic life cycle [51]. However, synchronization of development stages, which influences the timing of clinical symptoms, is not unusual in *Pf* infections [52]. The disease spectrum ranges from asymptomatic (termed "malaria infection" by WHO) to symptomatic (termed "malaria disease" by WHO) which can be further categorized as uncomplicated or complicated [53].

Asymptomatic malaria is characterized by the presence of *Plasmodium* without clinical symptoms, often going undetected due to the absence of medical consultation. These cases, however, contribute to the parasite reservoir, facilitating ongoing transmission. This is particularly true in semi-immune individuals in endemic regions, where parasitaemias are typically below 40 parasites/μL, under the pyrogenic threshold [49][54].

Uncomplicated malaria manifests through non-specific symptoms such as fever, chills, body aches, headache, cough, and diarrhoea. These symptoms often resemble other flu-like illnesses, making clinical diagnosis challenging, especially in non-endemic areas [55].

Severe *falciparum* malaria can affect various organ systems including the central nervous system (cerebral malaria), respiratory system, renal system (acute renal failure), or haematopoietic system (severe anaemia), and is usually linked to high parasite loads [56]. Young children and pregnant women are particularly vulnerable to severe forms like cerebral malaria, contributing significantly to malaria morbidity and mortality [57][58]. The case fatality rate for treated cerebral malaria is generally between 10 to 20%, rising to 50% in pregnant women [59]. There are noticeable differences in the presentation of severe malaria between children and adults; for instance, severe anaemia is more common in children,

who also frequently experience seizures, hypoglycaemia, and concomitant sepsis, while pulmonary oedema and renal failure are less common compared to adults [60].

Prompt identification and treatment of severe malaria cases are critical. To aid in this, the WHO has established a set of criteria for identifying severe malaria cases (Table 2) [61].

Table 2 Clinical and laboratory features of malaria

Clinical	**Description / Comment**
Impaired consciousness or unarousable coma	Glasgow Coma Scale ≤11 in adults; Blantyre Coma Scale <3 in children
Respiratory distress	Acidotic breathing
Multiple convulsions	More than two episodes in 24 hours
Prostration	Generalized weakness so that the patient is unable walk or sit up without assistance
Shock	Compensated shock is defined as capillary refill ≥3 s or temperature gradient on leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure <70 mm Hg in children or <80 mm Hg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill)
Pulmonary oedema	Radiologically confirmed, or oxygen saturation <92% on room air with a respiratory rate >30/min, often with chest indrawing and crepitations on auscultation
Abnormal bleeding	Including recurrent or prolonged bleeding from nose gums or venepuncture sites; haematemesis or melaena
Jaundice	Plasma or serum bilirubin > 3 mg/dL together with a parasite count >100.000/µL
Laboratory	**Description / Comment**
Severe anaemia	Haemoglobin <7 g/dL in adults, <5 g/dL in children
Hypoglycaemia	Blood or plasma glucose <2.2 mM (<40 mg/dL)
Acidosis	A base deficit of >8mEq/L or, if unavailable, a plasma bicarbonate of <15mmol/L or venous plasma lactate >5mmol/ L. Severe acidosis manifests clinically as respiratory distress – rapid, deep and laboured breathing
Hyperlactataemia	Lactate > 5 mmol/L
Renal impairment	Creatinine >3 mg/dL or urea >20 mmol/L)
Hyperparasitaemia	Asexual *Pf* parasitaemia >10% of infected red blood cells

Adapted from [61].

Hyperparasitaemia is recognized as a significant feature of severe malaria, correlating with an increased risk of mortality [62]. A distinctive characteristic of *Pf* that contributes to elevated total body parasite burdens is the sequestration of parasites in the capillary network [63]. The total biomass of *Pf* comprises both circulating and sequestered parasites, which can result in substantial overall parasite burdens, even in cases without high peripheral parasitaemia [64]. Clinical

observations reveal that some patients with parasitaemias of 10 - 20% can walk into health services, while others with only 1% parasitaemia may have unfavourable outcomes or even succumb to the disease [65]

Laboratory diagnosis is crucial for timely treatment initiation, with the goal of reducing parasite load and thereby lowering the risk of severe outcomes, including death [66]. Microscopy, often regarded as the gold standard, not only confirms the presence of parasites but also allows for the quantification of parasitaemia, guiding treatment decisions [67][68]. However, microscopy can only detect circulating parasites, leading to potential underestimation of the total parasite burden in *Pf* malaria, as it does not account for sequestered parasites [69][70]. Intriguingly, *Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP2), released during parasite multiplication, reflects the total parasite biomass more accurately. The levels of this protein are associated with disease severity and outcomes [71]. These insights establish a straightforward correlation: higher parasite counts are indicative of more severe disease manifestations, which could be expressed in a simple formula: (↑↑↑parasites burden = ↑↑↑severe disease).

1.5. Parasite related factors and disease severity

Microbes, in their evolutionary journey, have developed strategies to ensure adequate replication for establishment within hosts and successful transmission to new hosts [72]. Evolution for microbes often means optimizing their life cycles and modes of transmission to enhance survival and spread within a host population [73]. It involves a delicate balancing act between exploiting the host's resources for reproduction and avoiding host immune responses that could hinder their propagation. Over time, this evolutionary process has led to the diverse array of microbial strategies we observe today, from highly aggressive pathogens to those coexisting more benignly with their hosts [74].
This process, central to the concept of evolution, aims to maximize the spread of genetic material. Interestingly, pathogens inherently do not seek to harm or kill their hosts, as host death can impede further transmission, posing a dilemma between virulence and transmission [74]. The ideal pathogen strikes a balance, replicating at a rate sufficient for transmission without causing host mortality. This is exemplified in malaria infections in endemic areas (asymptomatic infection), where the parasite coexists with the host in a relatively balanced state [75].

However, many pathogens achieve higher transmission rates through rapid replication, often at the expense of the host's health. This replication can lead to

disease or even host death. Various microbes, most typically viruses, like human immunodeficiency virus (HIV), severe acute respiratory syndrome 2 (SARS-CoV-2), and rabies [76][77][78], exhibit this trait. In some cases, such as rabies, transmission efficiency is so high before host death that the pathogen has not evolved to preserve the host's life.

The severity of infection and the parasite's potential to cause severe disease are influenced by several inherent factors. These include:
(i) The infectious dose necessary for host infection. Studies in rodent malaria, for example, have shown that higher infectious doses can lead to severe malaria forms and death [79], potentially due to rapid attainment of high pathogen burdens triggering host overreaction, akin to sepsis.
(ii) The parasite's replication niche and load, varying with each specie's pathogenic potential [80]. For instance, *Pf*'s propensity to cause severe disease is higher than other species as it infects RBCs of all ages, unlike *Pv* which targets reticulocytes [81][82].
(iii) The presence of virulence factors that enhance replication and can result in severe outcomes. *Pf*, for example, exhibits unique adhesive features like cytoadherence and rosetting, contributing to its virulence [83][84].

Understanding these factors is crucial in comprehending the diverse clinical presentations of malaria and its potential severity.

1.5.1. Parasite load in the host

The concept of pathogen load, or the quantity of pathogens within a host's body, is a critical factor in determining the outcome of infectious diseases, as depicted in Table 3 [85]. This relationship can be attributed to the fact that a high burden of pathogens can result in organ damage, leading to more severe manifestations of the disease. For instance, in sepsis patients, mortality is often linked to an uncontrolled hyperinflammatory response of the host. This inflammatory state is believed to be influenced by the bacterial load and the interaction of microbial virulence factors with the immune system [86].
Moreover, the balance between the host's immune response and the pathogen's replication ability is pivotal in dictating the severity of the disease. In some cases, excessive pathogen replication, coupled with the host's failure to control the infection, can lead to fatal outcomes [87]. This dynamic can be summarized with the already mentioned straightforward equation: (↑↑↑parasites burden = ↑↑↑severe disease).

This principle not only highlights the importance of understanding pathogen dynamics within the host but also underscores the need for effective measures to control and reduce pathogen load as a means of mitigating disease severity.

Table 3 Pathogens load and disease outcomes

Pathogen	Disease	Pathogen load* related to disease outcome
Neisseria meningitidis	Meningitidis	Severity: Patients with severe disease had significantly higher pathogen loads compared to those with mild disease. Mortality: Patients who died had a significantly higher bacterial load compared to those who did survive.
Streptococcus pneumoniae	Pneumonia/ Invasive disease	Severity: Patients with severe outcomes had significantly higher bacterial loads compared to patients with uncomplicated outcomes. Mortality: The median bacterial load was significantly higher in non-survivors compared to survivors.
SARS-CoV-2	COVID-19	Mortality: Patients who died had significantly higher viral loads compared to patients who survived, in both nasopharyngeal swabs and plasma.
HIV-1	AIDS	Severity: - Patients with lower viral load presented lower likelihood of progressing to AIDS comparing with patients with high viral loads. - Infants with higher viral loads experienced a more rapid progression to the disease compared to infants with lower viral loads.
Leishmania infantum	Kala-azar	Patients who died exhibited significantly higher parasite loads in both bone marrow and blood samples.
Trypanosoma cruzi	Chagas	Both *T. cruzi* median DNA concentrations and parasitaemias were higher in seropositive Chagas patients with cardiomyopathy compared to those who did not develop cardiomyopathy.

* pathogen load determined by quantitative molecular methods. Based on references [88][89][90][91][92][93][94][95][96][97][98].

The development of severe malaria is closely associated with high parasite loads [99]. Such elevated burdens can lead to pathophysiological changes, including increased inflammation and vascular and endothelial dysfunction, contributing to the severity of the disease [100]. Although the disease is active during the asexual phase, making parasite load measurement seem straightforward [101], the microscopic examination of peripheral blood smears often fails to represent the total parasite burden. This is mainly due to the sequestration of *Pf* parasites in the capillaries, leading to a frequent underestimation of the actual total body parasite burden [102].

In benign forms of malaria, where sequestration does not occur, estimating the total number of parasites in the body is straightforward by multiplying the observed parasitaemia by the estimated blood volume. However, *Pf* infections present a unique challenge. Microscopic examination reveals only the initial stages of the asexual life cycle, with most parasitized RBCs becoming sequestered in the capillaries and venules during later stages [69][70]. This leads

to significant underestimations of the total parasite burden based on peripheral blood samples alone, causing confusion among clinicians. For instance, it is possible for two patients to present with identical levels of parasitaemia yet have vastly different total body parasite loads due to the extent of sequestration (Figure 3). This discrepancy underscores the complexity of assessing *Pf* malaria severity from peripheral parasitaemia alone due to the hidden burden of sequestered parasites [103].

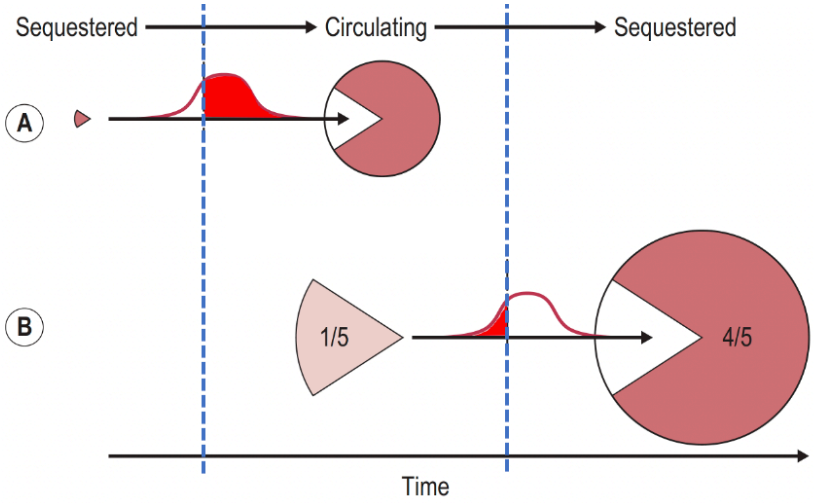


Figure 3 Difference between parasitaemia and total parasite burden
Comparison of Parasite Burden in two Patients. Patient A shows most parasites circulating (red shaded area), with only some pending schizont rupture (white shaded area). In Patient B, most parasites have already sequestered, leaving only 20% circulating (red shaded area) parasite count: >60 times higher than in Patient A. The key difference is in the maturity of circulating parasites, which are more advanced in Patient B, as indicated by the hatched lines. Adapted from [103].

Notably, the release of *Pf*HRP2 during parasite multiplication is strongly linked to disease severity (refer to Figure 4) [104]. Elevated *Pf*HRP2 plasma levels suggest a higher risk of developing severe malaria [105][106]. This phenomenon is not limited to *Pf*. In *Pv* infections, parasitaemia might also underestimate the total parasite burden due to endothelial adhesion phenomena, even though the total infected RBC count is restricted by the specific RBC type (reticulocytes) that *Pv* can infect [107]. In such scenarios, the plasma concentration of parasite lactate dehydrogenase (LDH) can act as an indicator of the total parasite load (as *Pv* does not produce HRP2) and is related to disease severity [102]. These findings further emphasize the relationship: (↑↑↑parasite burden = ↑↑↑severe disease).

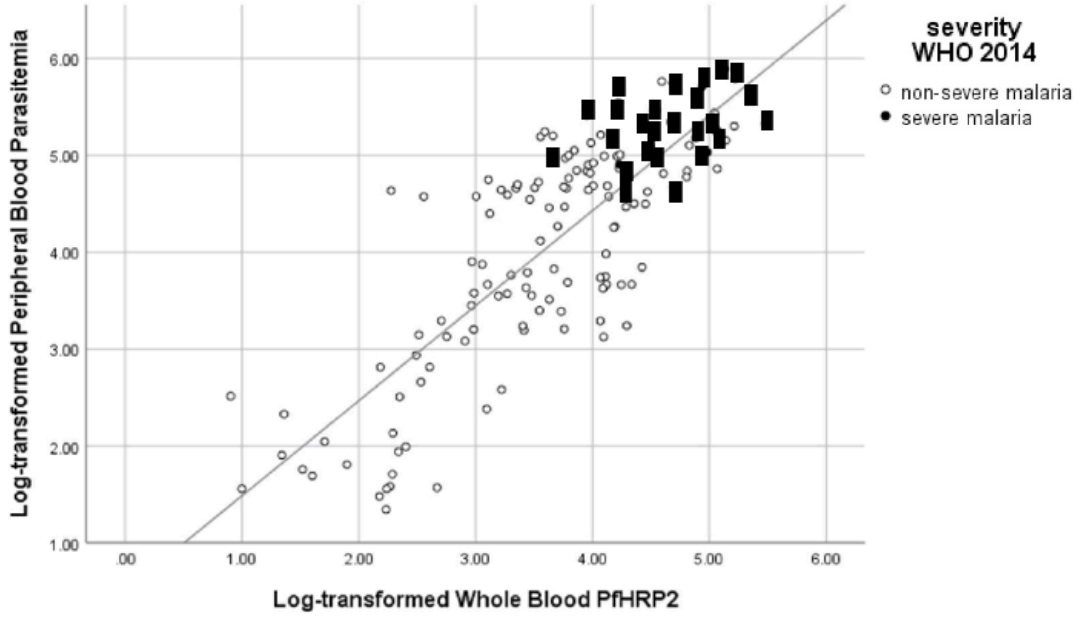


Figure 4 Correlation between parasitaemia, HRP2 and disease severity

A positive correlation between HRP2 levels and peripheral blood parasitaemia is evident, with non-severe cases having lower HRP2 (white dots, low parasitaemias) compared to severe cases (black squares, high parasitaemias). Adapted from [108]. HRP2: histidine-rich-protein 2.

The concept of tailoring treatment to disease severity is universally applicable across infectious diseases, notably recognized in managing sepsis. The precision in selecting, dosing, and timing antimicrobial therapy is pivotal in treating severe sepsis and septic shock, significantly influencing survival rates. This strategic approach is essential for enhancing pathogen clearance while addressing antimicrobial resistance, thereby improving patient outcomes [109].

Similarly, in malaria, prompt and appropriate treatment is vital to curb pathogen replication and reduce high parasite loads [110]. Timely administration of antimalarials can avert severe disease progression and potentially save lives [111]. Effective antimalarial therapy leads to a swift reduction in parasite burden, manifesting as an rapid improvement in patient health [112]. Most antimalarials, especially those from the quinoline group, act primarily on specific life cycle forms, such as mature trophozoites [113]. In contrast, artemisinin is notable for its broad-spectrum activity, targeting more life cycle stages, including ring-stage parasites, thus facilitating rapid parasite clearance from the bloodstream [114][115]. With artemisinin, the parasite's elimination from the bloodstream follows a first-order process, disrupting the development of stages critical for cytoadherence and the progression to severe disease (see Figure 5) [115][116][117].

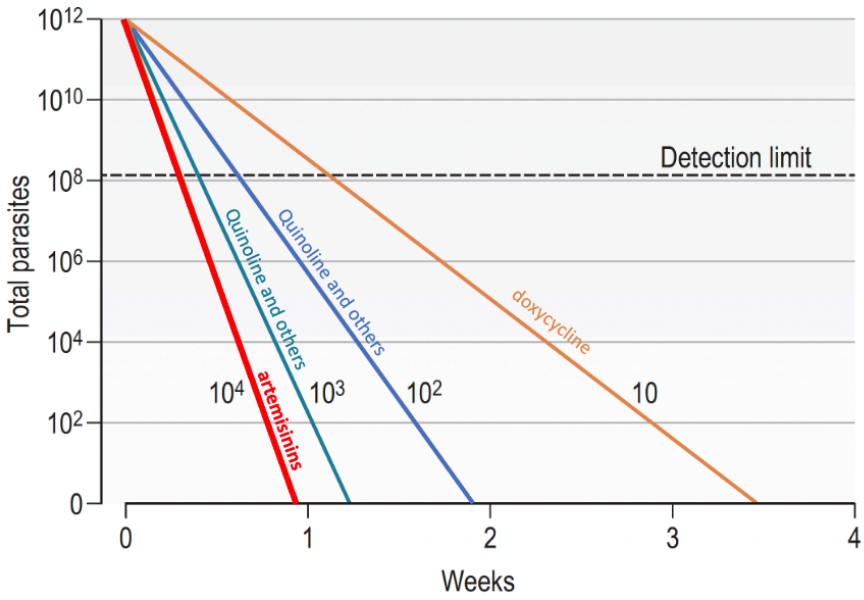


Figure 5 Parasite Reduction Rates - different antimalarial drugs
Parasite reduction rates (PRR) by different antimalarial drugs. Note that artemisinins have a PRR of 10^4 per sexual cycle, as compared of most other drugs. Doxycycline's low PRR of only 10 explains why it is not used as single drug treatment. Adapted from [103].

Consequently, the administration of intravenous artemisinin is particularly beneficial, leading to a faster reduction in parasite load compared to other treatments, such as intravenous quinine [118][119][120]. This efficacy is believed to be the primary reason why intravenous artesunate significantly outperforms traditional intravenous quinine in treating severe *falciparum* malaria, notably lowering mortality rates [121].
This underscores the critical role of antimalarial therapy in reducing parasite burden and, consequently, mitigating disease severity which could be expressed as: (↓↓↓parasite burden = ↓↓↓severe disease).

1.6. Host related factors and disease severity

Host factors, resulting from the co-evolution between the malaria parasite and humans, lead to complex molecular interactions between host and parasite. This evolutionary pressure has shaped diverse disease outcomes [122]. Factors such as gender, age, ethnicity, chronic conditions, and co-infections play significant roles in influencing disease severity [123]. Additionally, the interplay of environmental elements, genetic predispositions, and nutritional status of the host also significantly impacts disease outcomes [124].

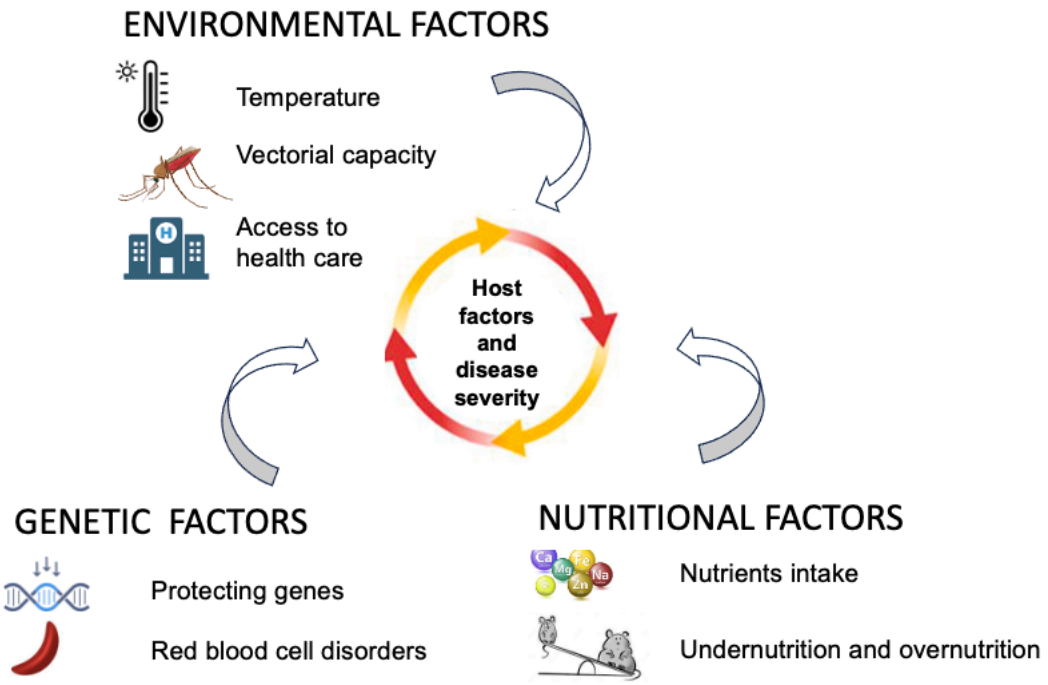


Figure 6 Three host-related factors and disease severity

Environmental influences, including temperature and vectorial capacity, along with the availability of healthcare infrastructure and effective antimalarial treatments, are key determinants of malaria's impact on humans [125]. Specifically, temperature influences malaria incidence rates; areas with warmer climates and higher humidity see elevated rates due to optimal conditions for vector development [126]. Furthermore, in regions where access to healthcare and effective treatments is limited, there is an increased risk of severe disease outcomes and higher mortality rates associated with malaria.

Genetic factors significantly influence individual susceptibility or resistance to infections, including malaria [127]. Genetic protection may be conferred by the absence of host receptors that bind to pathogen ligands [128]. For example, the chemokine receptor 5 (CCR5) is a co-receptor for HIV-1 entry into CD4 cells. Individuals with the CCR5-Δ32 deletion mutation exhibit resistance to HIV-1, resulting in a reduced viral load and often leading to asymptomatic or mild infection outcomes [129]. Similarly, genetic adaptations provide protection against highly virulent pathogens, a phenomenon seen in populations frequently exposed to such pathogens [130]. Approximately 25% of the risk of developing severe malaria is attributed to genetic factors [131].

Various genetic conditions have been linked to altered susceptibility or resistance to malaria. For instance, genetic variations affecting the glucose-6-phosphate dehydrogenase (G6PD) enzyme have been associated with malaria resistance

[132]. The prevalence of genetic traits conferring protection against severe malaria is notably higher in African regions endemic to the disease, reflecting an evolutionary adaptation to constant exposure to the malaria pathogen. This exemplifies the concept of "survival of the fittest" [132]. Among these genetic traits, the sickle cell anaemia trait stands out for providing substantial protection against severe malaria and related mortality, especially prevalent in African regions where malaria is endemic [133].

Nutritional status, spanning from undernutrition to overnutrition, plays a significant role in the pathogenesis of malaria.

Undernutrition, characterized by insufficient intake of essential nutrients like iron, vitamin A, and zinc, can weaken immune function, elevating the risk of severe malaria [134][135]. A study in Ghana's western region highlighted malnutrition's contribution to increased malaria-related morbidity and mortality. There's debate on malnutrition's role in malaria susceptibility; some view protein-energy malnutrition as predisposing individuals to infection and severe disease, while others believe it may offer some protection against *Plasmodium* infection. On one side, malnutrition may lead to severe disease by compromising the host's immune defence due to nutrient deficiencies [136]. Conversely, the parasite's growth and multiplication rely on host nutrients, suggesting a potential struggle for survival in malnourished hosts [136]. *Pf*, in particular, is believed to detect specific nutritional cues from the host, affecting its proliferation, antigenic variation, and transmission capabilities [137].

Research showed that caloric restriction in mice led to reduced body weight, glucose, and insulin levels, correlating with overall health improvement. When caloric-restricted mice were infected with *Plasmodium berghei*, a marked reduction in parasitaemia and lower parasite loads were observed compared to mice which were fed *ad libitum* [136]. This underscores the principle that lower pathogen levels result in milder disease manifestations.

This intricate relationship between nutritional status and malaria underscores the need for a holistic approach to prevention and treatment strategies, considering the nutritional well-being of individuals at risk or suffering from malaria.

Overnutrition and the rise in obesity rates, notably in the sub-Saharan African region, introduce significant health challenges, including an increased susceptibility to infectious diseases like malaria [138][139][140]. A case-control study in Ghana involving 1.466 adults revealed that those with type 2 diabetes faced a 46% heightened risk of *Pf* infection [141]. Furthermore, obesity and type 2 diabetes are not only predisposing factors for contracting *falciparum* malaria but also for the progression to its severe forms [141].

Supporting this, a Swedish study found that patients with severe malaria had a higher median body mass index (BMI) (29.3) compared to those with uncomplicated malaria (24.7) [142]. In this study, hyperparasitaemia (>5%) was more frequently observed in diabetic patients, indicating a strong link between diabetes and severe malaria outcomes. The increased disease severity in obese individuals may be due to an immunosuppressive state that hinders effective control of parasite replication [143]. Moreover, obesity is often accompanied by a chronic, low-grade inflammatory state, which, alongside other comorbidities, may exacerbate infection severity and contribute to the development of severe clinical syndromes [143]. This complex interplay highlights the critical need to address overnutrition and its associated health risks as part of comprehensive malaria management and prevention strategies.

1.6.1. Immune system

The primary role of the immune system is to protect the host from infections [144]. Initial defence is provided by the innate immune system, which not only activates immediate protective mechanisms but also facilitates the development of adaptive immunity aimed at controlling parasite replication to prevent severe disease [145].

In malaria, immunity involves antibodies that mediate the neutralization and opsonization of merozoites, reducing parasite load [146]. These malaria-specific antibodies also play crucial roles in inhibiting cytoadherence, blocking RBC invasion, and facilitating antibody-dependent cellular cytotoxicity and inhibition [147]. Constant exposure to the parasite in endemic regions leads to frequent re-infections [148], challenged by the polymorphisms and clonal antigenic variations of malaria parasite surface antigens [149]. This variability hinders the formation of sterilizing antibodies, maintaining a susceptibility to recurrent low-density parasitaemias [150].

Despite these challenges, repeated infections enable individuals to develop mechanisms to both limit the inflammatory responses responsible for acute symptoms (clinical immunity) and directly kill parasites (antiparasitic immunity). This is achieved through a repertoire of effective antiparasitic antibodies, resulting in lower parasite loads and milder disease manifestations, as depicted in Figure 7 [151][152]. The durability of this so-called “long-term immunity” is debated, with some suggesting it is transient and diminishes quickly upon leaving high-transmission areas, while others argue it can persist for years [151][152].

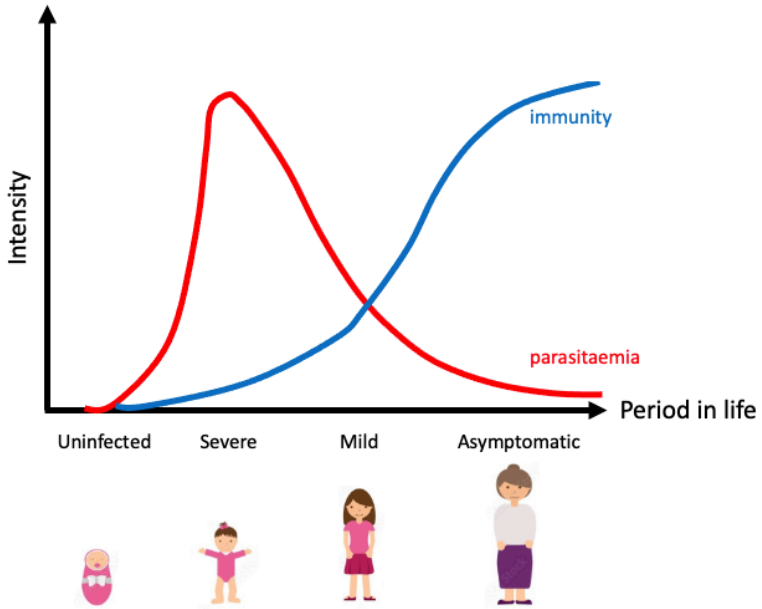


Figure 7 Malaria Outcomes: Immunity *versus* Parasite Load

Figure illustrates the dynamic between immunity (blue line) and parasite load (red line) in endemic areas. Initially, unexposed individuals have high parasitaemia and severe disease. Over time, with repeated exposure, immunity strengthens, reducing parasitaemia and severity, leading to asymptomatic outcomes in adults. Adapted from [152].

In Sub-Saharan Africa, children under five are particularly vulnerable to severe malaria, largely due to an underdeveloped immune response [155]. Conversely, in adults with established protective immunity, an excessive inflammatory response, or cytokine storm, can occur due to an unchecked reaction to the parasite [156]. This phenomenon is notably implicated in cerebral malaria, believed to be caused by both the sequestration of parasites in the brain's capillaries and an overactive immune response leading to excessive inflammatory cytokine production [157]. Clinical studies across diverse populations have shown a link between elevated tumour necrosis factor (TNF) levels and severe malaria manifestations, such as coma [158]. The increase in TNF can diminish brain oxygenation, contributing to cerebral coma [159]. In these instances, a lack of proper immune cell regulation leads to an overproduction of inflammatory proteins, potentially resulting in organ failure and death [160]. This dual nature of the immune response underscores the complexity of managing malaria, highlighting the need for treatments that can mitigate excessive inflammation while bolstering protective immunity.

Mild malaria cases often result from an immune-mediated inflammatory response, where fever and cytokine production help manage the infection, rather than from acquired immunity [161]. Severe malaria is notably associated with

insufficient immune defence, particularly affecting children under five, pregnant women, and travellers [162][163][164].

i) Children aged six months to five years are highly susceptible to severe malaria as they transition from maternal immunity without having developed their own specific immunity. Their relatively inexperienced immune systems lack the protective mechanisms to effectively counteract infection, leading to a heightened risk of acquiring clinical malaria and suffering severe disease outcomes due to elevated parasite burdens [165].

ii) Pregnancy brings about immunological adjustments that diminish a woman's resistance to malaria, making severe manifestations, including severe anaemia and cerebral malaria, more common. The mortality rate for cerebral malaria in pregnant women can be as high as 50%, underscoring the critical need for targeted care and treatment in this demographic [166].

iii) For travellers, malaria is a common acute and fatal imported tropical disease. The absence of immunity is a key risk factor [167]. Non-immune travellers with *falciparum* malaria face a case fatality rate of 0.6 – 3.8%, with severe cases constituting about 20% of incidents [168]. Travellers visiting friends and relatives (VFR) in their countries of origin have a lower risk of malaria complications (3.7%) and death (1.2%) compared to non-immune travellers, with semi-immune VFR travellers showing lower parasitaemias, faster parasite clearance, and shorter fever durations than their non-immune counterparts [169][170][171]. This advantage in individuals from endemic regions suggests a form of "long-term" semi-immunity acquired from extended exposure to malaria in areas of stable transmission [172].

The immune response plays a pivotal role in combating infections, where maintaining a balanced inflammatory reaction is essential for reducing the pathogen load and ensuring organ homeostasis [173]. Disruption in the equilibrium between the host's immune defences and the parasite's evasion strategies can lead to dire consequences. Factors contributing to this imbalance include immune system failure, which may allow the pathogen to multiply uncontrollably, leading to severe disease manifestations [174].

Thus, an effective immune response that successfully lowers the parasite burden is key to improving disease outcomes, reinforcing the principle that: (↓↓↓parasite burden = ↓↓↓severe disease).

1.6.2. The red blood cell – overview of structure and physiology

The human body contains an estimated 20-30 trillion (20-30 x 10^{13}) RBCs, the most numerous cell type [175]. During erythropoiesis, the process of RBC

synthesis, these cells undergo significant morphological changes, shedding most typical intracellular organelles such as nuclei, ribosomes, and mitochondria [176]. This adaptation allows RBCs to primarily store haemoglobin, the protein essential for oxygen delivery to tissues [177]. Mature RBCs are characterized by their high haemoglobin content (22-32 pg/cell), a flexible sub-membrane cytoskeleton, and the capacity to regulate their osmotic environment, crucial for efficient solute and ion transport [178]. These attributes underscore the RBC's fundamental role in oxygen transportation across the capillary networks [178].

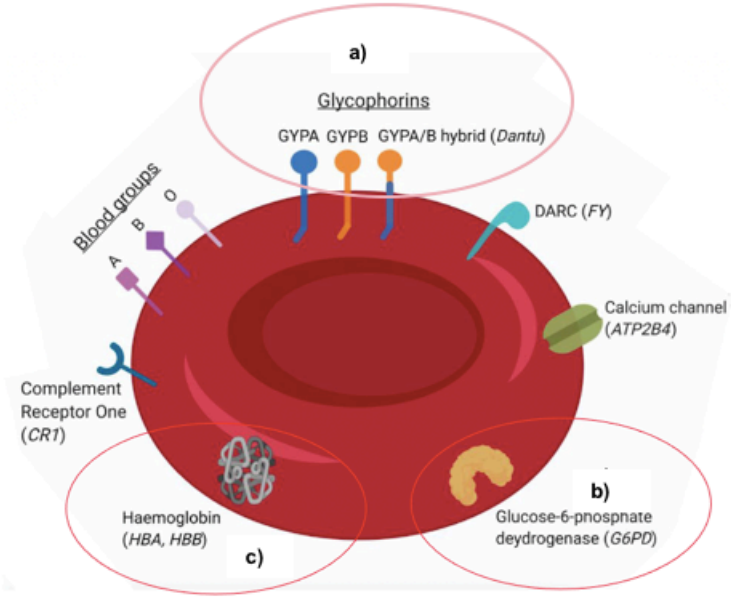


Figure 8 RBC Compartments Influencing Parasite Replication
Figure illustrates a red blood cell (RBC) and highlights three key compartments critical to parasite replication: a) the cell membrane and its associated receptors, b) metabolic pathways, with a focus on specific enzymes, and c) various types and variants of haemoglobin.

RBCs are central to the life cycle of the malaria parasite, providing both nourishment and a protective environment. The interaction between the parasite and RBCs, especially the adhesion properties of the parasite and the protective effects of RBC polymorphisms, plays a significant role in the virulence of malaria and the protection against severe manifestations of the disease (Figure 8) [179].

The RBC membrane is a critical component, showcasing several key features [180]:
i) Its biconcave shape enhances deformability, allowing RBCs to navigate through the narrowest blood capillaries and facilitating gas exchange;
ii) Comprised of a lipid bilayer of phospholipids and cholesterol, it includes transmembrane proteins essential for adhesion and structural integrity;

iii) Membrane proteins like the sodium-hydrogen antiporter 1 (NHE1), glycophorins A and C (GPA/C), band 3, Duffy antigen/chemokine receptor (DARC), and rhesus protein (Rh) are vital for interactions with various cell types;
iv) Anion exchanger proteins are predominant in the RBC membrane, critical for maintaining physiological function and homeostasis;
v) The absence of a nucleus and intracellular organelles maximizes space for haemoglobin storage;
vi) Selective permeability facilitates the passage of oxygen and carbon dioxide while restricting ions and other molecules;
vii) The glycocalyx, comprising glycoproteins and glycolipids on the RBC membrane, is essential for cell recognition and determining blood type.

Regarding energy metabolism, the primary role of RBCs is to transport oxygen to tissues and remove carbon dioxide. Given this function, RBCs do not engage in complex energy-generating processes typical of other cells. Throughout their lifespan, RBCs require energy for critical operations, including maintaining the electrolyte gradient, keeping haemoglobin iron in its functional ferrous state, and protecting metabolic enzymes, haemoglobin, and membrane proteins from oxidative damage [181].
RBCs depend on glucose as their sole energy source, metabolizing it anaerobically. Lacking mitochondria, RBCs are incapable of energy production through the Krebs cycle, unlike other body cells. Energy is generated primarily through the Embden-Meyerhof pathway, which anaerobically catabolizes glucose to pyruvate or lactate, and through glycolysis, which breaks down glucose into adenosine triphosphate (ATP) anaerobically [181]. Interestingly, although RBCs circulate in an oxygen-rich bloodstream, they function anaerobically and do not utilize oxygen for glycolysis. This unique metabolic feature ensures their efficiency in oxygen transport and carbon dioxide removal without consuming the oxygen they carry. Approximately 90% of glucose metabolism in RBCs occurs through these pathways, highlighting their specialized adaptation to fulfil their oxygen-transporting role effectively [181].
Haemoglobin (Hb) is an iron-containing protein crucial for oxygen transport in mammals [182]. RBCs are highly specialized for their oxygen-transporting role, containing large amounts of haemoglobin within their cytosol, the reasons RBCs shed their nuclei and other organelles [183]. In its operational state, iron is part of heme, a complex molecule with an iron ion at its core. A haemoglobin molecule comprises four subunits: two alpha and two beta chains, each equipped with a heme group. This structure enables an iron ion in each heme group to bind oxygen reversibly, facilitating efficient oxygen transport from the lungs to tissues

and carbon dioxide transport back to the lungs [184]. This reversible oxygen binding allows haemoglobin to change its oxidation state, optimizing oxygen delivery and CO_2 removal [185].

1.6.2.1. RBC membrane, parasite interaction and disease

The interaction between RBCs and the malaria parasite is intricate, involving a series of steps that induce significant changes in the RBC. These include the loss of the characteristic discoid shape, increased membrane rigidity, and enhanced permeability to ions and endothelial surfaces, all critical for the parasite's engagement, infiltration, growth, and eventual egress from the RBC, as depicted in Figure 9 [186].

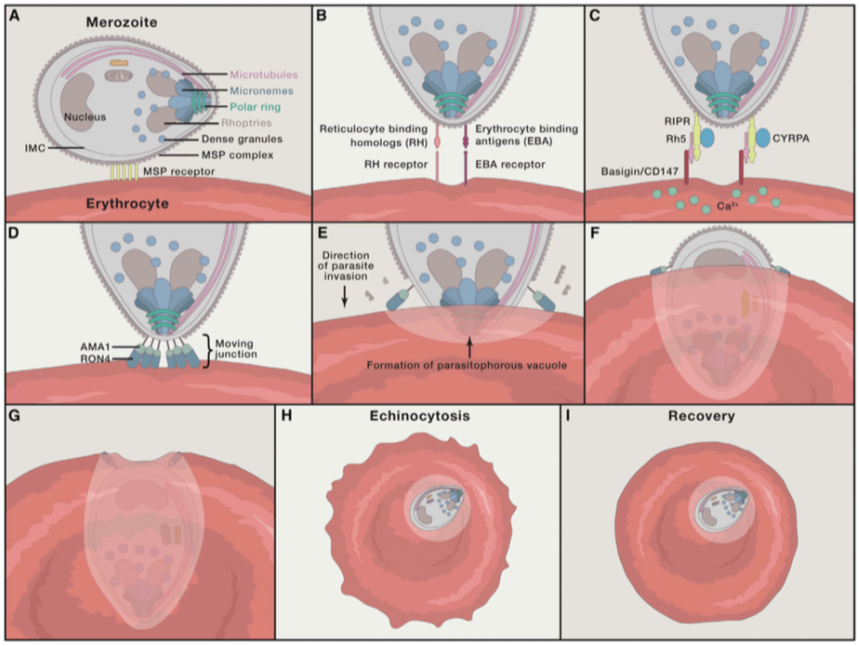


Figure 9 Red Blood Cell Invasion by Merozoites

The invasion process begins with Merozoite Surface Proteins (MSPs) facilitating initial contact with the red blood cell (RBC) (A). Merozoites then reorient their apical ends towards the RBC, with attachment mediated by specific ligands and receptors (B). Apical organelles, micronemes and rhoptries, release proteins, including proteases and phospholipases, to prepare the RBC membrane for invasion by softening and invaginating at the attachment point (B). This process, aided by membrane skeleton phosphorylation, reduces membrane rigidity, ensuring a stable junction for the merozoite's attachment against blood flow (B). Concurrently, parasite calcineurin, reacting to Ca2+ flux, strengthens the adhesion during contact (C). A moving junction forms, allowing the merozoite to enter the RBC, encased within the parasitophorous vacuole membrane (PMV) (D-E), followed by echinocytosis, where the RBC loses water (H). The merozoite will then stay enclosed in the PMV (F-G). The cell eventually regains homeostasis (I). Adapted from [187].

These morphological and kinetic alterations facilitate the parasite's invasion and survival, ultimately contributing to the virulence of the disease and the severity of malaria syndromes [188].

More than 400 parasite-encoded proteins are thought to be exported to the RBC cytoplasm, which means an impact in the RBC membrane and its interaction with

the parasite development [189]. Besides enabling invasion, this modifications also enable the parasite to shield itself against the immune system and consequently replicate within the RBC [190].

Pf merozoites are equipped with two significant secretory organelles at their apical end: micronemes and rhoptries, housing numerous proteins essential for invasion[191].Proteins from the erythrocyte binding-like (EBL) family and reticulocyte binding protein homologues (*Pf*Rh) are secreted from these organelles to bind specific receptors on the RBC surface, as detailed in Table 4 [192]. Crucially, following apical reorientation, the *Pf* ligand, *Pf*RH5, attaches to its corresponding receptor, basigin, on the RBC membrane [191]. This interaction initiates pore formation in the RBC membrane, essential for rhoptry release, moving junction formation, and subsequent parasite entry [191].

Table 4 RBC ligands / receptors involved parasite invasion

Parasite Ligand	RBC Receptor	Function
*Pf*AMA-1	RON2	Binds to RON2 at the tight junction, crucial for signalling to activate subsequent steps
EBA-175	Glycophorin A	Implicated in signalling the release of rhoptries, signalling to RBC making host membrane more deformable
EBA-181/JESBL	Trypsin-resistant erythrocytes receptor W	-
EBA-140/BAEBL	Glycophorin C	Implicated in signalling the release of rhoptries, signalling to RBC making host membrane more deformable
EBL1	Glycophorin B	Pseudogene in most of *Pf* parasites
*Pf*Ripr	Complex *Pf*Rh5/CyRPA at interface merozoite/RBC	Formation of pore with host cell
*Pf*Rh1	unknown receptor Y	Implicated in signalling for release of rhoptries; implicated in signalling to erythrocyte to activate a phosphorylation cascade making host membrane more deformable
*Pf*Rh2a	No demonstrated receptor	No demonstrated function
*Pf*Rh2b	Unknown receptor Z	implicated in signalling for release of rhoptries; implicated in signalling to erythrocyte to activate a phosphorylation cascade making host membrane more deformable
*Pf*Rh3	Probable transcribed pseudogene	-
*Pf*Rh4	Complement receptor 1 (CR1, CD35)	Implicated in signalling for release of rhoptries; implicated in signalling to erythrocyte to activate a phosphorylation cascade making host membrane more deformable
*Pf*Rh5	BASIGIN: RBC surface CyRPA: tripartite complex with *Pf*Ripr P113: membrane	Host tropism determinant and blood stage parasite´s growth

Adapted from [192].

Ligand-receptor interactions are key to parasite invasion into RBCs, initiating the intra-erythrocytic cycle [193]. EBL and Rh ligands, despite being functionally redundant, are crucial for the merozoite's apical reorientation and host cell deformation upon attachment, allowing parasites to invade RBCs through various ligands and receptors [194]. However, receptor polymorphisms can impair invasion. For example, polymorphisms in glycophorins (A, B, C) are linked to reduced parasite invasion due to altered RBC receptor interactions [186]. Complement Receptor 1 (CR-1) polymorphisms are associated with protection against severe malaria, particularly in African endemic regions [195]. CR-1 deficient RBCs show decreased rosette formation, leading to less microvascular obstruction and protection against severe disease [196]. Yet, a study in Thai adults found low CR1 expression linked to severe malaria, as CR-1 activates the complement immune response against parasites; its deficiency allows higher pathogen burdens and severe disease [197]. These discrepancies could stem from varying epidemiological patterns or interactions between CR-1 and other genetic factors.

Despite polymorphisms offering some protection against invasion, parasites can still enter RBCs due to: a) the plasticity of invasion phenotypes, adapting to acquired immunity against specific merozoite ligands, or b) the variability and abundance of RBC receptors, which allow alternative invasion pathways [197]. Host cell specificity remains incompletely understood, as parasites can utilize alternate pathways with different receptors for invasion. For instance, enzymatic treatment of RBCs, which removes receptor components like sialic acid from GPA, GPC and CR-1 (crucial for the sialic-acid invasion pathway), mimics the effects of genetic polymorphisms, yet invasion still occurs [197]. This underscores the complex interplay between parasite invasion strategies and host cellular defences.

Studies examining the relationship between the AB0 blood group system and malaria outcomes offer mixed findings, with some studies finding no correlation between *falciparum* infection and AB0 antigens [198][199], while others suggest that blood group A may be more susceptible to severe infections, with type 0 individuals showing protection through lower parasitaemias and milder disease manifestations [200]. *In-vitro* evidence also points to a preference of parasites for type 0 RBCs [200]. This complex interplay between AB0 blood groups and malaria susceptibility is further underscored by historical and geographical analyses suggesting that the AB0 blood group distribution, especially the prevalence of group 0, may have been shaped under the selective pressure of *Pf* malaria [201]. The geographic distribution of AB0 blood groups correlates with historical malaria

prevalence, indicating a survival advantage for individuals with group 0 blood in malaria-endemic areas. These findings collectively suggest that *Pf* infection has influenced the evolutionary distribution of AB0 blood groups, aligning with the hypothesis that group 0 provides a protective advantage in regions where malaria is or was endemic (Figure 10) [198][199][200][201].

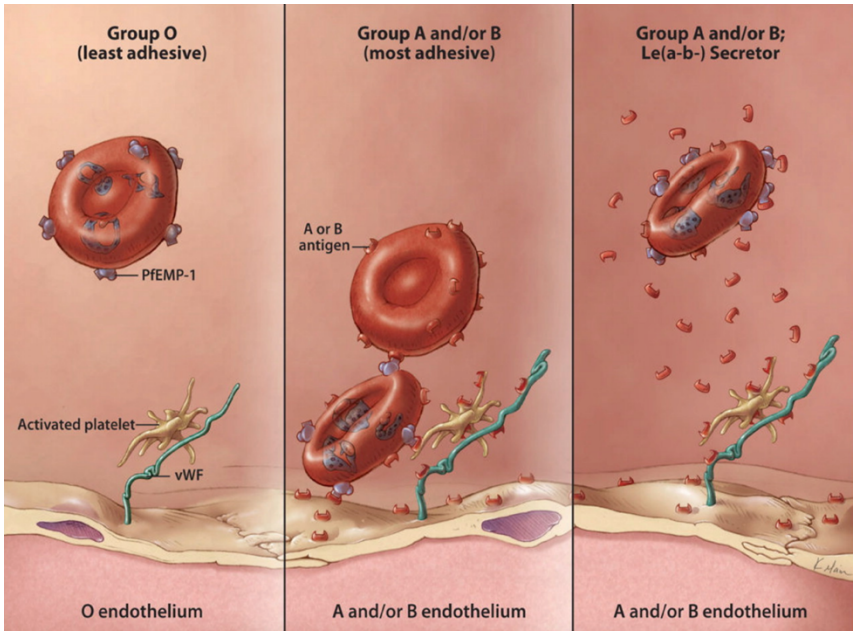


Figure 10 Blood groups and susceptibility to malaria.
Adapted from [201].

A and B antigens, which are targets for rosetting, are hypothesized to contribute to severe malaria outcomes in AA, BB, and AB phenotypes due to larger rosettes and increased microvascular obstructions compared to A0, B0, and 00 phenotypes, which seem to be protected against severe forms of the disease [202]. However, Theron *et al.*'s *in-vitro* study showed a *Pf* laboratory strain preference for type 0 RBCs over type A, suggesting differences in parasite behaviour between *in-vitro* and *in-vivo* conditions, particularly regarding virulent adhesion features like rosetting and cytoadherence [200]. Notably, rosetting facilitates parasite replication by binding infected RBCs to uninfected ones, while cytoadherence can obstruct blood flow, especially in cerebral capillaries, leading to severe malaria. Individuals with A or B blood groups, which express antigens that serve as receptors for *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) on the surface of infected red blood cells (iRBC), are more prone to these adhesive interactions and subsequent severe syndromes.

The GYPA and GYPB genes, responsible for encoding MNS blood group antigens GPA and glycophorin B (GPB) [203], play roles in RBC merozoite invasion [204]. Dantu polymorphisms, involving structural rearrangements in the glycophorin gene cluster, result in RBCs with increased cell tension and resistance to merozoite invasion, leading to lower pathogen burdens. Homozygous Dantu individuals exhibit a 74% protection against severe malaria [204].

The Knops blood group system is also implicated in malaria pathogenesis, with antigens like McCoy (McC) and Swain-Langley (Sla) located on the CR-1 receptor of RBC membranes [205]. CR1 is involved in the rosetting process by binding to *Pf*EMP1 on infected RBCs [206]. The common African CR-1 polymorphism (Sl/a) shows reduced *Pf*EMP1 adhesion, leading to decreased rosette formation and protection against severe malaria [207]. The higher frequencies of McC(b+) and Sl(a−) phenotypes in African populations compared to Europeans suggest an evolutionary adaptation to malaria [208].

Defects in RBC membrane structure, such as those seen in hereditary red blood cell membrane disorders like spherocytosis, elliptocytosis, and ovalocytosis, can significantly impact the interaction between the RBC and the malaria parasite. These conditions alter the membrane's structural organization, leading to the premature removal of these RBCs by the spleen [209]. Such disorders contribute to lower parasitaemias due to reduced expression of RBC surface antigens, providing resistance to merozoite invasion and, consequently, to the establishment of infection [209]. Thus, alterations in RBC structure represent a significant challenge for parasite invasion. Even when invasion occurs, affected RBCs are removed from circulation prematurely, preventing the accumulation of high parasite burdens within the host. This mechanism underscores the complex interplay between host genetic factors and malaria pathogenesis, highlighting the role of RBC membrane integrity in mediating resistance to *Pf* malaria [210].

1.6.2.2. RBC metabolism and the parasite

Malaria infection leads to significant changes in host RBCs, including the formation of new permeation pathways (NPPs) in iRBCs, enhancing nutrient uptake and waste expulsion, essential for the parasite's growth [211][212]. The parasite depends on host glucose, facilitated by the glucose transporter 1 (GLUT-1) at the RBC surface, part of the NPPs, for energy, which also facilitates the

transport of ions, isoleucine, glutamine, monosaccharides, peptides, nucleosides, and pantothenate[213][214]. Lacking energy reserves and the Krebs cycle, the parasite taps into the host glycolysis and uses anaerobic fermentation of pyruvate to lactate through the Embden-Meyerhof pathway, significantly increasing glucose consumption and lactate production. This metabolic shift can lead to hypoglycaemia and lactic acidosis in severe malaria, highlighting glucose's central role in parasite energy production and disease severity [215][216].
Lipid metabolism is essential for *Plasmodium* during the intra-erythrocytic cycle, as the parasite relies on exogenous fatty acids and cholesterol for growth, unable to synthesize these compounds *de novo* [217]. The invasion of iRBCs significantly boosts lipid metabolism, especially for membrane biosynthesis. *Plasmodium* utilizes both its metabolites (glycerides and phosphoglycerides) and lipids from human serum and RBC membranes [218]. Clinical evidence shows malaria infection alters the host's lipid profile, reducing serum high-density cholesterol (HDL-c) [219]. A study by Faucher *et al.*, found an increase in total and high-density lipoprotein cholesterol levels post-parasite clearance in patients with initial parasitaemia below 1000/µL [220].

Enzymatic activities in RBC, such as calcium signalling via transporters like ATPase Plasma Membrane Ca2+ Transporting 4 (ATP2B4), are crucial for *Plasmodium*'s life cycle, influencing development, fertilization, locomotion, and host cell infection [221]. Variants in ATP2B4 can disrupt calcium balance, impacting the parasite's development within RBCs. Polymorphisms in ATP2B4, particularly prevalent in African regions with high malaria rates, have been linked to protection against severe malaria [222]. While the protective mechanism of ATP2B4 polymorphisms is not fully understood, they are believed to reduce parasitaemia and lead to milder disease outcomes.

Red blood cell enzymopathies like G6PD and Pyruvate kinase (PK) deficiencies impact RBC integrity, reduce cellular lifespan, and can cause haemolytic anaemia, affecting *Pf* survival [223][224].
G6PD deficiency, the most widespread enzymopathy globally, especially in tropical and subtropical regions, is linked to a reduced risk of severe malaria [225]. This enzyme guards RBCs against oxidative damage; its deficiency can trigger haemolysis under stress conditions, such as infection or certain food and drug consumption. During malaria, oxidative stress induces haemolysis, lowering iRBCs and parasite load, leading to milder disease in G6PD-deficient individuals [226]. Yet, the protective mechanism against severe malaria remains unclear,

partly because G6PD deficiency manifests variably across genders and populations due to its sex-linked nature [227].

PK deficiency ranks as the second most common enzymopathy and is associated with non-spherocytic haemolytic anaemia. Mutations in the gene responsible for PK deficiency (PKLR) have been reported to present a higher frequency in those of African descent compared to those of Caucasian descent, suggesting an evolutionary adaptation [228]. PK plays a vital role in ATP production during glycolysis within the Embden-Meyerhof pathway, crucial for energy supply in RBCs (Figure 11) [229]. PK deficiencies lead to altered RBC metabolism, manifesting as anaemia and an impaired energy profile, which also contributes to decreased malaria susceptibility. Deficient RBCs lack the energy to maintain cell flexibility, becoming rigid and more susceptible to early haemolysis [229].

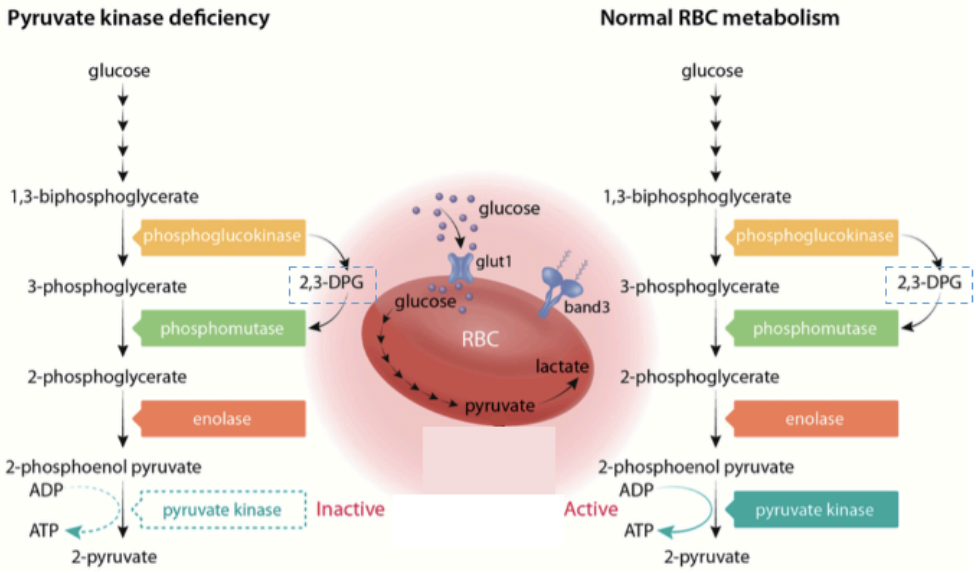


Figure 11 Enzymatic Glucose Metabolism in RBCs

Illustrates glycolytic glucose breakdown in red blood cells, comparing pyruvate kinase (PK)-deficient RBC metabolism against normal. Begins with 1,3-biphosphoglycerate formation, through enzymatic phosphorylation and rearrangements, to the intermediate 2,3-bisphosphoglyceric acid (2,3-BPG). The pathway's crucial step, converting phosphoenolpyruvate to pyruvate via pyruvate kinase—turning adenine diphosphate (ADP) to adenine triphosphate (ATP) (energy)—is compromised in PK-deficient RBCs, unlike in normal RBCs. Adapted from [229].

PK-deficient RBCs show reduced ATP levels linked to higher 2,3-diphosphoglycerate (2,3-DPG) concentrations, outlined in Figure 11 with a blue rectangle. Elevated 2,3-DPG levels diminish G6PD activity, weakening RBC antioxidant defences and altering cell membrane stability, leading to increased membrane instability and reduced RBC deformability [230]. This oxidative stress, coupled with high 2,3-DPG, creates an intraerythrocytic environment less

hospitable to parasites, affecting membrane proteins which might lead to enhanced RBC clearance and compromise parasite invasion [230]. Morais *et al.*, *in-vitro* study showed that: (i) 2,3-DPG influences intraerythrocytic parasite growth, (ii) alters iRBC metabolic profiles making them similar to uninfected RBCs, reducing progeny release, and (iii) impairs RBC membrane functionality, affecting parasite invasion [230] [231].

Further research underscores PK deficiency's impact: rodent models with PK deficiency exhibit malaria protection due to energy shortage and haemolysis [232]; human PK-deficient RBC malaria´s resistance may be attributed to ATP depletion affecting parasite invasion, *in-vitro* [233]. This depletion causes membrane protein cross-linking, impacting parasite lifecycle stages within RBCs.

1.6.2.3. RBC haemoglobin and the parasite

Haemoglobin is essential for parasite development within RBCs. Parasitic invasion leads to haemolysis, decreasing haemoglobin levels and potentially causing anaemia, a frequent malaria complication [234]. Inside the RBC, the parasite consumes about 70% of cytoplasmic haemoglobin for growth [235].
Haemoglobinopathies, alterations in haemoglobin due to genetic polymorphisms, serve as evolutionary adaptations against severe malaria in regions historically exposed to the disease. These changes can be structural (e.g., HbS, HbC, HbE) or quantitative, affecting alpha and beta chain production and leading to thalassaemias [236]. Traits like HbS and alpha-thalassaemia offer significant protection against severe malaria, particularly in African children, with heterozygous HbS (sickle cell trait) individuals seeing a 90% reduction in severe disease risk [237][238].
The sickle cell trait provides protection by creating a less hospitable environment for parasite growth due to cell deformation, impaired haemoglobin digestion, and increased splenic clearance [239][240][241]. Sickle cells (Hb AS) also promote reactive oxygen species (ROS) production, affecting the plasma membrane's configuration and disrupting the display of *Pf*EMP-1, a key molecule in *falciparum*'s endothelial adherence and severe malaria pathogenesis, especially in cerebral malaria through its interaction with intracellular adhesion molecule 1 (ICAM-1) ,Figure 12 [241][242].

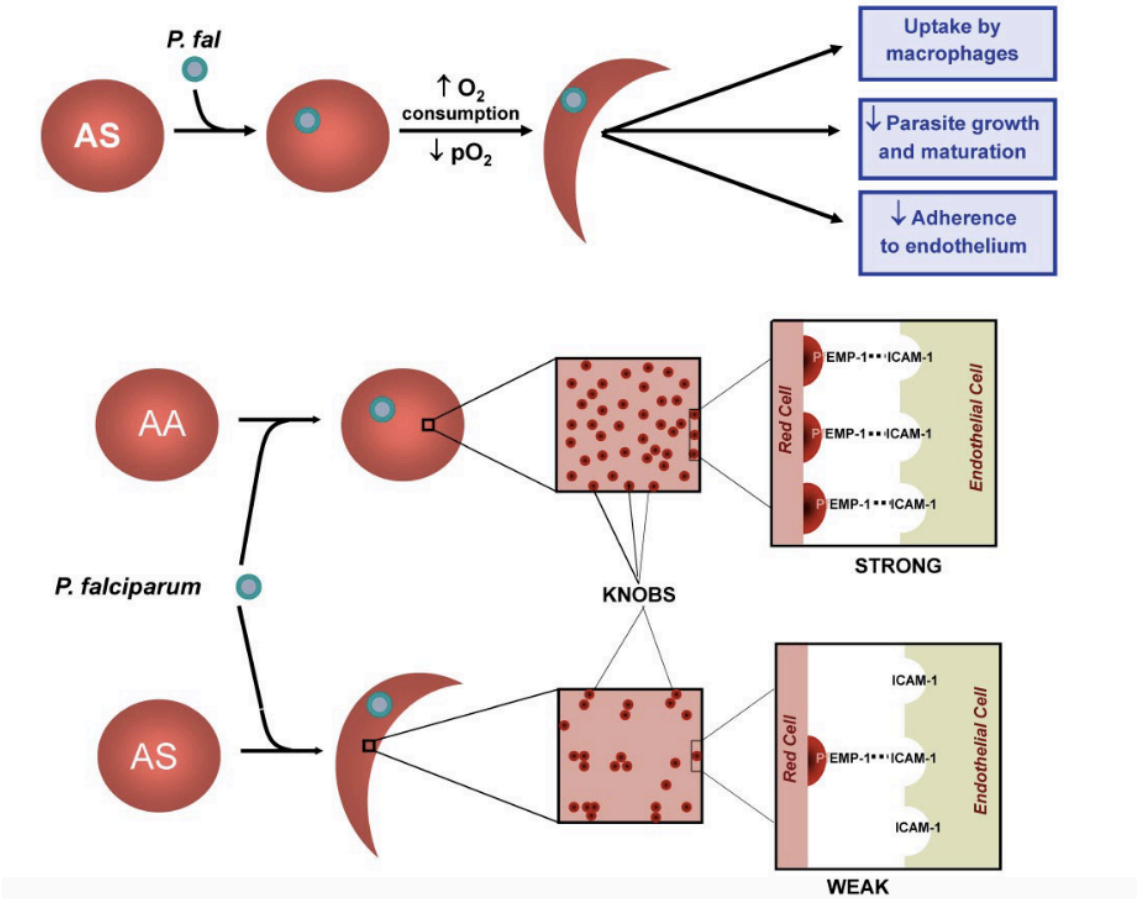


Figure 12 Protective mechanisms in AS RBCs against malaria

Parasitized AS (sickle cell trait) red blood cells exhibit increased oxygen consumption and reduced oxygen partial pressure (pO2), leading to haemoglobin sickling. This process alters cell membranes through oxidative stress, promoting macrophage uptake, inhibiting parasite growth and maturation, and reducing endothelial adherence. Infection further disrupts the distribution of surface knobs on these cells. The hypoxia-induced sickling weakens *Pf*EMP-1 and ICAM-1 interactions, crucial for cerebral malaria, by altering the iRBC surface topology. Adapted from [241].

Alpha-thalassaemia, a prevalent polymorphism offering protection against malaria, is notably frequent in African populations, with around 80% prevalence [243]. This condition results from the deletion of one or more genes coding for haemoglobin alpha chains. Heterozygotes (-α/αα) have one alpha chain gene copy deleted on one chromosome, while homozygotes (-α/-α) lack a gene copy on both chromosomes, often leading to mild microcytic anaemia [244]. Homozygous individuals exhibit considerable resistance to severe malaria, particularly severe malarial anaemia.

The study by Fowkes *et al.*, highlights this protection . Homozygous children in this study lost less haemoglobin compared to non-alpha-thalassaemia children and faced severe malarial anaemia with a 10% greater RBC reduction than healthy counterparts. Thus, alpha-thalassaemia's protective effect primarily

prevents severe anaemia, a critical manifestation of severe malaria, rather than directly affecting parasite invasion, metabolism, or growth [245].

Protection offered by inherited RBC disorders varies, with none against asymptomatic parasitaemia, moderate against uncomplicated malaria, and high against severe malaria [236]. Haemoglobinopathies likely alter malaria pathogenesis by disrupting *Pf*EMP1 export to the RBC surface, reducing adhesion crucial for severe malaria syndromes [237].

1.7. Parasite numbers: *in-vivo* and *in-vitro* observations

The immune system is a key player in controlling the growth rate of *Pf*, which directly affects parasite burden and the disease's varied outcomes [246]. However, disease progression can also be influenced by factors beyond the immune system, raising the question of whether different growth rates of the parasite are observed among individuals, especially those naive to malaria, where immunity plays little to no role.
This phenomenon was notably observed in malariotherapy experiments, which showed interindividual variability among malaria-naive subjects. Before the advent of antibiotics, syphilis, leading to conditions such as general paresis of the insane (GPI) due to neurosyphilis, was treated with malariotherapy [247]. This treatment, introduced by Austrian psychiatrist Wagner-Jauregg, involved inoculating syphilis patients with blood from individuals with *vivax* malaria to induce fever and eventually kill the *Treponema pallidum* spirochetes [247][248].
Pv was thought to be the most suitable species for this treatment due to its benign features and high and regular fever cycles [247].

Despite being naive to malaria, syphilis patients exhibited significant interindividual variability in several aspects. One report found that, out of 151 patients undergoing malariotherapy for general paralysis, 51% improved significantly, 23% returned to work, 16% showed no improvement, and 11% worsened [248]. Besides varying treatment outcomes, differences in parasitaemia levels were also recorded (Figure 13) [249]. Studies characterizing parasite growth dynamics revealed significant variability among patients, with parasite fold-change ranging from 5-fold to 12-fold, demonstrating a variability in patients infected with the same *Pf* species without undergoing malariotherapy [250].
Interindividual variability was notably observed in malariotherapy when patients were mistakenly injected with *Pf* instead of *Pv*, leading to three deaths out of four

patients. This outcome is attributed to *Pf*'s ability to infect RBCs of all ages, causing higher pathogen burdens and severe disease outcomes.

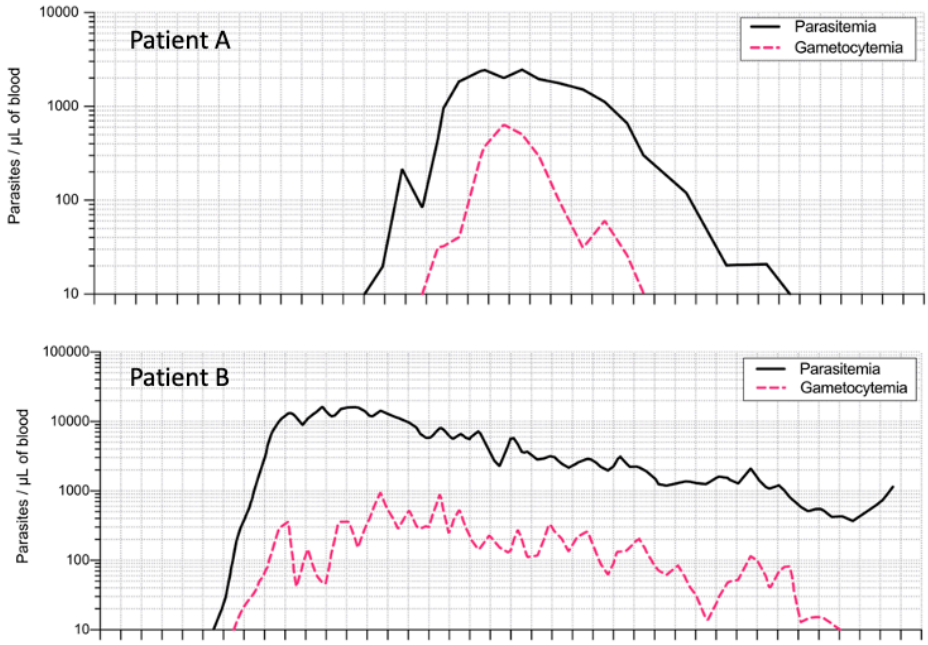


Figure 13 Timeline of *P. vivax* Infection in malariotherapy

This figure tracks parasitaemia and gametocytaemia over time following inoculation in two malariotherapy patients. Patient A exhibits delayed initial parasitaemia compared to Patient B (day 13 *vs* day 5), with a higher peak in parasitaemia. Patient A also cleared parasitaemia sooner than Patient B (~21 days *vs* ~30 days). In both cases, gametocytaemia patterns closely followed parasitaemia trends. The X-axis indicates days after inoculation, the Y-axis shows parasites/µL of blood. Retrieved from [249].

In the 1970s, to address the challenge of sourcing parasite infected samples solely from infected patients, Trager and Jensen achieved a breakthrough in 1976 by developing a method to culture *Pf in-vitro*, detailed in their landmark publication [251]. Following this, Vanderberg and Gwadz showed that female *Anopheles* mosquitoes feeding on these *in-vitro* cultures could effectively transmit *Plasmodium* [251]. This paved the way for the first human malaria challenge study in 1986 across various institutions, leading to WHO-developed guidelines and the establishment of the controlled human malaria infection (CHMI) model for *Pf* [251].

CHMI studies, crucial for evaluating malaria drugs and vaccines, involve deliberately infecting malaria-naive individuals. This can be done through exposure to laboratory-bred *Anopheles spp.* mosquitoes carrying *Pf* sporozoites or via direct injection of cryopreserved sporozoites. These studies aim to monitor the parasite's growth rate in the blood of the infected individuals [252][253][254].

CHMI studies using malaria-naive individuals also present interindividual variability. For instance, one study presented a model to address the important interindividual variation in vaccine trials (Figure 14) [255]. Also, parasitaemia levels in malaria-naive volunteers participating in IBSM (Infection Biology and Molecular Surveillance) studies exhibited significant fluctuations, underscoring the theme of interindividual variability once more, as shown in Figure 15 [256].

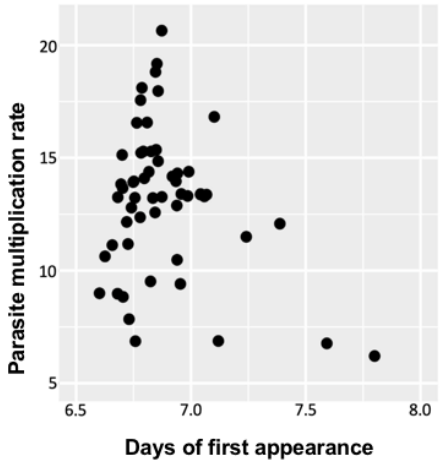


Figure 14 Parasite Multiplication Rate and First Appearance Day

This figure combines a matrix scatter plot of random effects highlighting sources of inter-individual variation in CHMI studies: day of first parasite appearance (X-axis) and parasite multiplication rate (Y-axis). Each black dot represents the point estimate for an individual CHMI volunteer (n=56). Adapted from [255].

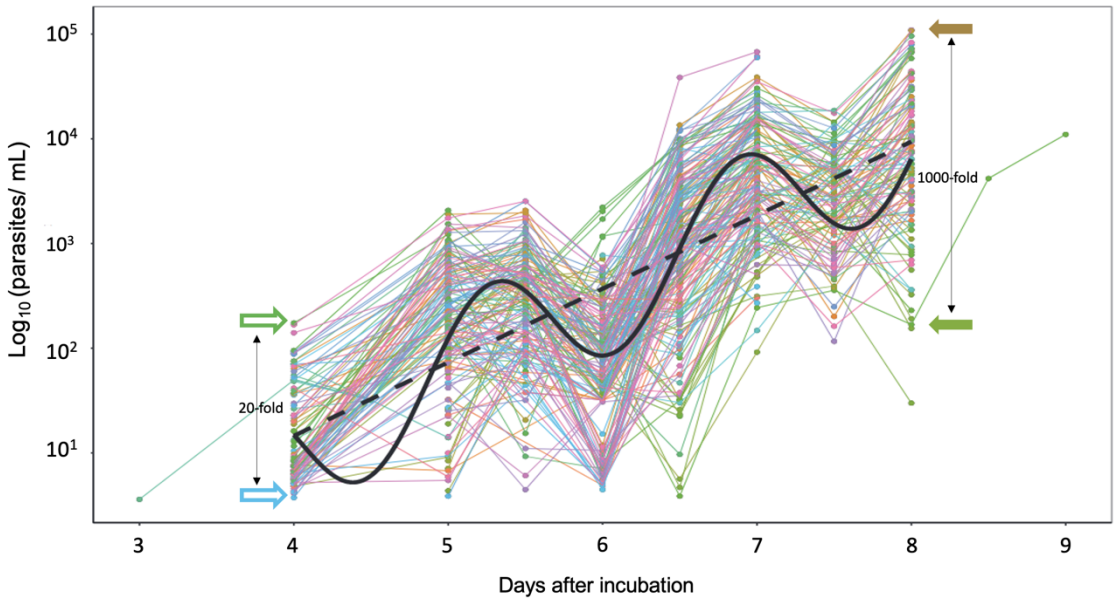


Figure 15 Parasitaemia Profiles in Malaria-Naive Volunteers (CHMI)

Shows parasitaemia levels of *Pf* (Note: log scale for parasites/μL) from 177 volunteers over 9 days post-inoculation. Each line colour tracks an individual's parasitaemia. On day 4, parasitaemia ranges from ~5 (blue arrow) to 10^2 (green arrow), indicating a 20-fold change. By the last day, the range shifts from 10^2 (green arrow) to 10^5 (brown arrow), marking a 1000-fold increase. Adapted from [256].

Assuming the immune system solely accounts for variations in parasite growth and disease outcomes, one might expect uniformity in parasite behaviour across *in-vitro* cultures of red blood cells from different individuals, where immune factors are absent [257][258].

Furthermore, previous work in the supervisor's laboratory with RBCs from volunteer donors had already led to the informal observations that the parasites tended to grow very well in the RBCs from some donors, while they grew very little in the RBCs from others (data not shown). This observation was/is constant and let to the use of RBCs from specific volunteers which showed the best growth rate.

One *in-vitro* study which explored the influence of carbohydrates on parasite growth also demonstrated interindividual variability. This study determined the *Pf* parasitaemias in the blood of 39 malaria-naive individuals, before and after a high glucose intake (chocolate bars) [259]. Cultures with RBCs of the study participants demonstrated variance in *Pf* growth rates, regardless of the glucose levels (Figure 16).

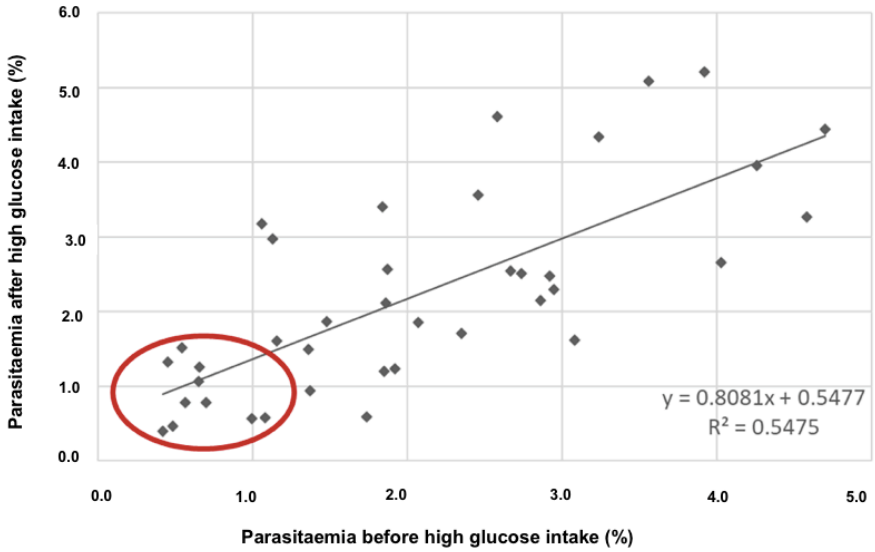


Figure 16 Variability in parasitaemia – *Pf* in *in-vitro* cultures

Growth of *Pf* in RBCs form 39 volunteers before and after a high glucose meal. Note the large inter-individual variability of parasitaemias observed between the volunteers, which does not seem to be related to glycaemia nor glycaemia variation, as illustrated by 10 individuals who present a consistently low final parasitaemia (red circle) before and after the high-glucose meal. Adapted from [259].

In summary all these observations lead to the question: What explains the different parasite growth rates between malaria-naive individuals *in-vitro*, particularly when infected with the same parasite strain in standard controlled conditions?

Pf is the only species of human *Plasmodium* which replicates well in cultures, therefore it is widely used in research, enabling the understanding of the malaria parasite [260]. Human red blood cells are required for *Pf* growth, while all other components are not – in fact, human serum which is also used has been widely replaced by bovine serum (Albumax).
Under *in-vitro* culture conditions, the human immune system does not interfere with parasite growth. Underlying differences in parasite growth are likely caused by host-related RBC factors. Thus, studying the growth in a large enough cohort of volunteers should allow to detect groups with different growth patterns. Once these cohorts have been established, they can be further investigated for possible associations linked to the growth pattern: low and high growth rate. This procedure does allow to not only investigate possible factors on (molecular/genetic) RBC level but allows to obtain clinical information and routine laboratory tests on everyone to eventually detect further associations.

2. AIM AND OBJECTIVES

Aim:

The primary goal of this study is to explore the variability in *Pf* growth rates *in-vitro* using RBCs from malaria-naive volunteers and to identify any potential correlations with low or high growth patterns.

Objectives:

1. To categorize growth patterns among a substantial cohort of volunteers, enabling the classification into two distinct groups based on growth rates: low and high. This classification aims to be reproducible and solely attributable to host-RBC factors.
2. To compile comprehensive data on each volunteer, covering demographic, clinical, dietary, and other relevant factors, to discern potential links to the observed growth patterns.
3. To analyse clinical-laboratory parameters, with a focus on metabolic and inflammatory statuses and RBC-specific metrics (e.g., Full Blood Count (FBC), iron status, haemoglobin variants), to uncover any associations with the growth patterns observed in the study.

3. MATERIAL AND METHODS

3.1. Volunteer Recruitment

The study involved 69 malaria-naive adult volunteers, primarily sourced from the community of the Faculty of Medicine, University of Lisbon (FMUL), and the Institute of Molecular Medicine (iMM). All participants provided informed consent prior to their involvement in the study.

3.2. Study Protocol Overview

This thesis is part of a broader study conducted with the collaboration of Dr. Sara Mateus Mahomed and medical students Ana Soares and Tomás Gonçalves, structured into three main phases:

i) Initial Assessment and Culture Setup: Volunteers first completed a questionnaire. Then, about 14 mL of blood was drawn from each, with immediate capillary glycaemia testing. Haemoglobin levels and AB0/Rh blood group were determined, and the blood was used to initiate cultures with the *Pf* 3D7 laboratory-adapted strain, not synchronized. Cultures were monitored over 96 hours, with parasitaemia assessed via light microscopy to determine individual parasite growth rates and classify volunteers into lower or higher growth cohorts.

ii) Reproduction of Results and Further Testing: Twenty-four volunteers provided additional blood samples to replicate initial findings and undergo routine haematological and biochemical tests. The aim was to explore any correlations between these parameters and the parasite growth patterns observed.

iii) Invasion Phenotype Exploration: In the final phase, eight volunteers contributed another blood sample for further analysis. Their RBCs were marked with Cell-Trace-Far-Red (CTFR) dye to examine potential impacts on parasite growth, focusing on *Pf*'s invasion phenotypes. Like the parasite cultures, variability in CTFR labelling among the volunteers' RBCs was noted.

3.3. Volunteer Questionnaire

Before their participation, all volunteers completed a comprehensive questionnaire designed to gather essential demographic and clinical information about themselves and their immediate family. This tool was crucial for both identifying potential factors influencing parasite growth and screening for eligibility. The questionnaire was organized into six key sections:

- Section A - Demographic Information: This section collected data on age, gender, blood group, nationality, birthplace, and the origins of non-Portuguese ancestors. Its primary objective was to explore any familial factors potentially affecting parasite growth.
- Section B - Blood Donation History: Questions in this section aimed to ascertain volunteers' blood donation status and the date of their last donation.
- Section C - Dietary Habits: This part of the questionnaire delved into the volunteers' dietary patterns, including the composition and timing of their last meal, dietary preferences (e.g., vegetarian, vegan, omnivorous), and any food intolerances, to investigate dietary influences on parasite growth.
- Section D - Malaria Exposure: Aimed to identify and exclude volunteers with a history of malaria.
- Section E - Anaemia History: Focused on gathering information about any personal or familial history of anaemia or other RBC disorders, including diagnoses, treatments, and any anaemia in direct family members, to assess potential impacts on parasite growth.
- Section F - Health and Disease History: Collected medical histories of the volunteers and their direct family members to exclude non-healthy participants and to explore whether any familial diseases, especially those related to RBCs, could correlate with the volunteer's parasite growth patterns.

3.4. Reagents

Malaria Complete Parasite Medium (MCM):
The foundation of our *in-vitro* experiments, Roswell Park Memorial Institute (RPMI) 1640 medium, was enriched with key supplements: 1M HEPES, gentamicin, L-glutamine solution (all from Life Technologies Europe, Bleiswijk, Netherlands), and Albumax II Lipid-Rich bovine serum albumin. Detailed composition and reagent proportions for MCM are provided in Table 5, with specific reagent ratios listed in Table 6.

To prepare a 200 mM L-glutamine stock solution, 14.6 g of L-glutamine was dissolved in 500 mL of distilled water. The Albumax solution was formulated with RPMI 1640 medium, Albumax II, gentamicin, HEPES (VWR, Haasrode Belgium),

sodium bicarbonate, D-(+)-Glucose, and hypoxanthine (all from Sigma-Aldrich, with the latter three sourced from both Dorset, UK, and St. Louis, USA). The exact proportions for the Albumax solution are detailed in Table 7.

For convenient use, both L-glutamine and Albumax solutions were aliquoted into 5 mL and 50 mL volumes, respectively, and stored at –20ºC until needed.

Table 5 Components of the Malaria Complete Parasite Medium

Reagent	Description
RPMI 1640 medium (without L-glutamine, with $NaHCO_3$)	Roswell Park Memorial Institute (RPMI) is a medium used for cell culture. It is formulated for use in an atmosphere of 5% CO_2. It contains glucose, phenol red as a pH indicator, salts, amino acids and vitamins. It must be supplemented with serum for cell culture.
Hepes 1M	Hepes is an organic chemical buffering agent used in cell culture. It enables manipulation of the culture outside a CO_2 incubator for long periods of time. It also can maintain the physiological pH despite CO_2 changes caused by cellular respiration.
Gentamicin (50mg/ml)	This antibiotic is effective against a wide range of gram-positive and gram-negative bacteria and thus it is used to avoid bacterial contamination in cell cultures. Also, its stability is independent on pH and temperature.
L-glutamine (200mM)	L-glutamine is an amino-acid required for cell culture. It enables the formation of purine and pyrimidine nucleotides, amino sugars, glutathione, L-glutamate and other amino acids as well as protein synthesis and glucose production.
Albumax II solution	Albumax solution is lipid-rich bovine serum albumin and is commonly used in malaria medium supplementation as a substitute for human serum.

Table 6 Formula used for Malaria Complete Medium

Malaria Complete Parasite Medium	
Reagent	Amount
RPMI 1640 medium (without L-glutamine, with $NaHCO_3$)	500 mL
Hepes 1M	12 mL
Gentamicin (50mg/ml)	500 µl
L-glutamine (200 mM)	5 mL
AlbuMax II solution	50 mL

Table 7 Reagents used for 500 mL of Albumax II solution

Albumax II solution	
Reagent	Amount
RPMI 1640 medium (no L-glutamine, with $NaHCO_3$)	5.2 g
Albumax II	25 g
Glucose	1 g
Gentamicin (50mg/ml)	10 µl
HEPES	2.98 g
Hypoxanthine	0.1 g
$NaHCO_3$	1.67 g

Reagents for solutions - Phosphate Buffered Saline (PBS): 1X PBS, pH 7,4 (Life Technologies Europe, Kwartsweg 2, 2665 NN Bleiswijk, Netherlands) solution was used.

Reagents for parasitaemia assessment using light microscopy:
Culture smears were fixed with absolute methanol (Merck, Darmstadt, Germany) and stained with Giemsa (Merck, Darmstadt, Germany) solution (1:10 in PBS 1X).

Reagents for haemoglobin measurement:
Drabkin´s reagent was used to determine haemoglobin levels. This reagent was provided by the Serviço de Patologia Clínica do Hospital de Santa Maria. It contained potassium ferricyanide, potassium cyanide, dihydrogen potassium phosphate and non-ionic detergent diluted in water.

Reagents for blood group determination:
Seraclone™ Anti-A, Anti-B, Anti-AB and Anti-D (Bio-Rad Medical Diagnostics GmbH, Dreieich Germany) antibodies were used to determine volunteers AB0 and Rhesus blood group.

3.5. Equipment/ materials

Equipment for *Pf* culture´s maintenance:
Pf cultures were kept in a Heracell™ incubator, at 5% CO_2 atmosphere and 37ºC. Cultures were manipulated in a class II biosafety safety cabinet (BSC).

Microscope and software programs for microscopy:
Smears were observed with an oil immersion objective (100x), Leica microscope (DM2800). Photographs from smears were taken using the Leica DFC480 digital camera and analysed with the Leica FireCam 3.4.1 (Leica Microsystems, Milton Keynes, United Kingdom) software. Parasitaemias were counted using ImageJ software, version 1.48g4 (National Institutes of Health, United States of America).

Flow cytometer
A BD Accuri™ C6 flow cytometry (BD Biosciences Oxford, United Kingdom) was used to detect labelled red blood cells with CTFR under a red laser (640 nm) and a 675/25 bandpass filter. Data was analysed using the program FlowJo (TreeStar, Ashland, Oregon), version 10.0.

Material for blood collection
Blood was collected with the S-Monovette ® system (Sarstedt, Nümbrecht, Germany). Sodium citrate was used for *Pf* cultures. EDTA K2 was used for haemaglobin measurements, blood phenotyping and haematology

determinations. Finally, a clot inducer was used to obtain serum to supplement the RPMI medium and also for biochemical determinations.

Equipment for glucose measurement
Glycaemia was determined with the OneTouch Select® Plus Flex glucometer (LifeScan Europe Zug, Switzerland) along with OneTouch Select® Plus strips.

Equipment for haemoglobin concentration measurement
A Spectronic™ Helios™ Gamma (Thermo Electron, Massachusetts, United States of America) spectrophotometer was used to determine haemoglobin concentration.

Equipment for RBC labelling
A Shaker Sanyo Orbital Shaker IOX402.XX1.C with a 5% CO_2 atmosphere and 37ºC was used to label RBC with CTRF.

3.6. Experimental Methods

Red Blood Cell preparation:
Blood sourced from the Portuguese Institute of Blood and Transplantation (IPST) and from volunteers was separated via centrifugation. IPST-provided blood sustained the primary cultures, while volunteers' blood was utilized for experimental cultures. The process involved centrifugation at 835 g for 10 minutes, removal of the supernatant and buffy coat, followed by three washes of the RBCs with RPMI. Post-final centrifugation, RBCs were stored at 4ºC, usable up to 6 days.

Serum preparation:
Following clot formation (around 30 minutes), serum was centrifuged at 4122 g for five minutes, then used immediately for supplementing volunteers' MCM or stored at -20ºC for up to 24 hours if not used immediately.

In-vitro cultures of *Pf*:
The 3D7 strain of *Pf* was cultured in T25 flasks with human blood from IPST, kept at 5% haematocrit with daily medium changes. Parasitaemia levels were checked every 48 hours, diluting with fresh RBCs for parasitaemia above 2%. Cultures were incubated at 37ºC in a 5% CO2 atmosphere.

Optimization of MCM protocol:

To better replicate human physiological conditions, a protocol was developed to replace bovine serum (Albumax) with human serum in the medium. This adjustment necessitated recalibrating the medium's reagent concentrations, including glucose supplementation. Details of the MCM formula with human serum are presented in Table 8, following optimization guidelines outlined in the literature [259].

Table 8 Reagents for MCM with human serum supplementation

MCM 10% Serum	
Reagent	Amount
RPMI 1640 medium (no L-glutamine, with $NaHCO_3$)	35,1 mL
HEPES 1M	100 µl
Gentamicin (50mg/ml)	36 µl
L-glutamine (200mM)	360 µl
20% glucose solution	360 µl
Serum	4 mL

Values for 40 mL of MCM supplemented with human serum.

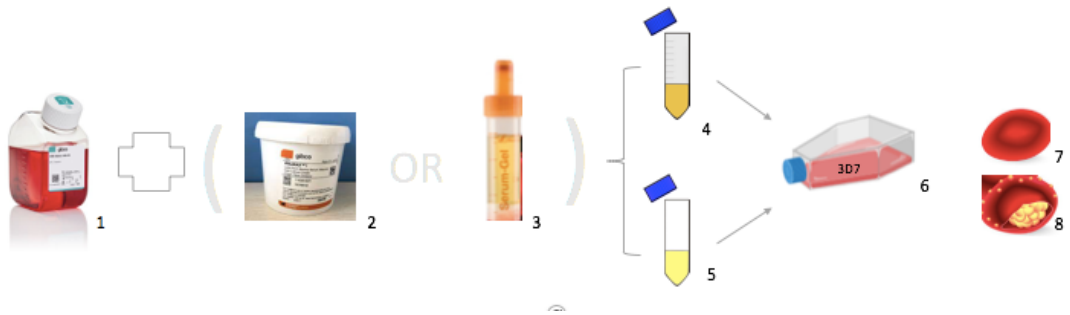


Figure 17 MCM optimization protocol with human serum

Culture medium was prepared with RPMI 1640(1). To supplement, either Albumax solution (2) or volunteer´s serum (3) was used. Volunteer´s RBC (7) and main culture infected RBC (8) were added to the medium to obtain a culture with a total of 5% haematocrit and a parasitaemia under 2%.

In-vitro cultures of *Pf* with volunteer´s blood

Culture medium was supplemented as referred previously. Cultures were performed in a six-well plate, with 3,8 mL of medium at a 5% haematocrit (Figure 18).

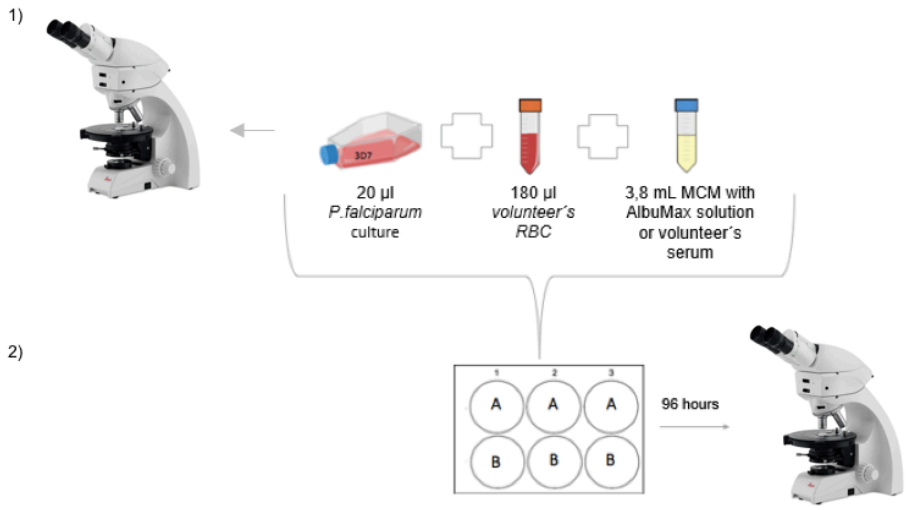


Figure 18 *Pf* culture protocol using volunteer's blood
Blood from ongoing cultures was mixed with the volunteer's blood in a medium supplemented either with Albumax (A) or the volunteer's own human serum (B) (1). Cultures were not synchronized. Each experimental setup was replicated in triplicate to ensure reliability. The cultures were then incubated for 96 hours at 37ºC in a 5% CO2 environment, with the medium refreshed daily (2). Parasitaemia levels were measured at the start (0 hours) from the continuous culture to establish initial levels (ranged from 0.5% and 1%) and at the conclusion (96 hours) from the volunteer's cultures using light microscopy to determine final parasitaemia.

3.7. Experimental procedures with volunteer's blood

(i) - Blood group determination:
The SeraClone™ rapid test was performed following the leaflet instructions to determine blood groups. In brief, it was performed on a microscope slide by adding a drop of reagent (Anti-A; Anti-B; Anti-AB; Anti-D) followed by 50 µl of volunteer's whole blood and observed for agglutination.
(ii) - Determination of haemoglobin concentration:
Twenty microliters of volunteer's blood was added to five millilitres of Drabkin´s reagent. This was homogenized and left to stand at room temperature for 10 minutes. Absorbances were read against the reagent blank in the spectrophotometer at 540 nanometres and the haemoglobin concentration was calculated form a standard curve.
(iii) - Haematological and biochemical parameters:
Haematological and biochemical parameters were determined in a certified clinical laboratory – Centro Hospitalar Universitário Lisboa Norte (CHULN). Some specific tests were performed such as glycated haemoglobin; haemoglobin, high-performance liquid chromatography (HPLC).
The chosen blood test parameters can be found in Table 9.

Table 9 Biochemical and haematological parameters

Biochemical parameters	Haematological parameters
Glucose	Full-Blood-Count
Total cholesterol	Haemoglobin
HbA1c	Haematocrit
C-reactive protein	MCV
Sedimentation Rate	MCH
Iron	MCHC
Ferritin	RCDW
Transferrin	Leukocytes
Folic acid	Platelets
Vitamin B12	Reticulocytes count
Lactate dehydrogenase (LDH)	
Total bilirubin	
Zinc	

Series of biochemical and haematological analysis performed on the 24 volunteers from the second-phase.

3.8. Labelling red blood cells with Cell-Trace-Far-Red (CTFR)

For the analysis, red blood cells labelled CTFR were examined using a flow cytometer configured to record data from a total of 40.000 events. The cytometer utilized a red laser (640 nm) for excitation and a 675/25 bandpass filter to detect the emitted fluorescence.

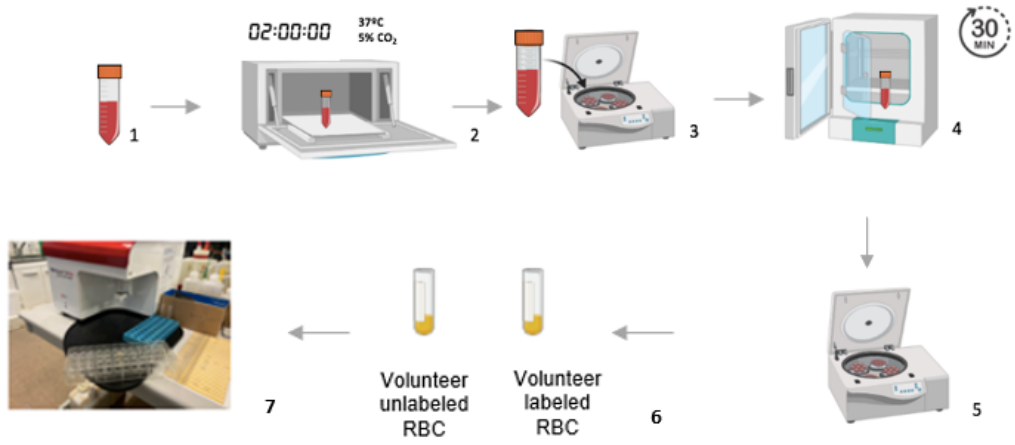


Figure 19 CTFR labelling Protocol for RBCs

This figure details the method for labelling red blood cells (RBCs) with Cell-Trace Far Red (CTFR) dye. The staining process involved a 2% haematocrit RBC suspension in 1X PBS, treated with a 10 µM concentration of the dye. The suspension was then incubated for 2 hours at 37ºC on an orbital shaker (1-2). Subsequently, the cell suspension underwent three washes with 1X PBS and a 30-minute incubation in RPMI to halt the labelling reaction (3-4). Following this, cells were centrifuged at 835 g for five minutes (5). The resulting pellet, comprising labelled RBCs and an unlabelled pellet (as a negative control) of RBCs, were re-suspended in 1 mL of 1X PBS for immediate flow cytometry analysis (6-7).

3.9. Assessment of parasitaemia

Parasitaemia from continuous cultures and from volunteer's cultures was determined using light microscopy and counted using ImageJ Software.

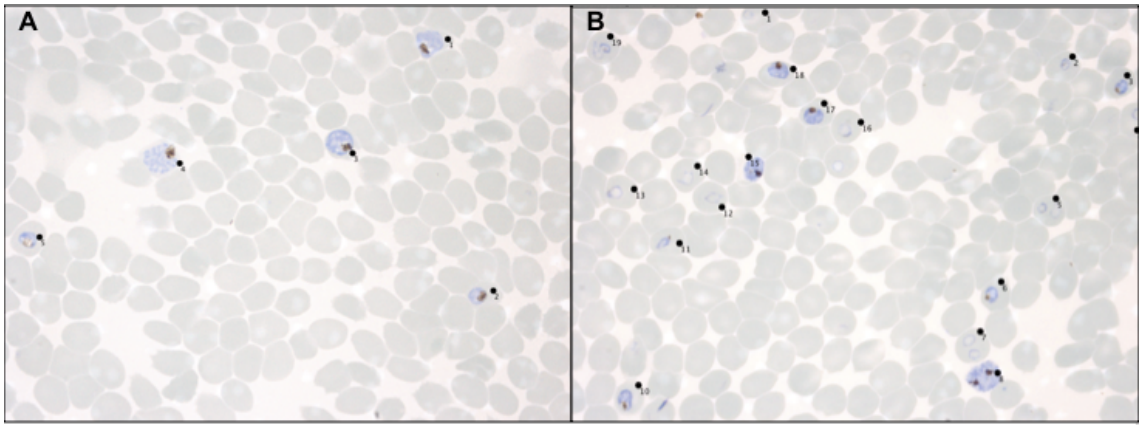


Figure 20 Manual counting of parasitaemia using ImageJ Software
Representative images of microscopic fields of continuous cultures (A) and cultures for experiments from volunteers (B). Parasitized cells are highlighted with black dots and sequential numbers for each image. Giemsa-stained cultures, 1000X magnification.

For each smear a minimum of 1.000 cells were counted. To determine parasitaemia, in percentage, the following formula was used:

(% parasitaemia) = (parasitized RBCs / total RBC).

Using the microscopy also enabled to monitor parasite´s status (healthy/ non - healthy).

3.10. Statistical analysis

Data were managed and analysed using Microsoft Excel and GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA). Categorical variables were presented as frequencies (n), and numerical variables were summarized as mean, range, and standard deviation. In specific cases, such as the CTFR experiments, the median was utilized.

Comparison in parasitaemia between groups was calculated as: (fold-change) = (final parasitaemia at 96h) / (initial parasitaemia at 0h).

Correlation between two numerical variables is expressed as R^2 with a 95% Confidence Interval (95% CI).

Effect size was calculated as Cohen's d Cohen's d = (M2 - M1)／SD_{pooled}, where SD_{pooled} = √((SD12 + SD22)／2) [261]. Interpreted as: small effect- d=0.2, medium effect - d=0.5, and large effect: d > 0.8.

Methods used for statistical interference to test the H_0 hypothesis were as follows: (i) categorial variables were compared using Fisher´s exact test;

(ii) numerical variables with a normal distribution and identical variances were compared using Student-t test (when comparing two variables) or;
(iii) one-way ANOVA test (when comparing three or more variables), while
(iv) for numerical variables which did not follow a normal distribution, a Mann-Whitney U test was performed to compare two variables.

3.11. Ethics

This work has been approved by the Ethics Committee (Number 496/18) from Centro Académico de Medicina de Lisboa.
Human blood samples were obtained from consenting healthy volunteers. Blood was also obtained from Instituto Português do Sangue e da Transplantação (IPST), under an established protocol that this institute has with iMM.
All information from volunteers was anonymized. Parasite growth results and the identity of their respective donors were only known to the study researchers and were all supervised by the study PI. The supervisor of this investigation is a trained physician (registered in the ODM) and supervised/performed all tasks regarding confidential information. All results were transmitted to each volunteer and in case of any doubts discussed confidentially and in person by the study supervisor.
All study investigators are proficient in the safe handling of blood samples. All relevant investigators have adequate training to perform blood collections.

4. RESULTS

4.1. Study population

For this study, results for *Plasmodium* culture were obtained from 69 malaria-naive participants, mainly recruited from the highly mobile and young student population of the Faculdade de Medicina or the Instituto de Medicina Molecular (Table 10).

Table 10 Demographic variables (n=69)

Demographic variables	(n)
Average age (range) in years	26 (18 – 56)
Gender (Female / Male)	55 (79.7%) / 14 (20.3%)
Nationality	
Portuguese	63 (91.3%)
Angolan	1 (1.4%)
Brazilian	1 (1.4%)
Cape Verdean	1 (1.4%)
German	1 (1.4%)
Mexican	1 (1.4%)
Spanish	1 (1.4%)

4.2. Interindividual variability of the *Pf in-vitro* cultures

The difference of growth of the *Pf* cultures was determined as fold-change after 96h culture (see Section 3.10). The results show a normal distribution with a mean of 10.0 (range: 3.0-19.3) and a Standard Deviation (SD) of 3.8 (Figure 21).

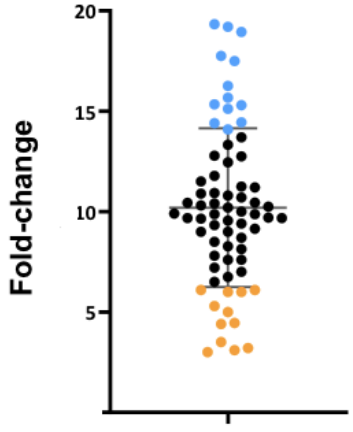


Figure 21 Fold-change in *Pf* growth among volunteers (0h to 96h)
Results are from 69 volunteers. They are normally distributed (mean = 10, SD = 3.8) and were divided in three groups: “normal growth” (n=44) within +/- 1 SD from the mean (black dots), “low growth” (LG) (n=12) (orange dots) and “high growth” (HG) (n=13) (blue dots), both >1SD from the mean, respectively.

Three groups were created: a) “low growth” (LG); b) “normal growth” (NG) and c) “high growth” (HG). A comparison of the results of each group is shown in Figure 22 and Table 11. Because groups were created based on their distance by >1SD

from the mean, a comparison of all three groups, but especially LG and HG was bound to show large effect sizes (Cohen's d=5.4) and significant P-values (<0.0001).

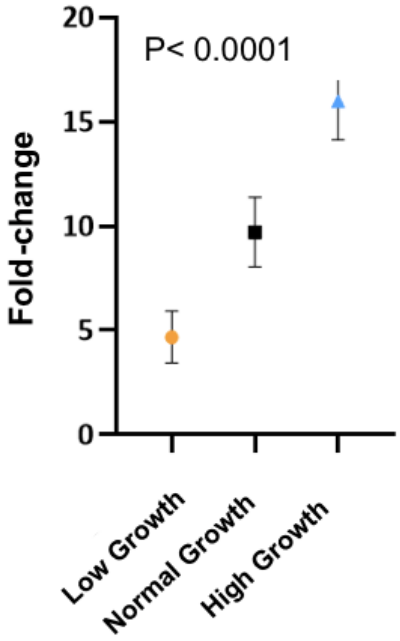


Figure 22 Comparison of results for each growth group
Fold-change (mean) is shown for low growth, normal growth, and high growth. The results from the three groups were statistically significantly different (one-way ANOVA test).

Table 11 Comparison of results for each growth group

	Low growth	Normal growth	High growth
Volunteers	12 (17.4%)	44 (63.8%)	13 (18.8%)
Mean	4.7	9.7	16.0
Range	3.0 – 6.1	6.5 – 13.3	13.8 – 19.3
Standard deviation	1.2	1.7	1.8

The difference between low growth and high growth showed a large effect size (Cohen's d=5.4, effect sizes: small - d=0.2, medium - d=0.5, and large: d> 0.8; P<0.0001.

4.3. Reproducibility of results in a subgroup of volunteers (n=24)

In prior experiments involving *Pf* cultures with blood from various donors (repeatedly used over 10 times), it was observed that the parasite growth pattern in individual donor´s blood appeared to be a consistent trait, although specific results from these experiments are not detailed here. To address the concern that the growth patterns observed in the initial study with 69 volunteers might be influenced by temporary or fluctuating factors, such as dietary intake, a follow-up test was conducted with a subset of 24 volunteers. The aim was to verify the stability and reproducibility of the identified growth patterns. The results from this subgroup confirmed a high degree of reproducibility, with a correlation coefficient

(R^2) of 0.99 and a Confidence Interval ranging from 0.9941 to 0.9991, as depicted in Figure 23.

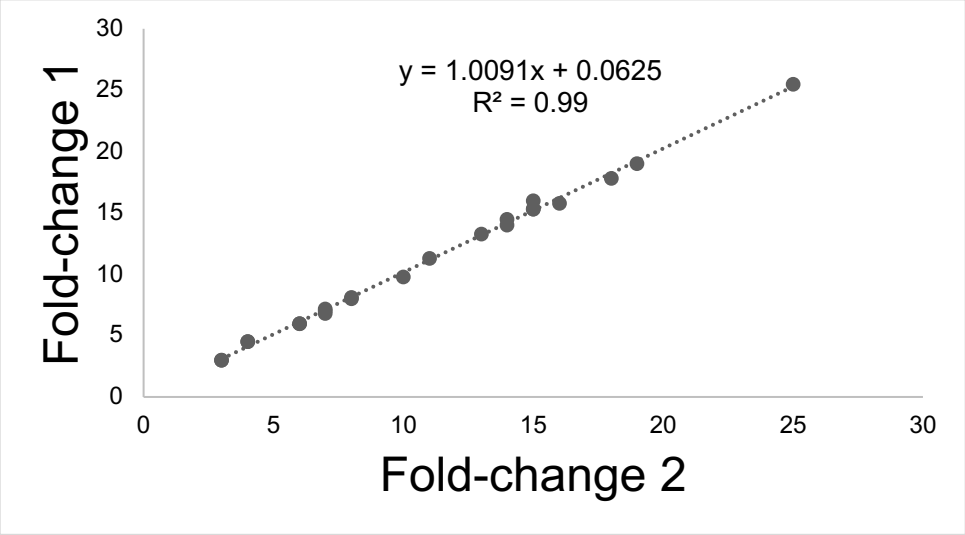


Figure 23 Reproducibility of growth pattern in a subgroup
The reproducibility of the growth pattern was tested in a subgroup (n=24). Fold-change from first culture series (fold-change 1) *vs* fold-change from repeated culture series (fold-change 2) show a high degree of reproducibility: R^2= 0.99 (95% Confidence Interval: 0.9941-0.9991).

4.4. Effect of autologous human serum on *in-vitro* parasite growth

In an investigation into the impact of serum type on *in-vitro Pf* growth, cultures from volunteers across all growth categories (low, normal, and high) were supplemented with either autologous human (n=14) serum or bovine serum (Albumax) (n=14). The findings indicated a marginally lower growth rate in cultures with human serum supplementation (Figure 24).

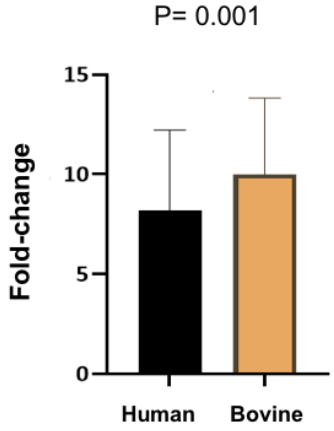


Figure 24 Growth in human or bovine serum supplemented cultures

Fold-changes of *Pf* growth after 96 hours in cultures supplemented with human serum (mean: 8.2, SD: 4) *versus* bovine (mean: 10, SD: 4). While the difference is significant (P=0.001) the Cohen's d effect size (0.4) is small.

Further analysis showed that within the same volunteer, parasite growth patterns (LG, NG, HG) remained consistent irrespective of the serum type used (Figure 25). However, the variation between growth groups was less pronounced in cultures supplemented with human serum. Additionally, there was an observable decrease in parasite viability in cultures with autologous human serum, as shown in Figure 26. This suggests that while the choice of serum can slightly affect the growth rate and viability of parasites, it does not fundamentally alter the inherent growth patterns established by host RBC factors (Figures 25, 26).

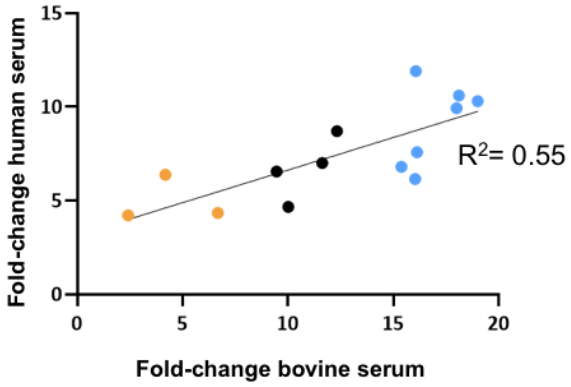


Figure 25 Growth rates in human or bovine serum supplement
Correlation between fold-changes of *Pf* growth rate after 96 hours with medium supplemented with either bovine serum or autologous human serum. Low growth (orange); normal growth (black); high growth (blue) (R^2=0.55; 95% Confidence Interval: 0.027 - 0.84).

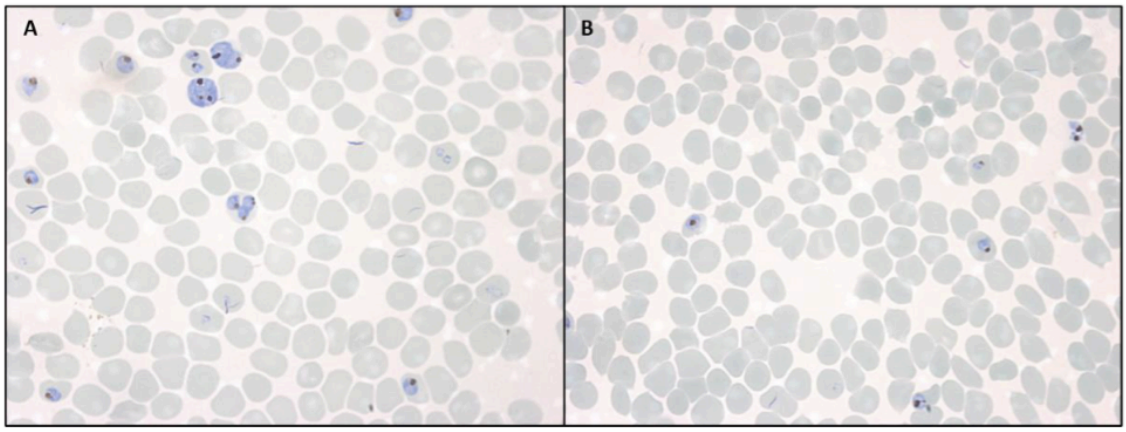


Figure 26 Morphology of *Pf* in bovine *vs* human serum supplemented cultures
Representative microscopic images of *Pf* smears from a single volunteer's culture, one supplemented with bovine serum (Panel A) and the other with human serum (Panel B). In the bovine serum culture, parasites exhibit a larger size, more distinct chromatin, and occurrences of cells hosting multiple parasites. Giemsa staining at a magnification of 1000X. The images highlight the morphological differences in parasite development influenced by the type of serum used in the culture medium.

Overall, these results indicate that the growth pattern (LG or HG) does not seem to depend on any factors present in the autologous serum. Rather, the autologous serum seems to have a slightly detrimental effect on parasite growth as compared to bovine serum.

4.5. Analysis of factors affecting low and high parasite growth patterns

In exploring the host-dependent distinctions between LG and HG patterns of *Pf*, an array of variables was scrutinized to identify potential correlations. These variables were organized into several categories for a comprehensive analysis:
1. Demographic Information: Including age, gender, and blood group, aimed at identifying familial or genetic influences on parasite growth.
2. Temporary Characteristics: Focused on factors like dietary habits, time since last meal, glucose and lipid levels, and inflammatory markers, which could temporarily affect parasite growth.
3. Clinical and Haematological Data: Encompassed volunteers' medical histories, particularly concerning anaemia or specific blood group traits, to assess their impact on parasite growth.
4. Laboratory Parameters: Detailed analysis of basic haematological and biochemical values such as FBC, iron status, and screening for haemoglobinopathies, to pinpoint any laboratory indicators potentially associated with growth patterns.

The investigation revealed a notable association with a history of anaemia and specific laboratory indicators suggesting borderline anaemia conditions. Interestingly, affected individuals did not report significant clinical symptoms at the time of sample collection, nor was there a clear history of diagnostic work-up or treatment for anaemia. This finding suggests that subtle variations in haematological health may influence the propensity for either LG or HG *Pf* growth patterns *in-vitro*, warranting further investigation into the nuanced interplay between host blood characteristics and parasite development.

4.5.1. Influence of demographic factors

Upon examining demographic variables such as age, gender, and the nationality of volunteers, no statistically significant differences were observed between the low growth and high growth groups. However, an intriguing trend was noted concerning the ancestral backgrounds of volunteers: a higher proportion of individuals in the LG group reported ancestries from malaria-endemic regions compared to those in the HG group. This difference approached statistical

significance, suggesting potential genetic or inherited factors linked to malaria resistance that warrant further exploration (refer to Table 12 for detailed statistics).

Table 12 Demographic variables (questionnaire) between LG and HG

Variables	Low growth (n=12)	High growth (n=13)	P – value[4]
Age in years - mean (range)	28 (21 – 48)	29 (19 – 56)	0.72
Gender			
Female	10 (83.3%)	9 (69.2%)	0.64
Male	2 (16.7%)	4 (30.8%)	
Nationality			
European[1]	10 (83.3%)	13 (100.0%)	0.22
Endemic-malaria country[2]	2 (16.7%)	0 (0.0%)	
Direct family native country[3]			
European[1]	6 (50.0%)	12 (92.3%)	0.03
Endemic-malaria country[2]	6 (50.0%)	1 (7.7)	

[1] European nationality: all Portuguese; one German (high growth group).
[2] Endemic malaria countries: Angola, Brazil, India, and Mozambique.
[3] Direct family: parents, grandparents and great-grandparents.
[4] P – value calculation: Statistical analysis - Section 3.10.

4.5.2. Comparison of basic (“temporary”) variables:

Analysis of the basic, or “temporary”, variables indicated that a considerable number of participants from both low and high growth groups had pre-existing medical conditions or were on regular medication, with a slight, though not statistically significant, predominance in the LG group (P=0.24). The prevalent medical conditions were related to allergies or asthma, and the most used medications included contraceptives and antihistamines. No link between these health conditions or medications and parasite growth rates was established.

Dietary habits prior to blood sample collection were uniform across volunteers, with all participants having consumed a main meal, incorporating carbohydrates, before providing blood samples. A noteworthy distinction between groups was the timing of the last meal; a higher proportion of volunteers in the LG group had their meal less than one hour before blood collection compared to the HG group. Despite this difference in meal timing, no significant variation in glucose levels was detected at the time of blood collection, Table 13.

When examining basic metabolic and inflammatory markers, no significant differences emerged between the LG and HG groups, as detailed in Table 13.

Table 13 Comparison of basic variables (LG and HG)[1]

GROUP – PHASE 1 (n=25)			
Variables	**Low growth (n=12)**	**High growth (n=13)**	**P – value[9]**
Concomitant underlying medical condition[2]	7 (58.3%)	4 (30.7%)	0.24
Regular medication[3]	8 (66.7%)	5 (38.5%)	0.24
Food pattern			
Omnivorous	11 (91.7%)	13 (100.0%)	0.48
Pesco-vegetarian[4]	1 (8.3%)	0 (0.0%)	
Food intolerance	3 (25.0%)	3 (23.1%)	0.99
Time since last meal before blood collection			
< 1 h	7 (58.3%)	2 (15.4%)	0.04
> 1h	5 (41.7%)	11 (84.6%)	
Glucose[5] (mg/dL)	103 (81 – 131)	99 (84 – 123)	0.52
Haemoglobin (g/dL)[6]	12.3 (10.6 – 15.1)	14.8 (13.0 – 19.0)	0.03
GROUP – PHASE 2 (n=15)			
Variables	**Low growth (n=6)**	**High growth (n=9)**	**P – value[9]**
Glucose[7] (mg/dL)	70 (64 – 76)	89 (60 – 140)	0.11
HbA1C[8] (%)	5.1 (4.7 – 5.3)	5.1 (4.9 – 5.6)	0.82
CRP[8] (mg/dL)	0.2 (0.03 – 0.7)	0.1 (0.03 – 0.5)	0.77
Sedimentation rate[8] (mm)	13 (2.0 – 30.0)	12 (4.0 – 23.0)	0.70
Total bilirubin[8] (mg/dL)	0.3 (0.2 – 0.5)	0.6 (0.2 – 0.7)	0.12
Total cholesterol[8] (mg/dL)	174 (135 – 237)	176 (108 – 271)	0.66
Zinc[8] (µmol/L)	10.5 (8.5 – 13.1)	9.7 (8.5 – 12.8)	0.88

Several volunteers reported concomitant underlying medical conditions and take regular medication. Food intake was significantly different between the two groups when 1h was used as cut-off. HbA1c: glycated haemoglobin; CRP: C-reactive protein.

[1] Phase 1 included all HG and LG (n=25), while Phase 2 is only a subgroup which returned for determination of laboratory parameters in a certified clinical laboratory (CHULN) (n=15).

[2] Medical condition: allergic rhinitis, asthma, arterial hypertension, and dyslipidaemia.

[3]Regular medication: combined hormonal contraceptive, antihistamine, antidepressant, antihypertensive and statins.

[4] Pesco-vegetarian: vegetarians that also consumed fish and seafood.

[5] Glucose (capillary) determined at time of blood collection for culture (mean, range) at the first round of culture experiments.

[6] Determined at time of blood collection for culture (mean, range) at the first round of culture experiments using Drabkin's reagent (Material and Methods Section – 3.7.).

[7] Glucose (venous – reference laboratory) determined at time of blood collection for culture (mean, range) at the second round (reproducibility) of culture experiments (reference value: 70-110 mg/dL)

[8] Determined at the second round (reproducibility) of culture experiments. (Reference range) HbA1C: 4.0 – 6.0%; C-reactive protein <0.5 mg/dL; sedimentation rate < 12 mm/h; total bilirubin: <1.2mg/dL; total cholesterol: <190mg/dL; Zinc: 10.7 – 18.3µmol/L.

[9] P – value calculation: Statistical analysis - Section 3.10. Cohen's effect size for Hb: -0.8 (medium to large).

4.5.3. Analysis of Haematological History and Variables

During the study period, none of the participants reported active anaemia cases. The observed low parasite growth in the LG group wasn't linked to overt anaemia symptoms (refer to Table 14 for details). Notably, five volunteers in the LG group had past anaemia instances: two discovered through medical diagnosis and three learned of low haemoglobin levels during blood donation attempts or routine screenings. None mentioned current anaemia problems when joining the study.
A higher incidence of anaemia history among family members was reported in the LG group compared to other groups.
Surprisingly, the AB0 blood group of participants did not significantly influence the parasite growth rates.

Table 14 Comparison of haematological variables (LG and HG).

Variables	**Low growth (n=12)**	**High growth (n=13)**	**P – value[3]**
Blood donors	5 (41.7%)	4 (30.8%)	0.99
Symptoms of anaemia[1] in the previous 2 years	2 (16.7%)	2 (15.4%)	0.99
Any previous history of anaemia[2]	5 (41.7%)	0 (0%)	0.01
Family history of anaemia	8 (66.7%)	3 (23.1%)	0.04
Blood group			
A	5 (41.6%)	4 (30.7%)	0.71
B	2 (16.7%)	2 (15.4%)	
AB	0 (0%)	2 (15.4%)	
0	5 (41.6%)	5 (38.5%)	

More volunteers in the LG group had a history and/or family history of anaemia, although in many cases this was not further investigated.
[1] Symptoms of anaemia: brittle nails, difficulty concentrating, dizziness, fatigue, feeling faint, hair loss, headaches, loss of appetite, low blood pressure, palpitations, and sleep alterations.
[2] Defined as at least one contact with healthcare services where anaemia was diagnosed (see text).
[3] P – value calculation: Statistical analysis - Section 3.10.

4.5.4. Laboratory Haematological Variable Analysis

For the HG group, all laboratory haematological variables fell within normal ranges (see Table 15 for detailed data). However, during the initial experiment phase, haemoglobin levels for three LG group volunteers were below the reference range's lower limit as determined using Drabkin's reagent (refer to Materials and Methods - Section 3.7.). Subsequent hospital laboratory analysis found four volunteers with haemoglobin levels below the reference minimum. Similar trends were observed for Mean Corpuscular Volume (MCV) and Mean

Corpuscular Haemoglobin (MCH), with some results bordering on the lower limit. Notably, iron levels differed significantly between groups, though still within normal limits. Conversely, ferritin levels were nearly identical across groups, without any inflammatory markers that might affect ferritin, suggesting comparable total iron storage.

Comparative analysis of FBC parameters and other metrics relevant to anaemia diagnosis highlighted significant disparities between groups. This included lower average haemoglobin, MCH, and MCV values, decreased reticulocyte counts, and reduced iron levels in the LG group, though ferritin levels remained consistent (refer to Table 15 for specifics).

Table 15 Haematological and biochemical variables (LG and HG) [1]

Variables	Low growth (n=6)	High growth (n=9)	P – value[2]
Red blood cells (10^{12}/L)	4.6 (4.1 – 5.2)	4.7 (4.1 – 5.4)	0.83
Haemoglobin (g/dL)[3]	12.7 (11.9 – 14.3)	13.9 (12.7 – 15.6)	*0.04*
Haematocrit (%)	39.4 (35.6 – 45.0)	40.1 (33.8 – 46)	0.77
MCV (fL)	83.8 (78.7 – 86.9)	86.3 (80.0 – 91.4)	0.08
MCH (pg)	27.4 (21.8 – 29.3)	29.4 (27.1 – 30.8)	0.08
MCHC (g/dL)	33.2 (30.8 – 34.2)	33.8 (32.7 – 34.5)	0.44
RCDW (CV%)	14.0 (13.1 – 17.4)	13.2 (12.2 – 17.4)	0.43
Reticulocytes (%)	0.9 (0.6 – 1.2)	1.2 (0.7 – 1.6)	0.07
Leucocytes (x10^9/L)	7.08 (4.20 – 8.80)	7.71 (5.60 – 10.1)	0.53
Platelets (x10^9/L)	277 (172 – 396)	267 (205 – 409)	0.90
LDH (U/L)	180 (164 – 204)	176 (126 – 285)	0.85
Iron (µg/dL)	75.5 (51.1 – 121.4)	103.1 (68.7 – 138.3)	0.07
Ferritin (Normal/mL)	64.6 (5.7 – 237)	66.8 (12.9 – 258)	0.61
Vitamin B12 (pg/mL)	424 (153 – 574)	586 (164 – 1056)	0.26
Folic acid (Normal/mL)	9.5 (7.2 – 12.4)	8.7 (4.1 – 20.0)	0.36

Note that several parameters in anaemia (MCV, MCH, reticulocytes and Iron) almost reached significantly difference while haemoglobin is significantly different among both groups (italics).

MCV: Mean corpuscular volume; MCH - Mean corpuscular haemoglobin; MCHC - Mean corpuscular haemoglobin concentration; RCDW - Red Cell Distribution Width; LDH: lactate dehydrogenase.

Red blood cells: 3.8 - 5.1 x 10^{12}/L; Haemoglobin: 12 - 15.3g/dL; Haematocrit: 36.0 – 15.3%; MCV: 80.0 – 97.0 fL; MCH: 27.0 – 33.0 pg; MCHC: 31.5 – 35.5 g/dL; RCDW: 11.5 – 14.5 CV%; Reticulocytes: 0.5 – 1.5%; Leucocytes: x10^9/L 4.0 – 11.0; Platelets: 150 – 450 x10^9/L; LDH, 100-500 U/L; Iron: 33 – 193 µg/dL; Ferritin: 13 – 150 Normal/mL; Vitamin B12: 195 – 770 pg/mL; Folic acid: 4.6 – 18.7 ng/mL.

[1] All parameters determined at the time of second blood collection for reproducibility of culture experiments at the Serviço de Patologia Clínica do Hospital de Santa Maria, except first haemoglobin results (determined at first round of culture experiments – see Section 3.7 - Material and Methods).

[2] P – value calculation: Statistical analysis - Section 3.10. Cohen's effect size for Hb: -0.33 (small to medium effect size).

[3] Note that Hb levels were determined on all 25 LG and HG (see Table 13 above), while the presented results are from a subgroup which returned for determination of laboratory parameters in a certified clinical laboratory (CHULN) (n=15). Note P-values almost identical.

4.5.5. Haemoglobin Variant Analysis

In the second phase of the study, all volunteers underwent screening for abnormal haemoglobin variants using HPLC, with results indicating no presence of haemoglobinopathies among participants (refer to Table 16 for detailed findings).

Table 16 Comparison of haemoglobinopathy screening

Growth	Haemoglobin F (%)	Haemoglobin A2 (%)	Variants (%)
Low	0.2	2.4	0
Low	0.2	2.8	0
Low	0.2	3.1	0
Low	0.3	2.4	0
Low	0.7	2.5	0
Low	0.8	2.8	0
Normal	0.3	2.5	0
Normal	0.4	2.1	0
Normal	0.5	2.7	0
Normal	0.6	2.3	0
Normal	0.6	2.5	0
Normal	0.6	2.9	0
Normal	0.7	2.6	0
Normal	0.8	2.8	0
Normal	1.0	2.4	0
High	0.2	2.3	0
High	0.3	2.6	0
High	0.3	2.6	0
High	0.4	2.3	0
High	0.4	2.6	0
High	0.4	2.6	0
High	0.5	2.5	0
High	0.5	2.8	0
High	0.6	2.3	0

Hb analysis of three subgroups: LG, NG and HG (n=24). Haemoglobin F reference range < 1%, Haemoglobin A2 reference range: 1.8 – 3.5%.

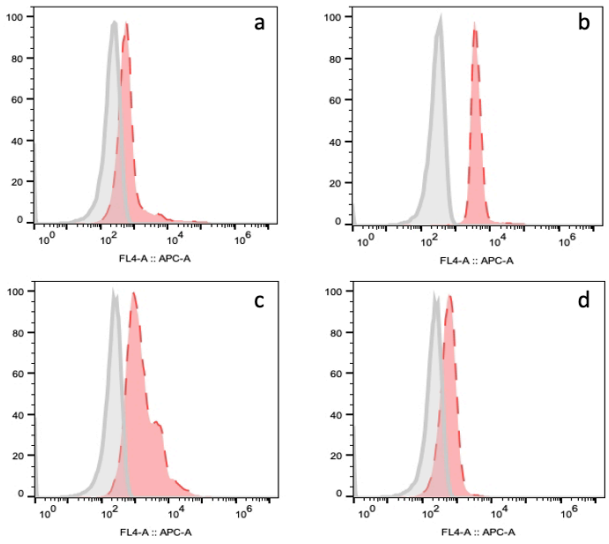
4.6. Co-culture experiments and CTFR labelling of RBCs

This section presents unexpected findings from attempts to establish a co-culture protocol.

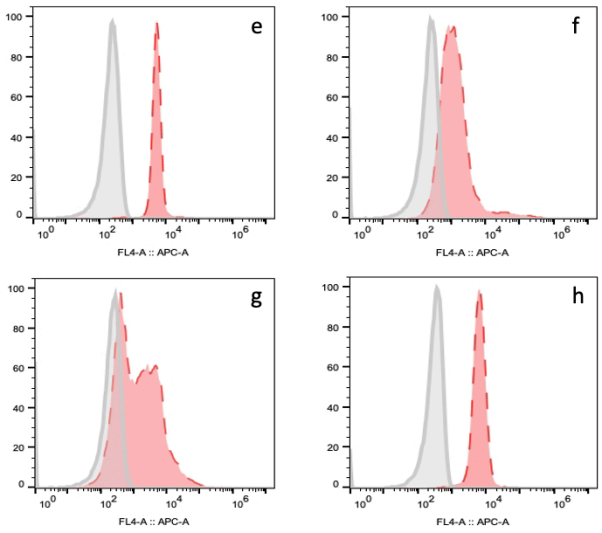
Efforts were made to develop a co-culture system using blood from volunteers, aiming to minimize variability observed in separate cultures from different individuals. The approach involved labelling one or both RBC populations with Cell-Trace-Far-Red (CTFR) before combining them into a single culture, based on literature suggesting CTFR does not affect culture viability [262][263]. Due to the staining method's specific requirements and cost, a modified protocol was necessary, particularly for measuring parasitaemia using flow cytometry and SYBR green, diverging from the standard parasitaemia assessment method of this thesis. These experiments did not yield consistent or replicable outcomes and were consequently discontinued.

4.6.1. CTFR staining intensity variations between growth groups

Analysis of CTFR-stained *versus* unstained RBCs from identical volunteers revealed fluorescence enhancements in all instances, though the degree of increase varied. Observations are graphically represented in histograms (Figure 27, panels A-E), with the x-axis on a logarithmic scale and adjacent tables detailing Median Fluorescent Intensity (MFI) preliminary data. A preliminary analysis indicated a higher average MFI in the HG group compared to the LG group. Nevertheless, given the small sample size (four individuals per group) and considerable standard deviations, these findings should be interpreted with caution for representativeness.



Low Growth (MFI)					
Volunteer	Unlabeled	CTFR	fold-change	mean	SD
A	210	553	2.6	6.0	4.4
B	282	3761	13.3		
C	216	1201	5.7		
D	207	546	2.6		



High Growth (MFI)					
Volunteer	Unlabeled	CTFR	fold-change	mean	SD
E	214	5018	23.5	13.3	8.4
F	219	1064	4.9		
G	214	1077	5.0		
H	309	6074	19.7		

Figure 27 CTFR-fluorescent intensity

Presents the fluorescence intensity of both unlabelled and CTFR-labelled red blood cells (RBCs), analysed in the FL4-APC channel (630-661nm) via a BD-Accuri flow cytometer. The histograms compare median fluorescent intensity (MFI) for CTFR-labelled (red dashed line) *versus* unlabelled (grey solid line) RBCs across volunteers. Data for individuals from low growth (LG) groups are depicted in panels a-d, while high growth (HG) groups are shown in panels e-h. The x-axis is on a logarithmic scale, spanning seven decades. The fold-change in MFI is calculated by dividing the MFI of labelled RBCs by the MFI of unlabelled RBCs.

The difference in MFI was calculated as: (fold-change MFI) = (MFI labelled RBC) / (MFI from unlabelled RBC). MFI: median fluoresce intensity, MFIM: geometrical mean of MFI, MFIM: STD Standard deviation of MFIM.

5. DISCUSSION

Any aspect that promotes parasite replication leads to a higher parasite burden and may contribute to a severe disease outcome (asymptomatic infection; uncomplicated complicated, or severe malaria) [264][265]. The balance between parasite and host factors is crucial in determining disease severity. Parasite factors such as antigenic variation, multiplication efficiency, adhesive virulence factors, immune evasion strategies, drug resistance, and red blood cell invasion spectrum significantly impact the host's susceptibility to harm [266][267][268][269][270][271][272][273][274]. Host factors, including the environment, genetics, nutritional status, and the immune system, similarly influence parasite replication [125][275][276][277][278][279][280].

In-vitro cultures are designed to minimize extrinsic variables, providing a controlled environment for parasite growth using human RBCs [260][281]. Because of the use of laboratory-adapted parasite strains and a controlled setup that includes nutrient supplementation with bovine serum for essential amino acids and glucose, one might anticipate minimal variation in parasite growth [282][283]. However, *in-vitro* studies reveal significant interindividual variability, underscoring the complex and multifaceted interactions between the parasite and host RBCs, even in seemingly uniform experimental conditions [249][255][256][259]. This variability is observed even in the absence of the immune system's influence and highlights the intricate nature of malaria pathogenesis and the importance of considering a wide array of factors.

Section 1.7. highlighted the significant interindividual variability observed in a CHMI study involving malaria-naive volunteers, where fold-changes in parasitaemia varied markedly between individuals at the end of the observation period (Figure 28 a) [256]. Similarly, a CHMI trial with healthy Kenyan individuals, who had varying levels of previous exposure to malaria (quantified by levels of anti-schizont and anti-merozoite surface protein 2 antibodies), revealed pronounced differences in parasitaemias (Figure 28 b) [284]. These observations suggest a wide interindividual parasitaemia response, although with a more pronounced effect in malaria-naive individuals. This observation could be explained by the immune system's pivotal role in modulating individual responses to *Plasmodium* infection, indicating that previous exposure and immune memory significantly influence parasitaemia levels and disease outcomes.

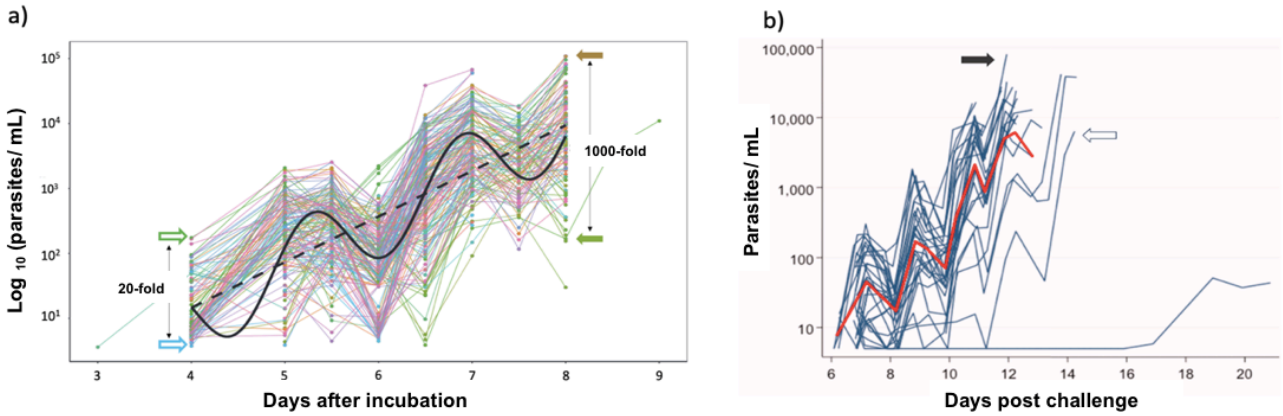


Figure 28 Parasite densities in non-immune *vs* immune individuals

The figure contrasts different parasite densities in CHMI studies (arrows). Panel a) depicts parasitaemia in 177 malaria-naïve volunteers over 9 days, with each color representing individual trajectories, highlighting a 20- to 1000-fold difference in parasitaemia on days 4 and 8. Panel b) shows parasite density variability among 28 malaria-exposed participants from initiation (day 6) to symptom onset (days 11-20), demonstrating a 10-fold variation in densities on days 12-14. Data are on a log scale, adapted from [256] and [284].

In-vitro cultures also reveal persistent interindividual differences, as illustrated by a study on invasion phenotypes [285], which highlighted significant variability in *Pf* parasitaemia levels across RBCs from various donors, evident with all six tested laboratory-adapted strains, as shown in Figure 29.

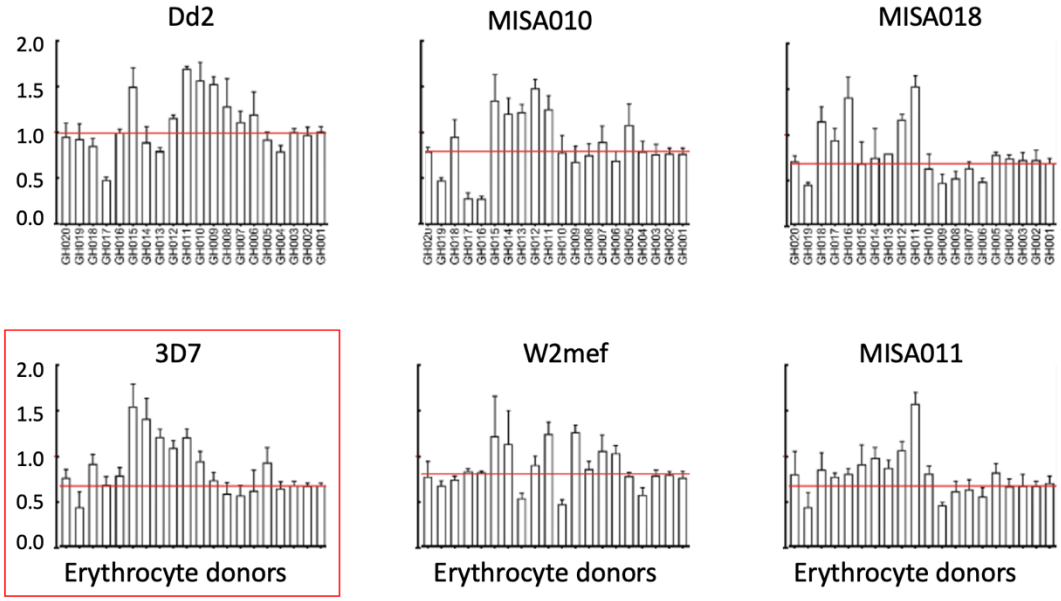


Figure 29 Variability in *in-vitro* parasitaemia across RBC donors

Fold-change in parasitaemia levels of RBCs relative to a reference sample (red line). Each bar represents one volunteer. Six laboratory adapted *Pf* strains, 3D7 (red square) used in current study. Adapted from [285].

The results of the current study, using the 3D7 laboratory-adapted strain to evaluate parasite growth rates across different individuals, show a fold-change range from 3.0 to 19.3 per cycle (~48h) (Figure 30). This contrasts with another study reporting fold-changes ranging from 0.5 to 2.5 among their donors, indicating less variability [285]. The larger sample size in our study (n=69) suggests that such a high variability might be more common and more pronounced than previously thought.

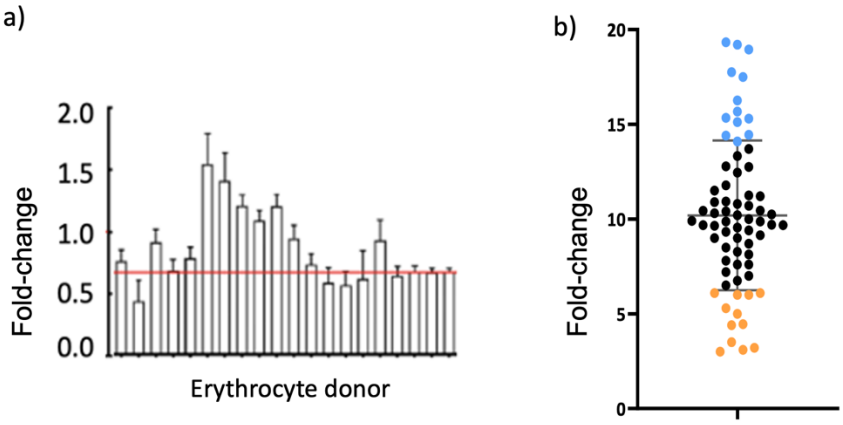


Figure 30 Parasitaemia in *in-vitro* cultures: literature *vs* current study
This figure offers a comparative visualization of fold-change in parasite growth rates: a) results from the literature with a smaller sample size (n=20) [285], and b) results from the current study with a larger cohort (n=69). The current study's larger volunteer pool and more pronounced fold changes highlight the extent of inter-individual variability observed.

Moreover, the current study not only highlights inter-individual variability but also clearly categorizes cohorts with low (LG) and high growth (HG) rates. Repeated cultures from 24 volunteers confirmed consistent growth patterns, with fold-changes across different time points showing a very high correlation (R^2: 0.99), underlining reproducibility and minimal impact from transient factors like dietary influences. Notably, one volunteer from each LG and HG group maintained their growth patterns over several cultures, serving as internal controls and further underscoring the stability of these growth patterns.

The literature indicates mixed results regarding the impact of culture supplementation with human *versus* bovine serum, with some studies showing no significant differences and others reporting enhanced growth with human serum in field isolates [286][287][258]. Contrarily, in our study, *Pf* cultures supplemented with autologous human serum demonstrated growth, albeit less pronounced than those supplemented with Albumax. Furthermore, microscopic examination revealed that parasites in cultures with autologous human serum

appeared smaller and less "healthy" compared to those in Albumax-supplemented cultures (Figure 26). This discrepancy could be due to the laboratory-adapted 3D7 strain's adaptation to Albumax, which might influence its growth dynamics in human serum.
Notably, (i) the distinct growth patterns of LG remained low, and HG remained high with both supplementations, and (ii) these observations suggest that serum factors may not directly account for the observed interindividual variability in parasite growth rates.

In this study, no significant influence of external factors such as sex, nationality, eating habits, and underlying medical conditions on LG and HG groups was identified. Interestingly, LG volunteers consumed their last meal less than an hour before blood collection. Despite similar meal contents across all volunteers, this proximity to blood collection did not lead to enhanced *in-vitro* culture growth, contrary to expectations – one might have expected to see higher nutrient levels. Furthermore, no significant correlation was found between parasite growth and glycemia. This is notable given that high glucose levels, as seen in diabetes, are associated with increased parasite proliferation and more severe disease outcomes [288]. Cholesterol levels, crucial for parasite invasion and growth, also did not significantly differ between LG and HG groups, contrary to observations from the literature on the parasite's use of host cholesterol [289].
These observations imply that the direct nutritional status prior to blood collection, including glucose and lipid levels, does not significantly affect parasite growth rates *in-vitro*. This reinforces the notion that the observed interindividual variability in parasite growth is not attributable to these factors, given that culture conditions effectively standardize them. Indeed, a study comparing parasite growth rates before and after glucose intake, which utilized autologous serum, reported varying growth rates [259], further supporting the minimal impact of these dietary factors on parasite growth within the controlled environment of *in-vitro* cultures in the current study.

The crucial question is thus: what causes the variability in parasite growth rates?

It appears that factors intrinsic to RBCs may be responsible. Variations in RBC surface antigens, cytoskeleton, enzymatic activities, or haemoglobin are known to influence malaria resistance [290][291][292][293][294]. Traits such as the sickle cell trait (HbAS) and α-thalassaemia, as well as specific enzymopathies provide significant protection against the disease [228][295][296]. These alterations in RBC structure and function play a vital role in disease resistance, affecting everything

from RBC membrane composition to intracellular signalling (Table 17). Such genetic variations lead to decreased parasite loads, thereby reducing disease severity, summarized by the principle: (↓↓↓parasite burden = ↓↓↓severe disease).

Table 17 Protective effect of alterations/polymorphisms in the RBC

Structure	Disease presentation	Mechanistic hypothesis of protection
Glycophorins (GYP ABC)	↓SM	Invasion inhibition; deficient intracellular growth
Complement receptor 1	↓SM	Reduced rosette formation
AB0	↓SM	Reduced rosette formation
MNS (Dantu phenotype)	↓SM	Dantu RBCs present a higher average cell tension in comparison with non-Dantu RBCs which leads to a higher resistance to merozoite invasion
Knops	↓SM	Reduced formation of rosettes
Ovalocytosis	↓SM	Invasion inhibition; deficient intracellular growth; premature splenic removal of iRBC
Eliptocytosis	↓SM	
Spherocytosis	↓SM	
G6PD	↓UM ;↓SM	Increased vulnerability to oxidant stress and protection against RBC parasitisation
Pyruvate kinase	↓ parasitaemia	RBC invasion defect and preferential clearance of ring-stage infected RBC
HbC	↓UM ↓SM	Reduces cytoadherence of infected RBC
HbE	↓SM; ↓ parasitaemia	Reduced RBC invasion; lower intra-erythrocytic growth; enhance iRBC phagocytosis
HbS	↓UM; ↓SM	Enhanced clearance of sickle RBCs; Reduced RBC invasion; growth inhibition of the parasite in micro vessels; Activation of innate and acquired immunity
α-thalassaemia	↓SM; ↓SMA	Reduced rosetting, reduced amount of haemoglobin loss and consequently protection against severe anaemia
ß- thalassaemia	↓SM	

G6PD: glucose-6-phospahate-deyhdrogenase, SM: severe malaria, SMA: severe malaria anaemia, UM: uncomplicated malaria, iRBC: infected RBC Adapted from [179][189][297][298].

Pf interacts with RBC membrane molecules, such as AB0 antigens [299]. However, the relationship between *Pf* and AB0 blood groups reveals contradictions, especially regarding parasite preference for different AB0 groups in both *in-vivo* and *in-vitro* studies [299]. Generally, blood group 0 individuals exhibit resistance to severe disease compared to A, B, or AB types, leading to milder clinical outcomes and reduced incidence of complications like cerebral malaria [300]. This difference is thought to stem from *Pf*'s ability to form rosettes more readily in RBCs of A, B, and AB groups, where A and B antigens are targets for rosetting [301]. However, contrary to this study, work with an *in-vitro* erythrocyte preference assay yielded findings reported the previously described protective effect of blood group 0 [200].

This study did not observe a direct link between blood group and parasite growth, as evidenced by a similar distribution of A, B, and 0 blood groups among LG and HG. This mirrors findings from a cross-sectional study in Ethiopia with 1065 febrile patients, which also found no significant AB0 group impact on malaria severity [302]. This suggests that the current *in-vitro* results might even align with field observations concerning AB0 blood groups.

Hereditary red blood cell membrane disorders, such as spherocytosis, elliptocytosis, and ovalocytosis, disrupt membrane structure and lead to early RBC removal by the spleen, reducing parasitaemia and enhancing resistance to merozoite invasion [303]. This results in lower risk of complicated malaria outcomes, as structural alterations in RBCs can impede parasite invasion or lead to early removal of infected cells, preventing significant parasite loads.
Haematological analysis in this study did not reveal significant RBC morphology alterations, and abnormal RBC shapes were rarely observed during microscopy. The low occurrence of these genetic disorders in the primarily Portuguese study population, where such conditions are less common compared to African and Asian populations, might explain this observation [304][305][306].

In this study, G6PD and pyruvate kinase (PK) deficiencies, conditions linked to haemolytic anaemias, did not show evidence of acute haemolytic episodes among volunteers, as indicated by normal lactate dehydrogenase (LDH) and bilirubin levels [307][308] (Table 15). It's important to highlight that this study did not conduct a detailed analysis of RBC metabolism in volunteers. G6PD and PK deficiencies, leading causes of non-spherocytic haemolytic anaemia, play significant roles in malaria outcomes, underscoring the importance of erythrocyte enzymopathies in influencing malaria susceptibility and progression [309].
G6PD deficiency, prevalent in regions historically affected by malaria, has been associated with evolutionary protection against the disease. Studies in Mali and Thailand suggest this protection may stem from increased oxidative stress susceptibility, leading to RBC lysis, rather than direct effects on parasite densities [225][310].
Similarly, PK deficiency has been shown to protect against malaria by impairing parasite invasion and promoting the clearance of infected cells [224]. Considering the genetic differentiation between African and Portuguese populations [228], investigating PK variants in volunteers with African ancestry could shed light on hereditary factors contributing to the LG pattern observed.
The extent to which erythrocyte enzymopathies explain the observed variability in parasite growth patterns within this study remains uncertain, especially when

considering the frequency of the LG (17%). Such a frequency could not be explained by enzyme deficiencies alone, because the frequency of G6PD (0.39%) and PK deficiencies (0.33%) in the Portuguese population are less than 0.5%, for each, this would mean that the occurrence of any of these deficiencies would affect less than 1 in 200 individuals in the general Portuguese population [311][312]. The discrepancy between the prevalence of specific RBC conditions in our cohort and their known distribution in general populations suggests that while it is possible that these deficiencies may have had a small contribution to variations in malaria susceptibility, other factors must have played a significant role in determining individual responses to *Pf* infection [309][311][312].

Although not reaching the 0.05 level for statistical significance, a tendency was observed where reticulocyte counts were lower in the LG group compared to the HG group (0.9% *vs* 1.2%; P=0.07), suggesting a link to the identified borderline anaemia in LG volunteers. This trend extends to iron levels, which were lower in the LG group compared to the HG group (75.5 µg/ dL *vs* 103.1 µg/ dL; P=0.07). Interestingly, supplementation with iron may increase malaria risk [313] and iron has been reported to be required for parasite proliferation [314], though the specifics of how and when the parasite acquires host iron, crucial for its proliferation, remain to be fully understood.

The principal discovery of this study centres on the aspect of borderline anaemia, revealing notable differences in history of past anaemia experiences (P=0.01) and familial anaemia history (P=0.04). While key RBCs parameters such as haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, ferritin, vitamin B12, and folic acid did not correlate with parasite growth, the LG group exhibited significantly lower haemoglobin levels than the HG group at both blood collection points (first phase: n=69, P=0.03 and second phase: n=24, P=0.04).

While the mechanism by which this influences parasite growth is not fully understood, it is significant that a study with 135 anaemic children showed anaemia reduced *Pf* invasion and growth in RBCs [315]. This study compared *Pf* growth *in-vitro* in RBCs from anaemic children, before and after iron supplementation, using flow cytometry-based assays. A key observation was that parasite growth increased 2.4-fold after 49 days of iron supplementation, indicating anaemia's protective role. Moreover, there was a direct correlation between anaemia and parasite growth rates, where a decrease in haemoglobin by one standard deviation corresponded to a 10% reduction in *Pf* growth. This

highlights a crucial biological interaction between host iron status and malaria pathogenesis, suggesting a strong link between parasitaemia and anaemia [312]. Although ferritin levels were lower in the LG cohort in this study, the LG group exhibits tendentially slightly lower iron levels and significantly reduced haemoglobin compared to HG. In fact, given that the 84% of the volunteers were young females and considering that slight iron deficiency (and resultant border-line anaemia) might be common, this link might warrant further investigation.
For example, exploring intra-erythrocytic iron could elucidate how the findings from Goheen *et al*. apply to our study. Although intra-erythrocytic iron determination has been explored in human haematopoietic cells, it hasn't been specifically applied to *Pf* invasion assays [316]. Perhaps, this novel approach could offer insights into the impact of intra-erythrocytic iron on parasite invasion efficiency. The protocol would need to be carefully designed to assess both haemoglobin-bound iron and the labile iron pool (LIP) [316], which includes iron not bound to haemoglobin. A proposed methodology might involve: (i) preparing RBCs from volunteers; (ii) measuring intra-erythrocytic iron, considering both haemoglobin-bound and LIP iron; (iii) using flow cytometry for precise quantification of iron within RBCs; (iv) culturing *Pf* and ensuring merozoite invasiveness; (v) validating cell viability to confirm the non-toxicity of the dyes to *Pf*; and (vi) incubating prepared RBCs with synchronized parasites, followed by flow cytometry to determine invasion rates. This comprehensive approach could potentially bridge the gap in understanding the interplay between erythrocytic iron levels and malaria pathogenesis.

Several studies highlight the correlation between haemoglobinopathies and protection against severe malaria syndromes, suggesting inherited forms of these conditions are prevalent in specific racial groups. For instance, sickle cell disease is more common in Central African populations, while thalassaemias are predominantly found in the Mediterranean, Middle East, and South Asia—regions corresponding to the ancestry of some low growth (LG) group volunteers [317][318]. This study found a significant association between individuals with ancestries from malaria-endemic countries in the LG group than those in the HG group (P=0.03), underscoring the impact of hereditary factors passed through generations on malaria susceptibility. However, common haemoglobinopathies were not detected, nor were any atypical curves observed in haemoglobin electrophoresis among LG group volunteers, suggesting other factors may be responsible.

The CTFR experiment yielded intriguing results, showing different labelling patterns among volunteer´s RBCs (Table 18), with LG group volunteers tending to exhibit lower median fluorescence intensity (MFI) compared to the HG group (Figure 27).

Table 18 Volunteers CTFR fold-change variability

Low Growth		High Growth	
MFI	**Fold-change**	**MFI**	**Fold-change**
533	2.6	5018	23.5
3761	13.3	1064	4.9
1201	5.7	1077	5.0
546	2.6	6074	19.7

This variation raises questions if RBC factors could be contributing to such differences and their potential link to observed interindividual variability. Interestingly, in the Goheen *et al,*. study [315], analyses of RBC surface markers related to cell age, membrane integrity, and susceptibility to merozoite invasion were conducted using CTFR dye in a flow cytometry assay [312]. This study revealed significant increases in markers such as GPA, CD47, CD35, and CD147, and a significant decrease in C3b deposition after iron supplementation at Day 49, indicating a shift towards younger and healthier RBCs. The fluorescence intensity of CTFR-stained cells can reflect cellular division history or changes in cellular characteristics, potentially influenced by membrane integrity markers like GPA and CD47. Changes in C3b deposition might also suggest alterations in immune recognition and clearance of RBCs, which could indirectly affect CTFR staining by altering cell population dynamics or the physical state of the cell surface. These findings suggest that CTFR fluorescence intensity could provide insights into the physiological state and age distribution of RBC populations, which may explain the diverse labeling patterns observed in our study. Future research could involve determining the age of volunteers' RBCs through Percoll-density centrifugation and analyzing surface markers with the CTFR assay to identify any correlation with the observed labeling patterns and parasite growth patterns.

Moreover,the Dantu blood group induces widespread alterations in the array of RBC surface proteins, including the reorganization of GYP genes, that encode the glycophorins, receptors that bind to *falciparum* ligands (Table 4). This alteration leads to an increased average tension within the RBCs, which is linked to enhanced resistance to merozoite invasion [204]. It could be speculated that volunteers with increased tension from Dantu RBCs might experience changes

in dye permeation, potentially explaining the lower labeling observed in LG volunteers. By analyzing the GYP genes of our volunteers, we could uncover a potential link to the varying CTFR labeling patterns observed.

Overall, the various observed labeling patterns are likely related to changes in the RBC surface markers, which affect dye uptake or retention. In turn, these markers are hypothesized to also play a role in the parasite's interaction with the RBC.

Despite the sample size limiting statistical robustness, the preliminary findings suggest a notable effect size (*Cohen's d:* 0.9), allowing for sample size estimation for future studies aiming to investigate this effect with an alpha of 0.05 and 80% power. Approximately 21 individuals per group (LG and HG) would be needed [261]. Considering that LG and HG were defined by a deviation of +/-1 SD from the mean, statistics mandate that both groups together can never be more than 32% of the whole study population. Therefore, a comprehensive study to definitively refute the null hypothesis of no difference in CTFR staining between groups would require at least 132 individuals, accounting for LG, NG, HG groups, without prior identification of their growth patterns.

In summary, although this study linked anaemia-related factors with the LG pattern and effectively excluded common causes like prevalent haemoglobinopathies, it didn't pinpoint a singular or unified mechanistic factor behind this pattern. The CTFR findings hint at membrane-related aspects being involved. However, any underlying mechanism identified must also explain the borderline anaemia observed, considering the frequency of this observation given the study's sample size (n=69).

The study has several limitations. The initial goal to recruit 100 volunteers was not met due to technical challenges and COVID-19 restrictions, resulting in only 69 participants. This shortfall particularly affected the sample size in later study phases. The demographic skew towards young women may limit the finding´s applicability across different genders and age groups. A more diverse sample, including equal numbers of males and females and eventually individuals from regions with varied malaria exposure, like sub-Saharan Africa, would have enriched the insights into protective polymorphisms. Moreover, the study did not delve into RBCs associated polymorphisms, missing out on an in-depth characterization of volunteer´s RBCs in terms of membrane, enzyme, and

haemoglobin deficiencies, which should include all structures listed in table 17, and perhaps go beyond. Another limitation is the *in-vitro* nature of the observations, raising questions to what extend the observed growth patterns would mirror real-life conditions.

6. Conclusion

This study has demonstrated the reproducible variability in the growth rates of *Pf in-vitro* cultures, showing that such variability is independent of immunological influences, demographics, or AB0 blood group factors. This finding underscores the importance of acknowledging interindividual variability in the context of *in-vitro* studies of malaria. Consequently, it becomes imperative to establish specific criteria for selecting RBC donors for *in-vitro* cultures. Such criteria are essential to minimize this variability and ensure the reliability of research results.

Furthermore, the impaired growth of *Pf* seems to be associated with connections to malaria-endemic regions, personal or family history of anaemia, and cases of borderline anaemia that cannot be explained by other haematological and biochemical parameters, including common pathologic haemoglobin variants. However, no common underlying mechanism could be identified.

By focusing on the variability in a large cohort of human volunteers, this thesis paves the way for future investigations to elucidate the possible RBC dependent mechanisms which cause parasite growth variability. It would be important to involve a larger and more diverse population in the research to ensure that the observations are representative and, for example, the CTFR label assay can be performed robustly. Additionally, conducting invasion assays could provide valuable insights into whether the parasite exhibits a preference for volunteers from low growth and high growth groups.
An exhaustive characterization of RBC membrane receptors and enzymes implicated in malaria, trough genomic and proteomics approaches along with the inclusion of alpha-thalassaemia in the haemoglobin study, would significantly enhance our understanding of the intricate dynamics between the malaria parasite and its host. These expanded studies could offer a more comprehensive picture of the factors influencing malaria parasite growth and potentially lead to more targeted and effective interventions for malaria prevention and treatment.

7. REFERENCES

[1] R. Tuteja, 'Malaria − an overview: Malaria − an overview', *FEBS Journal*, vol. 274, no. 18, pp. 4670–4679, Sep. 2007, doi: 10.1111/j.1742-4658.2007.05997.x.

[2] S. Sato, 'Plasmodium—a brief introduction to the parasites causing human malaria and their basic biology', *J Physiol Anthropol*, vol. 40, no. 1, p. 1, Jan. 2021, doi: 10.1186/s40101-020-00251-9.

[3] F. E. Cox, 'History of the discovery of the malaria parasites and their vectors', *Parasites Vectors*, vol. 3, no. 1, p. 5, 2010, doi: 10.1186/1756-3305-3-5.

[4] M. A. Boualam, B. Pradines, M. Drancourt, and R. Barbieri, 'Malaria in Europe: A Historical Perspective', *Front. Med.*, vol. 8, p. 691095, Jun. 2021, doi: 10.3389/fmed.2021.691095.

[5] R. Carter and K. N. Mendis, 'Evolutionary and Historical Aspects of the Burden of Malaria', *CLIN. MICROBIOL. REV.*, vol. 15, 2002.

[6] G. Majori, 'SHORT HISTORY OF MALARIA AND ITS ERADICATION IN ITALY', *Mediterr J Hematol Infect Dis*, vol. 4, no. 1, p. e2012016, Mar. 2012, doi: 10.4084/mjhid.2012.016.

[7] M. Dobson, '"Marsh fever"—The geography of malaria in England', *Journal of Historical Geography*, vol. 6, no. 4, pp. 357–389, Oct. 1980, doi: 10.1016/0305-7488(80)90145-0.

[8] L. J. Bruce-Chwatt and J. D. Zulueta, 'Malaria eradication in Portugal', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 71, no. 3, pp. 232–240, Jan. 1977, doi: 10.1016/0035-9203(77)90014-1.

[9] B. J. Brabin, 'Malaria's contribution to World War One – the unexpected adversary', *Malar J*, vol. 13, no. 1, p. 497, Dec. 2014, doi: 10.1186/1475-2875-13-497.

[10] E. T. Piperaki and G. L. Daikos, 'Malaria in Europe: emerging threat or minor nuisance?', *Clinical Microbiology and Infection*, vol. 22, no. 6, pp. 487–493, Jun. 2016, doi: 10.1016/j.cmi.2016.04.023.

[11] J. A. Nájera, M. González-Silva, and P. L. Alonso, 'Some Lessons for the Future from the Global Malaria Eradication Programme (1955–1969)', *PLoS Med*, vol. 8, no. 1, p. e1000412, Jan. 2011, doi: 10.1371/journal.pmed.1000412.

[12] A. Monroe, N. A. Williams, S. Ogoma, C. Karema, and F. Okumu, 'Reflections on the 2021 World Malaria Report and the future of malaria control', *Malar J*, vol. 21, no. 1, p. 154, Dec. 2022, doi: 10.1186/s12936-022-04178-7.

[13] N. J. White, 'Antimalarial drug resistance', *J. Clin. Invest.*, vol. 113, no. 8, pp. 1084–1092, Apr. 2004, doi: 10.1172/JCI21682.

[14] M. J. Donnelly, A. T. Isaacs, and D. Weetman, 'Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors', *Trends in Parasitology*, vol. 32, no. 3, pp. 197–206, Mar. 2016, doi: 10.1016/j.pt.2015.12.001.

[15] C. Caminade *et al.*, 'Impact of climate change on global malaria distribution', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 111, no. 9, pp. 3286–3291, Mar. 2014, doi: 10.1073/pnas.1302089111.

[16] K. Molina Gómez *et al.*, 'Characterizing the malaria rural-to-urban transmission interface: The importance of reactive case detection', *PLoS*

Negl Trop Dis, vol. 11, no. 7, p. e0005780, Jul. 2017, doi: 10.1371/journal.pntd.0005780.
[17] W. P. O'Meara, A. Noor, H. Gatakaa, B. Tsofa, F. E. McKenzie, and K. Marsh, 'The impact of primary health care on malaria morbidity - defining access by disease burden', *Tropical Medicine & International Health*, vol. 14, no. 1, pp. 29–35, Jan. 2009, doi: 10.1111/j.1365-3156.2008.02194.x.
[18] M. M. Zadeh, K. Shahandeh, S. Bigdeli, and R. Basseri, 'Conflict in Neighboring Countries, a Great Risk for Malaria Elimination in Southwestern Iran: Narrative Review Article', vol. 43, 2014.
[19] C. Lynch and C. Roper, 'The Transit Phase of Migration: Circulation of Malaria and Its Multidrug-Resistant Forms in Africa', *PLoS Med*, vol. 8, no. 5, p. e1001040, May 2011, doi: 10.1371/journal.pmed.1001040.
[20] M. Al-Awadhi, S. Ahmad, and J. Iqbal, 'Current Status and the Epidemiology of Malaria in the Middle East Region and Beyond', *Microorganisms*, vol. 9, no. 2, p. 338, Feb. 2021, doi: 10.3390/microorganisms9020338.
[21] T. Manyangadze, M. J. Chimbari, M. Macherera, and S. Mukaratirwa, 'Micro-spatial distribution of malaria cases and control strategies at ward level in Gwanda district, Matabeleland South, Zimbabwe', *Malar J*, vol. 16, no. 1, p. 476, Dec. 2017, doi: 10.1186/s12936-017-2116-1.
[22] C. By-Nc-Sa, 'World malaria report 2023'.
[23] M. Thellier *et al.*, 'Changes in malaria epidemiology in France and worldwide, 2000–2015', *Médecine et Maladies Infectieuses*, vol. 50, no. 2, pp. 99–112, Mar. 2020, doi: 10.1016/j.medmal.2019.06.002.
[24] P. J. Rosenthal, 'Malaria in 2022: Challenges and Progress', *The American Journal of Tropical Medicine and Hygiene*, vol. 106, no. 6, pp. 1565–1567, Jun. 2022, doi: 10.4269/ajtmh.22-0128.
[25] T. Gondwe *et al.*, 'Epidemiological Trends of Malaria in Five Years and under Children of Nsanje District in Malawi, 2015–2019', *IJERPH*, vol. 18, no. 23, p. 12784, Dec. 2021, doi: 10.3390/ijerph182312784.
[26] 'Malaria - world map'. Accessed: Feb. 12, 2024. [Online]. Available: https://www.wanda.be/en/a-z-index/malaria-world-map/
[27] M. Roser and H. Ritchie, 'Malaria', *Our World in Data*, Nov. 2019, Accessed: Jul. 06, 2023. [Online]. Available: https://ourworldindata.org/malaria
[28] G. Ruiz Lopez del Prado *et al.*, 'Malaria in developing countries', *J Infect Dev Ctries*, vol. 8, no. 01, pp. 001–004, Jan. 2014, doi: 10.3855/jidc.4610.
[29] '5-5-18-403.pdf'.
[30] C. A. Moxon, M. P. Gibbins, D. McGuinness, D. A. Milner, and M. Marti, 'New Insights into Malaria Pathogenesis', *Annu. Rev. Pathol. Mech. Dis.*, vol. 15, no. 1, pp. 315–343, Jan. 2020, doi: 10.1146/annurev-pathmechdis-012419-032640.
[31] J. K. Balikuddembe, J. D. Reinhardt, W. Zeng, H. Tola, and B. Di, 'Public health priorities for Sino-Africa cooperation in Eastern Africa in context of flooding and malaria burden in Children: a tridecadal retrospective analysis', *BMC Public Health*, vol. 23, no. 1, p. 1331, Jul. 2023, doi: 10.1186/s12889-023-16220-7.
[32] A. Lalremruata *et al.*, 'Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing', *Malar J*, vol. 16, no. 1, p. 398, Dec. 2017, doi: 10.1186/s12936-017-2044-0.

[33] P. Ruiz Cuenca, S. Key, K. A. Lindblade, I. Vythilingam, C. Drakeley, and K. Fornace, 'Is there evidence of sustained human-mosquito-human transmission of the zoonotic malaria Plasmodium knowlesi? A systematic literature review', *Malar J*, vol. 21, no. 1, p. 89, Mar. 2022, doi: 10.1186/s12936-022-04110-z.
[34] A. Kantele and T. S. Jokiranta, 'Review of Cases With the Emerging Fifth Human Malaria Parasite, Plasmodium knowlesi', *Clinical Infectious Diseases*, vol. 52, no. 11, pp. 1356–1362, Jun. 2011, doi: 10.1093/cid/cir180.
[35] S. Basu and P. K. Sahi, 'Malaria: An Update', *Indian J Pediatr*, vol. 84, no. 7, pp. 521–528, Jul. 2017, doi: 10.1007/s12098-017-2332-2.
[36] A. F. Cowman and B. S. Crabb, 'Invasion of Red Blood Cells by Malaria Parasites', *Cell*, vol. 124, no. 4, pp. 755–766, Feb. 2006, doi: 10.1016/j.cell.2006.02.006.
[37] A. F. Cowman and B. S. Crabb, 'Invasion of Red Blood Cells by Malaria Parasites', *Cell*, vol. 124, no. 4, pp. 755–766, Feb. 2006, doi: 10.1016/j.cell.2006.02.006.
[38] L. M. Smith *et al.*, 'An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*', *Science*, vol. 368, no. 6492, pp. 754–759, May 2020, doi: 10.1126/science.aba4357.
[39] D. H. Kerlin and M. L. Gatton, 'Preferential Invasion by Plasmodium Merozoites and the Self-Regulation of Parasite Burden', *PLoS ONE*, vol. 8, no. 2, p. e57434, Feb. 2013, doi: 10.1371/journal.pone.0057434.
[40] K. Venugopal, F. Hentzschel, G. Valkiūnas, and M. Marti, 'Plasmodium asexual growth and sexual development in the haematopoietic niche of the host', *Nat Rev Microbiol*, vol. 18, no. 3, pp. 177–189, Mar. 2020, doi: 10.1038/s41579-019-0306-2.
[41] S. C. Wassmer *et al.*, 'Investigating the Pathogenesis of Severe Malaria: A Multidisciplinary and Cross-Geographical Approach', *The American Journal of Tropical Medicine and Hygiene*, vol. 93, no. 3_Suppl, pp. 42–56, Sep. 2015, doi: 10.4269/ajtmh.14-0841.
[42] P. Wilairatana, N. Tangpukdee, and S. Krudsood, 'Definition of hyperparasitemia in severe falciparum malaria should be updated', *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 7, p. 586, Jul. 2013, doi: 10.1016/S2221-1691(13)60119-7.
[43] D. D. Laishram *et al.*, 'The complexities of malaria disease manifestations with a focus on asymptomatic malaria', *Malar J*, vol. 11, no. 1, p. 29, Dec. 2012, doi: 10.1186/1475-2875-11-29.
[44] L. Chan, M. H. Dietrich, W. Nguitragool, and W. Tham, '*Plasmodium vivax* Reticulocyte Binding Proteins for invasion into reticulocytes', *Cellular Microbiology*, vol. 22, no. 1, Jan. 2020, doi: 10.1111/cmi.13110.
[45] P. G. McQueen and F. E. McKenzie, 'Age-structured red blood cell susceptibility and the dynamics of malaria infections', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 101, no. 24, pp. 9161–9166, Jun. 2004, doi: 10.1073/pnas.0308256101.
[46] H. Li, Z. L. Liu, L. Lu, P. Buffet, and G. E. Karniadakis, 'How the spleen reshapes and retains young and old red blood cells: A computational investigation', *PLoS Comput Biol*, vol. 17, no. 11, p. e1009516, Nov. 2021, doi: 10.1371/journal.pcbi.1009516.

[47] N. M. Anstey *et al.*, ‘Knowlesi malaria: Human risk factors, clinical spectrum, and pathophysiology’, in *Advances in Parasitology*, vol. 113, Elsevier, 2021, pp. 1–43. doi: 10.1016/bs.apar.2021.08.001.
[48] J.-W. Hang, F. Tukijan, E.-Q.-H. Lee, S. R. Abdeen, Y. Aniweh, and B. Malleret, ‘Zoonotic Malaria: Non-Laverania Plasmodium Biology and Invasion Mechanisms’, *Pathogens*, vol. 10, no. 7, p. 889, Jul. 2021, doi: 10.3390/pathogens10070889.
[49] B. Galatas, Q. Bassat, and A. Mayor, ‘Malaria Parasites in the Asymptomatic: Looking for the Hay in the Haystack’, *Trends in Parasitology*, vol. 32, no. 4, pp. 296–308, Apr. 2016, doi: 10.1016/j.pt.2015.11.015.
[50] K. A. Koram and M. E. Molyneux, ‘When Is “Malaria” Malaria? The Different Burdens of Malaria Infection, Malaria Disease, and Malaria-Like Illnesses’, *The American Journal of Tropical Medicine and Hygiene*, vol. 77, no. 6_Suppl, pp. 1–5, Dec. 2007, doi: 10.4269/ajtmh.77.6.suppl.1.
[51] *Manson´s Tropical disease*.
[52] M. A. Greischar, A. F. Read, and O. N. Bjørnstad, ‘Synchrony in Malaria Infections: How Intensifying Within-Host Competition Can Be Adaptive’, *The American Naturalist*, vol. 183, no. 2, pp. E36–E49, Feb. 2014, doi: 10.1086/674357.
[53] K. M. Kimenyi, K. Wamae, and L. I. Ochola-Oyier, ‘Understanding P. falciparum Asymptomatic Infections: A Proposition for a Transcriptomic Approach’, *Front. Immunol.*, vol. 10, p. 2398, Oct. 2019, doi: 10.3389/fimmu.2019.02398.
[54] N. R. Noordin *et al.*, ‘Prevalence of Asymptomatic and/or Low-Density Malaria Infection among High-Risk Groups in Peninsular Malaysia’, *The American Journal of Tropical Medicine and Hygiene*, vol. 103, no. 3, pp. 1107–1110, Sep. 2020, doi: 10.4269/ajtmh.20-0268.
[55] A. Bartoloni and L. Zammarchi, ‘CLINICAL ASPECTS OF UNCOMPLICATED AND SEVERE MALARIA’, *Mediterr J Hematol Infect Dis*, vol. 4, no. 1, p. e2012026, May 2012, doi: 10.4084/mjhid.2012.026.
[56] A. Trampuz, M. Jereb, I. Muzlovic, and R. M. Prabhu, ‘[No title found]’, *Crit Care*, vol. 7, no. 4, p. 315, 2003, doi: 10.1186/cc2183.
[57] M. Meremikwu, E. Ezedinachi, and J. E. Ehiri, ‘Malaria in Women and Children’, in *Maternal and Child Health*, J. Ehiri, Ed., Boston, MA: Springer US, 2009, pp. 205–223. doi: 10.1007/b106524_12.
[58] X. Song *et al.*, ‘Cerebral malaria induced by plasmodium falciparum: clinical features, pathogenesis, diagnosis, and treatment’, *Front. Cell. Infect. Microbiol.*, vol. 12, p. 939532, Jul. 2022, doi: 10.3389/fcimb.2022.939532.
[59] E. A. Ashley, A. Pyae Phyo, and C. J. Woodrow, ‘Malaria’, *The Lancet*, vol. 391, no. 10130, pp. 1608–1621, Apr. 2018, doi: 10.1016/S0140-6736(18)30324-6.
[60] K. Maitland, ‘Management of severe paediatric malaria in resource-limited settings’, *BMC Med*, vol. 13, no. 1, p. 42, Dec. 2015, doi: 10.1186/s12916-014-0263-6.
[61] ‘Severe Malaria’, *Trop Med Int Health*, vol. 19, pp. 7–131, Sep. 2014, doi: 10.1111/tmi.12313_2.
[62] S. Seneviratne *et al.*, ‘A malaria death due to an imported Plasmodium falciparum infection in Sri Lanka during the prevention of re-establishment phase of malaria’, *Malar J*, vol. 22, no. 1, p. 243, Aug. 2023, doi: 10.1186/s12936-023-04681-5.

[63] A. R. Jensen, Y. Adams, and L. Hviid, ‘Cerebral *Plasmodium falciparum* malaria: The role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent it’, *Immunol Rev*, vol. 293, no. 1, pp. 230–252, Jan. 2020, doi: 10.1111/imr.12807.
[64] N. J. White, ‘Severe malaria’, *Malar J*, vol. 21, no. 1, p. 284, Oct. 2022, doi: 10.1186/s12936-022-04301-8.
[65] N. Tangpukdee, S. Krudsood, S. Kano, and P. Wilairatana, ‘Falciparum malaria parasitemia index for predicting severe malaria: PARASITEMIA INDEX FOR FALCIPARUM MALARIA’, *International Journal of Laboratory Hematology*, vol. 34, no. 3, pp. 320–327, Jun. 2012, doi: 10.1111/j.1751-553X.2011.01398.x.
[66] M. A. Oboh, E. C. Oriero, T. Ndiaye, A. S. Badiane, D. Ndiaye, and A. Amambua-Ngwa, ‘Comparative analysis of four malaria diagnostic tools and implications for malaria treatment in southwestern Nigeria’, *International Journal of Infectious Diseases*, vol. 108, pp. 377–381, Jul. 2021, doi: 10.1016/j.ijid.2021.05.049.
[67] A. Mbanefo and N. Kumar, ‘Evaluation of Malaria Diagnostic Methods as a Key for Successful Control and Elimination Programs’, *TropicalMed*, vol. 5, no. 2, p. 102, Jun. 2020, doi: 10.3390/tropicalmed5020102.
[68] H. Reyburn, J. Ruanda, O. Mwerinde, and C. Drakeley, ‘The contribution of microscopy to targeting antimalarial treatment in a low transmission area of Tanzania’, *Malar J*, vol. 5, no. 1, p. 4, Dec. 2006, doi: 10.1186/1475-2875-5-4.
[69] J. Rek *et al.*, ‘Characterizing microscopic and submicroscopic malaria parasitaemia at three sites with varied transmission intensity in Uganda’, *Malar J*, vol. 15, no. 1, p. 470, Dec. 2016, doi: 10.1186/s12936-016-1519-8.
[70] A. R. Berendt, D. J. P. Ferguson, and C. I. Newbold, ‘Sequestration in Plasmodium falciparum malaria: Sticky cells and sticky problems’, *Parasitology Today*, vol. 6, no. 8, pp. 247–254, Aug. 1990, doi: 10.1016/0169-4758(90)90184-6.
[71] A. M. Dondorp *et al.*, ‘Estimation of the Total Parasite Biomass in Acute Falciparum Malaria from Plasma PfHRP2’, *PLoS Med*, vol. 2, no. 8, p. e204, Aug. 2005, doi: 10.1371/journal.pmed.0020204.
[72] B. B. Finlay and S. Falkow, ‘Common themes in microbial pathogenicity’.
[73] J. W. Peterson, ‘Bacterial Pathogenesis’, in *Medical Microbiology*, 4th ed., S. Baron, Ed., Galveston (TX): University of Texas Medical Branch at Galveston, 1996. Accessed: Apr. 12, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK8526/
[74] Á. Kun, A. G. Hubai, A. Král, J. Mokos, B. Á. Mikulecz, and Á. Radványi, ‘Do pathogens always evolve to be less virulent? The virulence–transmission trade-off in light of the COVID-19 pandemic’, *BIOLOGIA FUTURA*, vol. 74, no. 1–2, pp. 69–80, Jun. 2023, doi: 10.1007/s42977-023-00159-2.
[75] T. Bousema, L. Okell, I. Felger, and C. Drakeley, ‘Asymptomatic malaria infections: detectability, transmissibility and public health relevance’, *Nat Rev Microbiol*, vol. 12, no. 12, pp. 833–840, Dec. 2014, doi: 10.1038/nrmicro3364.
[76] C. Zhou *et al.*, ‘Higher Risk of Mortality and Virologic Failure in HIV-Infected Patients With High Viral Load at Antiretroviral Therapy Initiation: An

Observational Cohort Study in Chongqing, China', *Front. Public Health*, vol. 10, p. 800839, Feb. 2022, doi: 10.3389/fpubh.2022.800839.
[77] M. E. Soria *et al.*, 'High SARS-CoV-2 viral load is associated with a worse clinical outcome of COVID-19 disease', *Access Microbiology*, vol. 3, no. 9, Sep. 2021, doi: 10.1099/acmi.0.000259.
[78] B. Dodet *et al.*, 'Rabies awareness in eight Asian countries', *Vaccine*, vol. 26, no. 50, pp. 6344–6348, Nov. 2008, doi: 10.1016/j.vaccine.2008.09.003.
[79] R. Timms, N. Colegrave, B. H. K. Chan, and A. F. Read, 'The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*', *Parasitology*, vol. 123, no. 1, pp. 1–11, Jul. 2001, doi: 10.1017/S0031182001008083.
[80] L. Schofield, 'Intravascular infiltrates and organ-specific inflammation in malaria pathogenesis', *Immunol Cell Biol*, vol. 85, no. 2, pp. 130–137, Feb. 2007, doi: 10.1038/sj.icb.7100040.
[81] A. A. Escalante, A. S. Cepeda, and M. A. Pacheco, 'Why Plasmodium vivax and Plasmodium falciparum are so different? A tale of two clades and their species diversities', *Malar J*, vol. 21, no. 1, p. 139, Dec. 2022, doi: 10.1186/s12936-022-04130-9.
[82] I. A. Clark and W. B. Cowden, 'Why is the Pathology of Falciparum Worse than that of Vivax Malaria?', *Parasitology Today*, vol. 15, no. 11, pp. 458–461, Nov. 1999, doi: 10.1016/S0169-4758(99)01535-5.
[83] A. J. Cunnington, M. T. Bretscher, S. I. Nogaro, E. M. Riley, and M. Walther, 'Comparison of parasite sequestration in uncomplicated and severe childhood Plasmodium falciparum malaria', *Journal of Infection*, vol. 67, no. 3, pp. 220–230, Sep. 2013, doi: 10.1016/j.jinf.2013.04.013.
[84] J. C. Reeder *et al.*, 'The adhesion of *Plasmodium falciparum* -infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 96, no. 9, pp. 5198–5202, Apr. 1999, doi: 10.1073/pnas.96.9.5198.
[85] H. W. Kingston *et al.*, 'Disease Severity and Effective Parasite Multiplication Rate in Falciparum Malaria', *Open Forum Infectious Diseases*, vol. 4, no. 4, p. ofx169, Oct. 2017, doi: 10.1093/ofid/ofx169.
[86] T. van der Poll and S. M. Opal, 'Host–pathogen interactions in sepsis', *The Lancet Infectious Diseases*, vol. 8, no. 1, pp. 32–43, Jan. 2008, doi: 10.1016/S1473-3099(07)70265-7.
[87] R. Medzhitov, D. S. Schneider, and M. P. Soares, 'Disease Tolerance as a Defense Strategy', *Science*, vol. 335, no. 6071, pp. 936–941, Feb. 2012, doi: 10.1126/science.1214935.
[88] S. J. Hackett, 'Meningococcal bacterial DNA load at presentation correlates with disease severity', *Archives of Disease in Childhood*, vol. 86, no. 1, pp. 44–46, Jan. 2002, doi: 10.1136/adc.86.1.44.
[89] T. Darton *et al.*, 'Severity of Meningococcal Disease Associated with Genomic Bacterial Load', *CLIN INFECT DIS*, vol. 48, no. 5, pp. 587–594, Mar. 2009, doi: 10.1086/596707.
[90] J. Rello *et al.*, 'Severity of Pneumococcal Pneumonia Associated With Genomic Bacterial Load', *Chest*, vol. 136, no. 3, pp. 832–840, Sep. 2009, doi: 10.1378/chest.09-0258.
[91] E. D. Carrol *et al.*, 'High Pneumococcal DNA Loads Are Associated With Mortality in Malawian Children With Invasive Pneumococcal Disease',

Pediatric Infectious Disease Journal, vol. 26, no. 5, pp. 416–422, May 2007, doi: 10.1097/01.inf.0000260253.22994.61.
[92] E. Pujadas *et al.*, 'Comparison of SARS-CoV-2 detection from nasopharyngeal swab samples by the Roche cobas 6800 SARS-CoV-2 test and a laboratory-developed real-time RT-PCR test', *J Med Virol*, vol. 92, no. 9, pp. 1695–1698, Sep. 2020, doi: 10.1002/jmv.25988.
[93] J. Fajnzylber *et al.*, 'SARS-CoV-2 viral load is associated with increased disease severity and mortality', *Nat Commun*, vol. 11, no. 1, p. 5493, Oct. 2020, doi: 10.1038/s41467-020-19057-5.
[94] J. W. Mellors, 'Plasma Viral Load and CD4+ Lymphocytes as Prognostic Markers of HIV-1 Infection', *Ann Intern Med*, vol. 126, no. 12, p. 946, Jun. 1997, doi: 10.7326/0003-4819-126-12-199706150-00003.
[95] W. T. Shearer *et al.*, 'Viral Load and Disease Progression in Infants Infected with Human Immunodeficiency Virus Type 1', *N Engl J Med*, vol. 336, no. 19, pp. 1337–1342, May 1997, doi: 10.1056/NEJM199705083361901.
[96] C. H. N. Costa *et al.*, 'Bone Marrow Parasite Burden among Patients with New World Kala-Azar is Associated with Disease Severity', *The American Journal of Tropical Medicine and Hygiene*, vol. 90, no. 4, pp. 621–626, Apr. 2014, doi: 10.4269/ajtmh.13-0376.
[97] E. C. Sabino *et al.*, 'Detection of *Trypanosoma cruzi* DNA in blood by PCR is associated with Chagas cardiomyopathy and disease severity: *T. cruzi* PCR status correlates with clinical disease', *Eur J Heart Fail*, vol. 17, no. 4, pp. 416–423, Apr. 2015, doi: 10.1002/ejhf.220.
[98] A. L. Basquiera, 'Risk progression to chronic Chagas cardiomyopathy: influence of male sex and of parasitaemia detected by polymerase chain reaction', *Heart*, vol. 89, no. 10, pp. 1186–1190, Oct. 2003, doi: 10.1136/heart.89.10.1186.
[99] P. A. Buffet *et al.*, 'The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology', *Blood*, vol. 117, no. 2, pp. 381–392, Jan. 2011, doi: 10.1182/blood-2010-04-202911.
[100] H. J. Lee *et al.*, 'Integrated pathogen load and dual transcriptome analysis of systemic host-pathogen interactions in severe malaria', *SCIENCE TRANSLATIONAL MEDICINE*, 2018.
[101] D. A. Milner, 'Malaria Pathogenesis', *Cold Spring Harb Perspect Med*, vol. 8, no. 1, p. a025569, Jan. 2018, doi: 10.1101/cshperspect.a025569.
[102] A. J. Cunnington, 'The Importance of Pathogen Load', *PLoS Pathog*, vol. 11, no. 1, p. e1004563, Jan. 2015, doi: 10.1371/journal.ppat.1004563.
[103] J. Farrar *et al.*, *Manson's Tropical Diseases E-Book*. Elsevier Health Sciences, 2023.
[104] I. C. E. Hendriksen *et al.*, 'Diagnosing Severe Falciparum Malaria in Parasitaemic African Children: A Prospective Evaluation of Plasma PfHRP2 Measurement', *PLoS Med*, vol. 9, no. 8, p. e1001297, Aug. 2012, doi: 10.1371/journal.pmed.1001297.
[105] M. P. Rubach *et al.*, 'Plasma Plasmodium falciparum Histidine-Rich Protein-2 Concentrations Are Associated with Malaria Severity and Mortality in Tanzanian Children', *PLoS ONE*, vol. 7, no. 5, p. e35985, May 2012, doi: 10.1371/journal.pone.0035985.
[106] L. L. Fox *et al.*, 'Histidine-Rich Protein 2 Plasma Levels Predict Progression to Cerebral Malaria in Malawian Children With Plasmodium

falciparum Infection', *The Journal of Infectious Diseases*, vol. 208, no. 3, pp. 500–503, Aug. 2013, doi: 10.1093/infdis/jit176.
[107] B. O. Carvalho *et al.*, 'On the Cytoadhesion of *Plasmodium vivax* – Infected Erythrocytes', *J INFECT DIS*, vol. 202, no. 4, pp. 638–647, Aug. 2010, doi: 10.1086/654815.
[108] J. D. Kwak, J. J. Young, A. C. Stuij, R. Koelewijn, J. J. Van Hellemond, and P. J. J. Van Genderen, 'A comparative study of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) blood levels and peripheral blood parasitemia as parameters of disease severity in individuals with imported falciparum malaria', *Travel Medicine and Infectious Disease*, vol. 42, p. 102076, Jul. 2021, doi: 10.1016/j.tmaid.2021.102076.
[109] S. Y. Liang and A. Kumar, 'Empiric Antimicrobial Therapy in Severe Sepsis and Septic Shock: Optimizing Pathogen Clearance', *Curr Infect Dis Rep*, vol. 17, no. 7, p. 36, Jul. 2015, doi: 10.1007/s11908-015-0493-6.
[110] H. Tripathi *et al.*, 'Malaria therapeutics: are we close enough?', *Parasites Vectors*, vol. 16, no. 1, p. 130, Apr. 2023, doi: 10.1186/s13071-023-05755-8.
[111] N. J. White, 'Malaria parasite clearance', *Malar J*, vol. 16, no. 1, p. 88, Dec. 2017, doi: 10.1186/s12936-017-1731-1.
[112] N. White, 'The parasite clearance curve', *Malar J*, vol. 10, no. 1, p. 278, Dec. 2011, doi: 10.1186/1475-2875-10-278.
[113] N. J. White, 'The assessment of antimalarial drug efficacy', *Trends in Parasitology*, vol. 18, no. 10, pp. 458–464, Oct. 2002, doi: 10.1016/S1471-4922(02)02373-5.
[114] G. A. Balint, 'Artemisinin and its derivatives: an important new class of antimalarial agents', *Pharmacology & Therapeutics*, vol. 90, no. 2–3, pp. 261–265, May 2001, doi: 10.1016/S0163-7258(01)00140-1.
[115] N. Day *et al.*, 'Clearance kinetics of parasites and pigment-containing leukocytes in severe malaria', *Blood*, vol. 88, no. 12, pp. 4694–4700, Dec. 1996, doi: 10.1182/blood.V88.12.4694.bloodjournal88124694.
[116] K. Chotivanich *et al.*, 'Central Role of the Spleen in Malaria Parasite Clearance', *J INFECT DIS*, vol. 185, no. 10, pp. 1538–1541, May 2002, doi: 10.1086/340213.
[117] F. A. Fatih *et al.*, 'Cytoadherence and virulence - the case of Plasmodium knowlesi malaria', *Malar J*, vol. 11, no. 1, p. 33, Dec. 2012, doi: 10.1186/1475-2875-11-33.
[118] F. Abanyie, S. D. Acharya, I. Leavy, M. Bowe, and K. R. Tan, 'Safety and Effectiveness of Intravenous Artesunate for Treatment of Severe Malaria in the United States—April 2019 Through December 2020', *Clinical Infectious Diseases*, vol. 73, no. 11, pp. 1965–1972, Dec. 2021, doi: 10.1093/cid/ciab570.
[119] M. Eder, H. Farne, T. Cargill, A. Abbara, and R. N. Davidson, 'Intravenous artesunate versus intravenous quinine in the treatment of severe falciparum malaria: a retrospective evaluation from a UK centre', *Pathogens and Global Health*, vol. 106, no. 3, pp. 181–187, Jul. 2012, doi: 10.1179/2047773212Y.0000000032.
[120] T. Zoller *et al.*, 'Intravenous Artesunate for Severe Malaria in Travelers, Europe', *Emerg. Infect. Dis.*, vol. 17, no. 5, pp. 771–777, May 2011, doi: 10.3201/eid1705.101229.

[121] Q. Li and P. Weina, ‘Artesunate: The Best Drug in the Treatment of Severe and Complicated Malaria’, *Pharmaceuticals*, vol. 3, no. 7, pp. 2322–2332, Jul. 2010, doi: 10.3390/ph3072322.
[122] X. Su, C. Zhang, and D. A. Joy, ‘Host-Malaria Parasite Interactions and Impacts on Mutual Evolution’, *Front. Cell. Infect. Microbiol.*, vol. 10, p. 587933, Oct. 2020, doi: 10.3389/fcimb.2020.587933.
[123] B. B. Andrade and M. Barral-Netto, ‘Biomarkers for susceptibility to infection and disease severity in human malaria’, *Mem. Inst. Oswaldo Cruz*, vol. 106, no. suppl 1, pp. 70–78, Aug. 2011, doi: 10.1590/S0074-02762011000900009.
[124] T. N. Williams, ‘Human red blood cell polymorphisms and malaria’, *Current Opinion in Microbiology*, vol. 9, no. 4, pp. 388–394, Aug. 2006, doi: 10.1016/j.mib.2006.06.009.
[125] M. C. Castro, ‘Malaria Transmission and Prospects for Malaria Eradication: The Role of the Environment’, *Cold Spring Harb Perspect Med*, vol. 7, no. 10, p. a025601, Oct. 2017, doi: 10.1101/cshperspect.a025601.
[126] W. Leal Filho, J. May, M. May, and G. J. Nagy, ‘Climate change and malaria: some recent trends of malaria incidence rates and average annual temperature in selected sub-Saharan African countries from 2000 to 2018’, *Malar J*, vol. 22, no. 1, p. 248, Aug. 2023, doi: 10.1186/s12936-023-04682-4.
[127] D. Kwiatkowski, ‘Science, medicine, and the future: Susceptibility to infection’, *BMJ*, vol. 321, no. 7268, pp. 1061–1065, Oct. 2000, doi: 10.1136/bmj.321.7268.1061.
[128] A. Alcaïs, L. Abel, and J.-L. Casanova, ‘Human genetics of infectious diseases: between proof of principle and paradigm’, *J. Clin. Invest.*, vol. 119, no. 9, pp. 2506–2514, Sep. 2009, doi: 10.1172/JCI38111.
[129] G. Alkhatib, ‘The biology of CCR5 and CXCR4’:, *Current Opinion in HIV and AIDS*, vol. 4, no. 2, pp. 96–103, Mar. 2009, doi: 10.1097/COH.0b013e328324bbec.
[130] I. C. Withrock *et al.*, ‘Genetic diseases conferring resistance to infectious diseases’, *Genes & Diseases*, vol. 2, no. 3, pp. 247–254, Sep. 2015, doi: 10.1016/j.gendis.2015.02.008.
[131] M. J. Mackinnon, T. W. Mwangi, R. W. Snow, K. Marsh, and T. N. Williams, ‘Heritability of Malaria in Africa’, *PLoS Med*, vol. 2, no. 12, p. e340, Nov. 2005, doi: 10.1371/journal.pmed.0020340.
[132] P. W. Hedrick, ‘Population genetics of malaria resistance in humans’, *Heredity*, vol. 107, no. 4, pp. 283–304, Oct. 2011, doi: 10.1038/hdy.2011.16.
[133] S. Eridani, ‘Sickle Cell Protection from Malaria’, *Hematology Reports*, vol. 3, no. 3, p. e24, Nov. 2011, doi: 10.4081/hr.2011.e24.
[134] V. Nussenblatt and R. D. Semba, ‘Micronutrient malnutrition and the pathogenesis of malarial anemia’, *Acta Tropica*, vol. 82, no. 3, pp. 321–337, Jun. 2002, doi: 10.1016/S0001-706X(02)00049-9.
[135] S. Ehrhardt *et al.*, ‘Malaria, Anemia, and Malnutrition in African Children—Defining Intervention Priorities’, *J INFECT DIS*, vol. 194, no. 1, pp. 108–114, Jul. 2006, doi: 10.1086/504688.
[136] L. Mancio-Silva *et al.*, ‘Nutrient sensing modulates malaria parasite virulence’, *Nature*, vol. 547, no. 7662, pp. 213–216, Jul. 2017, doi: 10.1038/nature23009.

[137] M. Kumar, K. Skillman, and M. T. Duraisingh, ‘Linking nutrient sensing and gene expression in *Plasmodium falciparum* blood-stage parasites’, *Mol Microbiol*, vol. 115, no. 5, pp. 891–900, May 2021, doi: 10.1111/mmi.14652.
[138] S. Biadgilign, T. Mgutshini, D. Haile, B. Gebremichael, Y. Moges, and K. Tilahun, ‘Epidemiology of obesity and overweight in sub-Saharan Africa: a protocol for a systematic review and meta-analysis’, *BMJ Open*, vol. 7, no. 11, p. e017666, Nov. 2017, doi: 10.1136/bmjopen-2017-017666.
[139] J. H. Goedecke and A. E. Mendham, ‘Pathophysiology of type 2 diabetes in sub-Saharan Africans’, *Diabetologia*, vol. 65, no. 12, pp. 1967–1980, Dec. 2022, doi: 10.1007/s00125-022-05795-2.
[140] L. M. A. J. Muller *et al.*, ‘Increased Risk of Common Infections in Patients with Type 1 and Type 2 Diabetes Mellitus’, *Clinical Infectious Diseases*, vol. 41, no. 3, pp. 281–288, Aug. 2005, doi: 10.1086/431587.
[141] I. Danquah, G. Bedu-Addo, and F. P. Mockenhaupt, ‘Type 2 Diabetes Mellitus and Increased Risk for Malaria Infection’, *Emerg. Infect. Dis.*, vol. 16, no. 10, pp. 1601–1604, Oct. 2010, doi: 10.3201/eid1610.100399.
[142] K. Wyss *et al.*, ‘Obesity and Diabetes as Risk Factors for Severe Plasmodium falciparum Malaria: Results From a Swedish Nationwide Study’, *Clinical Infectious Diseases*, vol. 65, no. 6, pp. 949–958, Sep. 2017, doi: 10.1093/cid/cix437.
[143] B. Sobha Kumari and R. K. Chandra, ‘Overnutrition and immune responses’, *Nutrition Research*, vol. 13, pp. S3–S18, Jan. 1993, doi: 10.1016/S0271-5317(05)80281-7.
[144] P. J. Delves, ‘The Immune System’, *ADVANCES IN IMMUNOLOGY*, 2000.
[145] A. Iwasaki and R. Medzhitov, ‘Control of adaptive immunity by the innate immune system’, *Nat Immunol*, vol. 16, no. 4, pp. 343–353, Apr. 2015, doi: 10.1038/ni.3123.
[146] A. D. Douglas *et al.*, ‘Substantially Reduced Pre-patent Parasite Multiplication Rates Are Associated With Naturally Acquired Immunity to Plasmodium falciparum’, *The Journal of Infectious Diseases*, vol. 203, no. 9, pp. 1337–1340, May 2011, doi: 10.1093/infdis/jir033.
[147] K. Artavanis-Tsakonas, J. E. Tongren, and E. M. Riley, ‘The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology’, *Clinical and Experimental Immunology*, vol. 133, no. 2, pp. 145–152, Jul. 2003, doi: 10.1046/j.1365-2249.2003.02174.x.
[148] J. Illingworth *et al.*, ‘Chronic Exposure to *Plasmodium falciparum* Is Associated with Phenotypic Evidence of B and T Cell Exhaustion’, *The Journal of Immunology*, vol. 190, no. 3, pp. 1038–1047, Feb. 2013, doi: 10.4049/jimmunol.1202438.
[149] P. Liehl *et al.*, ‘Innate Immunity Induced by Plasmodium Liver Infection Inhibits Malaria Reinfections’, *Infect Immun*, vol. 83, no. 3, pp. 1172–1180, Mar. 2015, doi: 10.1128/IAI.02796-14.
[150] G. J. Wright and J. C. Rayner, ‘Plasmodium falciparum Erythrocyte Invasion: Combining Function with Immune Evasion’, *PLoS Pathog*, vol. 10, no. 3, p. e1003943, Mar. 2014, doi: 10.1371/journal.ppat.1003943.
[151] L. Molineaux, ‘*Plasmodium falciparum* malaria: some epidemiological implications of parasite and host diversity’, *Annals of Tropical Medicine & Parasitology*, vol. 90, no. 4, pp. 379–393, Jan. 1996, doi: 10.1080/00034983.1996.11813067.

[152] V. R. D. Mendonça and M. Barral-Netto, ‘Immunoregulation in human malaria: the challenge of understanding asymptomatic infection’, *Mem. Inst. Oswaldo Cruz*, vol. 110, no. 8, pp. 945–955, Dec. 2015, doi: 10.1590/0074-02760150241.
[153] P. Schlagenhauf, R. Steffen, and L. Loutan, ‘Migrants as a Major Risk Group for Imported Malaria in European Countries’, *Journal of Travel Medicine*, vol. 10, no. 2, pp. 106–107, Mar. 2006, doi: 10.2310/7060.2003.31764.
[154] J. Langhorne, F. M. Ndungu, A.-M. Sponaas, and K. Marsh, ‘Immunity to malaria: more questions than answers’, *Nat Immunol*, vol. 9, no. 7, pp. 725–732, Jul. 2008, doi: 10.1038/ni.f.205.
[155] A. Abossie, T. Yohanes, A. Nedu, W. Tafesse, and M. Damitie, ‘Prevalence of Malaria and Associated Risk Factors Among Febrile Children Under Five Years: A Cross-Sectional Study in Arba Minch Zuria District, South Ethiopia’, *IDR*, vol. Volume 13, pp. 363–372, Feb. 2020, doi: 10.2147/IDR.S223873.
[156] I. A. Clark, L. M. Alleva, A. C. Budd, and W. B. Cowden, ‘Understanding the role of inflammatory cytokines in malaria and related diseases’, *Travel Medicine and Infectious Disease*, vol. 6, no. 1–2, pp. 67–81, Jan. 2008, doi: 10.1016/j.tmaid.2007.07.002.
[157] I. A. Clark, L. M. Alleva, A. C. Mills, and W. B. Cowden, ‘Pathogenesis of Malaria and Clinically Similar Conditions’, *Clin Microbiol Rev*, vol. 17, no. 3, pp. 509–539, Jul. 2004, doi: 10.1128/CMR.17.3.509-539.2004.
[158] I. A. Clark *et al.*, ‘Tissue distribution of migration inhibitory factor and inducible nitric oxide synthase in falciparum malaria and sepsis in African children’, *Malaria Journal*, 2003.
[159] I. A. Clark and B. Vissel, ‘The meteorology of cytokine storms, and the clinical usefulness of this knowledge’, *Semin Immunopathol*, vol. 39, no. 5, pp. 505–516, Jul. 2017, doi: 10.1007/s00281-017-0628-y.
[160] E. Mortaz, P. Tabarsi, M. Varahram, G. Folkerts, and I. M. Adcock, ‘The Immune Response and Immunopathology of COVID-19’, *Front. Immunol.*, vol. 11, p. 2037, Aug. 2020, doi: 10.3389/fimmu.2020.02037.
[161] T. Smith, I. Felger, M. Tanner, and H.-P. Beck, ‘11. Premunition in Plasmodium falciparum infection: insights from the epidemiology of multiple infections’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 93, pp. 59–64, Feb. 1999, doi: 10.1016/S0035-9203(99)90329-2.
[162] J. O. Sarfo *et al.*, ‘Malaria amongst children under five in sub-Saharan Africa: a scoping review of prevalence, risk factors and preventive interventions’, *Eur J Med Res*, vol. 28, no. 1, p. 80, Feb. 2023, doi: 10.1186/s40001-023-01046-1.
[163] J. Schantz-Dunn and N. M. Nour, ‘Malaria and Pregnancy: A Global Health Perspective’.
[164] D. G. Lalloo and D. R. Hill, ‘Preventing malaria in travellers’, *BMJ*, vol. 336, no. 7657, pp. 1362–1366, Jun. 2008, doi: 10.1136/bmj.a153.
[165] L. M. Cohee and M. K. Laufer, ‘Malaria in Children’, *Pediatric Clinics of North America*, vol. 64, no. 4, pp. 851–866, Aug. 2017, doi: 10.1016/j.pcl.2017.03.004.

[166] E. N. Takem and U. D'Alessandro, 'MALARIA IN PREGNANCY', *Mediterr J Hematol Infect Dis*, vol. 5, no. 1, p. e2013010, Jan. 2013, doi: 10.4084/mjhid.2013.010.
[167] E. T. Ryan and K. C. Kain, 'Illness after International Travel', *The New England Journal of Medicine*, 2002.
[168] K. C. Kain, M. A. Harrington, S. Tennyson, and J. S. Keystone, 'Imported Malaria: Prospective Analysis of Problems in Diagnosis and Management', *CLIN INFECT DIS*, vol. 27, no. 1, pp. 142–149, Jul. 1998, doi: 10.1086/514616.
[169] T. Jelinek *et al.*, 'Imported Falciparum Malaria in Europe: Sentinel Surveillance Data from the European Network on Surveillance of Imported Infectious Diseases', *Clinical Infectious Diseases*, vol. 34, no. 5, pp. 572–576, 2002.
[170] R. Romi, G. Sabatinelli, and G. Majori, 'Malaria Epidemiological Situation in Italy and Evaluation of Malaria Incidence in Italian Travelers', *Journal of Travel Medicine*, vol. 8, no. 1, pp. 6–011, Mar. 2006, doi: 10.2310/7060.2001.5140.
[171] A. Pavli and H. C. Maltezou, 'Malaria and travellers visiting friends and relatives', *Travel Medicine and Infectious Disease*, vol. 8, no. 3, pp. 161–168, May 2010, doi: 10.1016/j.tmaid.2010.01.003.
[172] T. Pistone, A. Diallo, M. Mechain, M.-C. Receveur, and D. Malvy, 'Epidemiology of imported malaria give support to the hypothesis of "long-term" semi-immunity to malaria in sub-Saharan African migrants living in France', *Travel Medicine and Infectious Disease*, vol. 12, no. 1, pp. 48–53, Jan. 2014, doi: 10.1016/j.tmaid.2013.08.006.
[173] L. C. Rankin and D. Artis, 'Beyond Host Defense: Emerging Functions of the Immune System in Regulating Complex Tissue Physiology', *Cell*, vol. 173, no. 3, pp. 554–567, Apr. 2018, doi: 10.1016/j.cell.2018.03.013.
[174] K. Deroost, T.-T. Pham, G. Opdenakker, and P. E. Van den Steen, 'The immunological balance between host and parasite in malaria', *FEMS Microbiology Reviews*, vol. 40, no. 2, pp. 208–257, Mar. 2016, doi: 10.1093/femsre/fuv046.
[175] H. L. Anderson, I. E. Brodsky, and N. S. Mangalmurti, 'The Evolving Erythrocyte: Red Blood Cells as Modulators of Innate Immunity', *The Journal of Immunology*, vol. 201, no. 5, pp. 1343–1351, Sep. 2018, doi: 10.4049/jimmunol.1800565.
[176] M. Moras, S. D. Lefevre, and M. A. Ostuni, 'From Erythroblasts to Mature Red Blood Cells: Organelle Clearance in Mammals', *Front. Physiol.*, vol. 8, p. 1076, Dec. 2017, doi: 10.3389/fphys.2017.01076.
[177] T. Yoshida, M. Prudent, and A. D'Alessandro, 'Red blood cell storage lesion: causes and potential clinical consequences', *Blood Transfusion*, no. Blood Transfusion-1 2019 (January-February), pp. 27–52, Jan. 2019, doi: 10.2450/2019.0217-18.
[178] R. van Wijk and W. W. van Solinge, 'The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis', *Blood*, vol. 106, no. 13, pp. 4034–4042, Dec. 2005, doi: 10.1182/blood-2005-04-1622.
[179] T. N. Williams, 'Human red blood cell polymorphisms and malaria', *Current Opinion in Microbiology*, vol. 9, no. 4, pp. 388–394, Aug. 2006, doi: 10.1016/j.mib.2006.06.009.

[180] V. Pretini *et al.*, 'Red Blood Cells: Chasing Interactions', *Front. Physiol.*, vol. 10, p. 945, Jul. 2019, doi: 10.3389/fphys.2019.00945.
[181] R. van Wijk and W. W. van Solinge, 'The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis', *Blood*, vol. 106, no. 13, pp. 4034–4042, Dec. 2005, doi: 10.1182/blood-2005-04-1622.
[182] D. A. Gell, 'Structure and function of haemoglobins', *Blood Cells, Molecules, and Diseases*, vol. 70, pp. 13–42, May 2018, doi: 10.1016/j.bcmd.2017.10.006.
[183] T. Yoshida, M. Prudent, and A. D'Alessandro, 'Red blood cell storage lesion: causes and potential clinical consequences', *Blood Transfusion*, no. Blood Transfusion-1 2019 (January-February), pp. 27–52, Jan. 2019, doi: 10.2450/2019.0217-18.
[184] A. J. Marengo-Rowe, 'Structure-Function Relations of Human Hemoglobins', *Baylor University Medical Center Proceedings*, vol. 19, no. 3, pp. 239–245, Jul. 2006, doi: 10.1080/08998280.2006.11928171.
[185] Y. Ma *et al.*, 'Structural optimization and prospect of constructing hemoglobin oxygen carriers based on hemoglobin', *Heliyon*, vol. 9, no. 9, p. e19430, Sep. 2023, doi: 10.1016/j.heliyon.2023.e19430.
[186] D. Gaur, D. C. G. Mayer, and L. H. Miller, 'Parasite ligand–host receptor interactions during invasion of erythrocytes by Plasmodium merozoites', *International Journal for Parasitology*, vol. 34, no. 13–14, pp. 1413–1429, Dec. 2004, doi: 10.1016/j.ijpara.2004.10.010.
[187] A. F. Cowman, J. Healer, D. Marapana, and K. Marsh, 'Malaria: Biology and Disease', *Cell*, vol. 167, no. 3, pp. 610–624, Oct. 2016, doi: 10.1016/j.cell.2016.07.055.
[188] B. M. Cooke, N. Mohandas, and R. L. Coppel, 'Malaria and the red blood cell membrane', *Seminars in Hematology*, vol. 41, no. 2, pp. 173–188, Apr. 2004, doi: 10.1053/j.seminhematol.2004.01.004.
[189] N. Mohandas and X. An, 'Malaria and human red blood cells', *Med Microbiol Immunol*, vol. 201, no. 4, pp. 593–598, Nov. 2012, doi: 10.1007/s00430-012-0272-z.
[190] J. Delaunay, 'Molecular Basis of Red Cell Membrane Disorders', *Acta Haematol*, vol. 108, no. 4, pp. 210–218, 2002, doi: 10.1159/000065657.
[191] B. Baro, 'Plasmodium falciparum exploits CD44 as a co-receptor for erythrocyte invasion'.
[192] A. F. Cowman, C. J. Tonkin, W.-H. Tham, and M. T. Duraisingh, 'The Molecular Basis of Erythrocyte Invasion by Malaria Parasites', *Cell Host & Microbe*, vol. 22, no. 2, pp. 232–245, Aug. 2017, doi: 10.1016/j.chom.2017.07.003.
[193] L. I. Ochola-Oyier *et al.*, 'Few Plasmodium falciparum merozoite ligand and erythrocyte receptor pairs show evidence of balancing selection', *Infection, Genetics and Evolution*, vol. 69, pp. 235–245, Apr. 2019, doi: 10.1016/j.meegid.2019.02.004.
[194] W.-H. Tham, J. Healer, and A. F. Cowman, 'Erythrocyte and reticulocyte binding-like proteins of Plasmodium falciparum', *Trends in Parasitology*, vol. 28, no. 1, pp. 23–30, Jan. 2012, doi: 10.1016/j.pt.2011.10.002.
[195] I. A. Cockburn *et al.*, 'A human complement receptor 1 polymorphism that reduces *Plasmodium falciparum* rosetting confers protection against severe malaria', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 101, no. 1, pp. 272–277, Jan. 2004, doi: 10.1073/pnas.0305306101.

[196] I. A. Cockburn *et al.*, 'A human complement receptor 1 polymorphism that reduces *Plasmodium falciparum* rosetting confers protection against severe malaria', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 101, no. 1, pp. 272–277, Jan. 2004, doi: 10.1073/pnas.0305306101.
[197] P. W. Bowyer *et al.*, 'Variation in Plasmodium falciparum Erythrocyte Invasion Phenotypes and Merozoite Ligand Gene Expression across Different Populations in Areas of Malaria Endemicity', *Infect Immun*, vol. 83, no. 6, pp. 2575–2582, Jun. 2015, doi: 10.1128/IAI.03009-14.
[198] A. Degarege, M. T. Gebrezgi, G. Ibanez, M. Wahlgren, and P. Madhivanan, 'Effect of the ABO blood group on susceptibility to severe malaria: A systematic review and meta-analysis', *Blood Reviews*, vol. 33, pp. 53–62, Jan. 2019, doi: 10.1016/j.blre.2018.07.002.
[199] T. Zerihun, A. Degarege, and B. Erko, 'Association of ABO blood group and Plasmodium falciparum malaria in Dore Bafeno Area, Southern Ethiopia', *Asian Pacific Journal of Tropical Biomedicine*, vol. 1, no. 4, pp. 289–294, Aug. 2011, doi: 10.1016/S2221-1691(11)60045-2.
[200] M. Theron, N. Cross, P. Cawkill, L. Y. Bustamante, and J. C. Rayner, 'An in vitro erythrocyte preference assay reveals that Plasmodium falciparum parasites prefer Type O over Type A erythrocytes', *Sci Rep*, vol. 8, no. 1, p. 8133, May 2018, doi: 10.1038/s41598-018-26559-2.
[201] C. M. Cserti and W. H. Dzik, 'The ABO blood group system and Plasmodium falciparum malaria', *Blood*, vol. 110, no. 7, pp. 2250–2258, Oct. 2007, doi: 10.1182/blood-2007-03-077602.
[202] D. H. Opi *et al.*, 'Non-O ABO blood group genotypes differ in their associations with Plasmodium falciparum rosetting and severe malaria', *PLoS Genet*, vol. 19, no. 9, p. e1010910, Sep. 2023, doi: 10.1371/journal.pgen.1010910.
[203] G. H. Lopez, C. A. Hyland, and R. L. Flower, 'Glycophorins and the MNS blood group system: a narrative review', *Ann Blood*, vol. 6, pp. 39–39, Dec. 2021, doi: 10.21037/aob-21-9.
[204] S. N. Kariuki *et al.*, 'Red blood cell tension protects against severe malaria in the Dantu blood group', *Nature*, vol. 585, no. 7826, pp. 579–583, Sep. 2020, doi: 10.1038/s41586-020-2726-6.
[205] V. Thathy, J. M. Moulds, B. Guyah, W. Otieno, and J. A. Stoute, 'Complement receptor 1 polymorphisms associated with resistance to severe malaria in Kenya', *Malar J*, vol. 4, no. 1, p. 54, Dec. 2005, doi: 10.1186/1475-2875-4-54.
[206] H. H. Hansson *et al.*, 'Human genetic polymorphisms in the Knops blood group are not associated with a protective advantage against Plasmodium falciparum malaria in Southern Ghana', *Malar J*, vol. 12, no. 1, p. 400, Dec. 2013, doi: 10.1186/1475-2875-12-400.
[207] J. A. Rowe, J. M. Moulds, C. I. Newbold, and L. H. Miller, 'P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor', vol. 388, 1997.
[208] J. Moulds *et al.*, 'Identification of complement receptor one (CR1) polymorphisms in West Africa', *Genes Immun*, vol. 1, no. 5, pp. 325–329, Jun. 2000, doi: 10.1038/sj.gene.6363676.
[209] T. N. Williams, 'Red blood cell defects and malaria', *Molecular and Biochemical Parasitology*, vol. 149, no. 2, pp. 121–127, Oct. 2006, doi: 10.1016/j.molbiopara.2006.05.007.

[210] X. An and N. Mohandas, ‘Disorders of red cell membrane’, *Br J Haematol*, vol. 141, no. 3, pp. 367–375, May 2008, doi: 10.1111/j.1365-2141.2008.07091.x.
[211] T. F. de Koning-Ward, M. W. A. Dixon, L. Tilley, and P. R. Gilson, ‘Plasmodium species: master renovators of their host cells’, *Nat Rev Microbiol*, vol. 14, no. 8, pp. 494–507, Aug. 2016, doi: 10.1038/nrmicro.2016.79.
[212] S. G. Tewari, R. P. Swift, J. Reifman, S. T. Prigge, and A. Wallqvist, ‘Metabolic alterations in the erythrocyte during blood-stage development of the malaria parasite’, *Malar J*, vol. 19, no. 1, p. 94, Dec. 2020, doi: 10.1186/s12936-020-03174-z.
[213] K. Slavic, S. Krishna, E. T. Derbyshire, and H. M. Staines, ‘Plasmodial sugar transporters as anti-malarial drug targets and comparisons with other protozoa’, *Malar J*, vol. 10, no. 1, p. 165, Dec. 2011, doi: 10.1186/1475-2875-10-165.
[214] V. Polonais and D. Soldati-Favre, ‘Versatility in the acquisition of energy and carbon sources by the Apicomplexa’, *Biology of the Cell*, vol. 102, no. 8, pp. 435–445, Aug. 2010, doi: 10.1042/BC20100005.
[215] R. J. W. Allen and K. Kirk, ‘The Membrane Potential of the Intraerythrocytic Malaria Parasite Plasmodium falciparum’, *Journal of Biological Chemistry*, vol. 279, no. 12, pp. 11264–11272, Mar. 2004, doi: 10.1074/jbc.M311110200.
[216] D. D. Van Niekerk, F. Du Toit, K. Green, D. Palm, and J. L. Snoep, ‘A detailed kinetic model of glycolysis in Plasmodium falciparum-infected red blood cells for antimalarial drug target identification’, *Journal of Biological Chemistry*, vol. 299, no. 9, p. 105111, Sep. 2023, doi: 10.1016/j.jbc.2023.105111.
[217] G. G. Holz, ‘Lipids and the malarial parasite’.
[218] T. Mitamura and N. M. Q. Palacpac, ‘Lipid metabolism in Plasmodium falciparum-infected erythrocytes: possible new targets for malaria chemotherapy’, *Microbes and Infection*, vol. 5, no. 6, pp. 545–552, May 2003, doi: 10.1016/S1286-4579(03)00070-4.
[219] B. J. Visser, R. W. Wieten, I. M. Nagel, and M. P. Grobusch, ‘Serum lipids and lipoproteins in malaria - a systematic review and meta-analysis’, *Malar J*, vol. 12, no. 1, p. 442, Dec. 2013, doi: 10.1186/1475-2875-12-442.
[220] J.-F. Faucher, E. Ngou-Milama, M. Missinou, R. Ngomo, M. Kombila, and P. Kremsner, ‘The impact of malaria on common lipid parameters’, *Parasitology Research*, vol. 88, no. 12, pp. 1040–1043, Dec. 2002, doi: 10.1007/s00436-002-0712-6.
[221] L. S. De Oliveira *et al.*, ‘Calcium in the Backstage of Malaria Parasite Biology’, *Front. Cell. Infect. Microbiol.*, vol. 11, p. 708834, Jul. 2021, doi: 10.3389/fcimb.2021.708834.
[222] F. Joof *et al.*, ‘Genetic variations in human ATP2B4 gene alter Plasmodium falciparum in vitro growth in RBCs from Gambian adults’, *Malar J*, vol. 22, no. 1, p. 5, Jan. 2023, doi: 10.1186/s12936-022-04359-4.
[223] E. C. Mbanefo *et al.*, ‘Association of glucose-6-phosphate dehydrogenase deficiency and malaria: a systematic review and meta-analysis’, *Sci Rep*, vol. 7, no. 1, p. 45963, Apr. 2017, doi: 10.1038/srep45963.

[224] K. Ayi *et al.*, 'Pyruvate Kinase Deficiency and Malaria', *N Engl J Med*, vol. 358, no. 17, pp. 1805–1810, Apr. 2008, doi: 10.1056/NEJMoa072464.
[225] A. Guindo, R. M. Fairhurst, O. K. Doumbo, T. E. Wellems, and D. A. Diallo, 'X-Linked G6PD Deficiency Protects Hemizygous Males but Not Heterozygous Females against Severe Malaria', *PLoS Med*, vol. 4, no. 3, p. e66, Mar. 2007, doi: 10.1371/journal.pmed.0040066.
[226] S. J. Harcke, D. Rizzolo, and H. T. Harcke, 'G6PD deficiency: An update', *JAAPA*, vol. 32, no. 11, pp. 21–26, Nov. 2019, doi: 10.1097/01.JAA.0000586304.65429.a7.
[227] A. L. Peters and C. J. F. V. Noorden, 'Glucose-6-phosphate Dehydrogenase Deficiency and Malaria: Cytochemical Detection of Heterozygous G6PD Deficiency in Women', *J Histochem Cytochem.*, vol. 57, no. 11, pp. 1003–1011, Nov. 2009, doi: 10.1369/jhc.2009.953828.
[228] P. Machado *et al.*, 'Pyruvate Kinase Deficiency in Sub-Saharan Africa: Identification of a Highly Frequent Missense Mutation (G829A;Glu277Lys) and Association with Malaria', *PLoS ONE*, vol. 7, no. 10, p. e47071, Oct. 2012, doi: 10.1371/journal.pone.0047071.
[229] A. I. Alayash, 'Targeting the red cell enzyme pyruvate kinase with a small allosteric molecule AG-348 may correct underlying pathology of a glycolytic enzymopathy', *haematol*, vol. 106, no. 1, pp. 9–11, Jan. 2021, doi: 10.3324/haematol.2020.266585.
[230] M. Carvalho *et al.*, '2,3-Diphosphoglycerate and the Protective Effect of Pyruvate Kinase Deficiency against Malaria Infection—Exploring the Role of the Red Blood Cell Membrane', *IJMS*, vol. 24, no. 2, p. 1336, Jan. 2023, doi: 10.3390/ijms24021336.
[231] I. Morais *et al.*, 'Synthetic Red Blood Cell-Specific Glycolytic Intermediate 2,3-Diphosphoglycerate (2,3-DPG) Inhibits Plasmodium falciparum Development In Vitro', *Front. Cell. Infect. Microbiol.*, vol. 12, p. 840968, Mar. 2022, doi: 10.3389/fcimb.2022.840968.
[232] G. Min-Oo, A. Fortin, M.-F. Tam, P. Gros, and M. Stevenson, 'Phenotypic expression of pyruvate kinase deficiency and protection against malaria in a mouse model', *Genes Immun*, vol. 5, no. 3, pp. 168–175, May 2004, doi: 10.1038/sj.gene.6364069.
[233] P. M. Durand and T. L. Coetzer, 'Pyruvate kinase deficiency protects against malaria in humans', *Haematologica*, vol. 93, no. 6, pp. 939–940, Jun. 2008, doi: 10.3324/haematol.12450.
[234] R. Kyeremeh, S. Antwi-Baffour, M. Annani-Akollor, J. K. Adjei, O. Addai-Mensah, and M. Frempong, 'Comediation of Erythrocyte Haemolysis by Erythrocyte-Derived Microparticles and Complement during Malaria Infection', *Advances in Hematology*, vol. 2020, pp. 1–5, Aug. 2020, doi: 10.1155/2020/1640480.
[235] L. Tilley, M. W. A. Dixon, and K. Kirk, 'The Plasmodium falciparum-infected red blood cell', *The International Journal of Biochemistry & Cell Biology*, vol. 43, no. 6, pp. 839–842, Jun. 2011, doi: 10.1016/j.biocel.2011.03.012.
[236] S. M. Taylor, C. M. Parobek, and R. M. Fairhurst, 'Haemoglobinopathies and the clinical epidemiology of malaria: a systematic review and meta-analysis', *The Lancet Infectious Diseases*, vol. 12, no. 6, pp. 457–468, Jun. 2012, doi: 10.1016/S1473-3099(12)70055-5.

[237] S. M. Taylor, C. Cerami, and R. M. Fairhurst, ‘Hemoglobinopathies: Slicing the Gordian Knot of Plasmodium falciparum Malaria Pathogenesis’, *PLoS Pathog*, vol. 9, no. 5, p. e1003327, May 2013, doi: 10.1371/journal.ppat.1003327.
[238] G. Pasvol, ‘Does α +-Thalassaemia Protect against Malaria?’, *PLoS Med*, vol. 3, no. 5, p. e235, May 2006, doi: 10.1371/journal.pmed.0030235.
[239] S. A. S. Diakité *et al.*, ‘Stage-dependent fate of Plasmodium falciparum-infected red blood cells in the spleen and sickle-cell trait-related protection against malaria’, *Malar J*, vol. 15, no. 1, p. 482, Dec. 2016, doi: 10.1186/s12936-016-1522-0.
[240] M. Aidoo *et al.*, ‘Protective effects of the sickle cell gene against malaria morbidity and mortality’, *The Lancet*, vol. 359, no. 9314, pp. 1311–1312, Apr. 2002, doi: 10.1016/S0140-6736(02)08273-9.
[241] H. F. Bunn, ‘The triumph of good over evil: protection by the sickle gene against malaria’, *Blood*, vol. 121, no. 1, pp. 20–25, Jan. 2013, doi: 10.1182/blood-2012-08-449397.
[242] F. Lennartz *et al.*, ‘Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria’, *Cell Host & Microbe*, vol. 21, no. 3, pp. 403–414, Mar. 2017, doi: 10.1016/j.chom.2017.02.009.
[243] S. Wambua *et al.*, ‘The Effect of α +-Thalassaemia on the Incidence of Malaria and Other Diseases in Children Living on the Coast of Kenya’, *PLoS Med*, vol. 3, no. 5, p. e158, Apr. 2006, doi: 10.1371/journal.pmed.0030158.
[244] F. P. Mockenhaupt *et al.*, ‘α+-thalassemia protects African children from severe malaria’, *Blood*, vol. 104, no. 7, pp. 2003–2006, Oct. 2004, doi: 10.1182/blood-2003-11-4090.
[245] F. J. I. Fowkes, S. J. Allen, A. Allen, M. P. Alpers, D. J. Weatherall, and K. P. Day, ‘Increased Microerythrocyte Count in Homozygous α+-Thalassaemia Contributes to Protection against Severe Malarial Anaemia’, *PLoS Med*, vol. 5, no. 3, p. e56, Mar. 2008, doi: 10.1371/journal.pmed.0050056.
[246] K. Deroost, T.-T. Pham, G. Opdenakker, and P. E. Van den Steen, ‘The immunological balance between host and parasite in malaria’, *FEMS Microbiology Reviews*, vol. 40, no. 2, pp. 208–257, Mar. 2016, doi: 10.1093/femsre/fuv046.
[247] D. R. C. Freitas, J. B. Santos, and C. N. Castro, ‘Healing with malaria: a brief historical review of malariotherapy for neurosyphilis, mental disorders and other infectious diseases’, *Rev. Soc. Bras. Med. Trop.*, vol. 47, no. 2, pp. 260–261, Apr. 2014, doi: 10.1590/0037-8682-0209-2013.
[248] C. Austin, ‘The History of Malariotherapy for Neurosyphilis’.
[249] S. Bantuchai, H. Imad, and W. Nguitragool, ‘Plasmodium vivax gametocytes and transmission’, *Parasitology International*, vol. 87, p. 102497, Apr. 2022, doi: 10.1016/j.parint.2021.102497.
[250] J. A. Simpson, L. Aarons, W. E. Collins, G. M. Jeffery, and N. J. White, ‘Population dynamics of untreated *Plasmodium falciparum* malaria within the adult human host during the expansion phase of the infection’, *Parasitology*, vol. 124, no. 3, pp. 247–263, Mar. 2002, doi: 10.1017/S0031182001001202.
[251] D. I. Stanisic, J. S. McCarthy, and M. F. Good, ‘Controlled Human Malaria Infection: Applications, Advances, and Challenges’, *Infect Immun*, vol. 86, no. 1, pp. e00479-17, Jan. 2018, doi: 10.1128/IAI.00479-17.

[252] M. Spring, M. Polhemus, and C. Ockenhouse, 'Controlled Human Malaria Infection', *Journal of Infectious Diseases*, vol. 209, no. suppl 2, pp. S40–S45, Jun. 2014, doi: 10.1093/infdis/jiu063.
[253] M. Roestenberg *et al.*, 'Controlled Human Malaria Infections by Intradermal Injection of Cryopreserved Plasmodium falciparum Sporozoites', *The American Journal of Tropical Medicine and Hygiene*, vol. 88, no. 1, pp. 5–13, Jan. 2013, doi: 10.4269/ajtmh.2012.12-0613.
[254] B. Mordmüller *et al.*, 'Direct venous inoculation of Plasmodium falciparum sporozoites for controlled human malaria infection: a dose-finding trial in two centres', *Malar J*, vol. 14, no. 1, p. 117, Dec. 2015, doi: 10.1186/s12936-015-0628-0.
[255] L. E. Coffeng, C. C. Hermsen, R. W. Sauerwein, and S. J. De Vlas, 'The Power of Malaria Vaccine Trials Using Controlled Human Malaria Infection', *PLoS Comput Biol*, vol. 13, no. 1, p. e1005255, Jan. 2017, doi: 10.1371/journal.pcbi.1005255.
[256] L. F. Wockner *et al.*, 'Growth Rate of Plasmodium falciparum: Analysis of Parasite Growth Data from Malaria Volunteer Infection Studies', *The Journal of Infectious Diseases*, p. jiz557, Nov. 2019, doi: 10.1093/infdis/jiz557.
[257] M. LeRoux, V. Lakshmanan, and J. P. Daily, 'Plasmodium falciparum biology: analysis of in vitro versus in vivo growth conditions', *Trends in Parasitology*, vol. 25, no. 10, pp. 474–481, Oct. 2009, doi: 10.1016/j.pt.2009.07.005.
[258] S. Duffy and V. M. Avery, 'Routine In Vitro Culture of Plasmodium falciparum: Experimental Consequences?', *Trends in Parasitology*, vol. 34, no. 7, pp. 564–575, Jul. 2018, doi: 10.1016/j.pt.2018.04.005.
[259] FenixEdu, 'FenixEdu™', FenixEdu. Accessed: Feb. 09, 2024. [Online]. Available: http://www.fenixedu.org
[260] F. L. Schuster, 'Cultivation of *Plasmodium* spp', *Clin Microbiol Rev*, vol. 15, no. 3, pp. 355–364, Jul. 2002, doi: 10.1128/CMR.15.3.355-364.2002.
[261] P. Elis, *The essential guide to effect sizes: statistical power, meta-analysis, and the interpretation of research results*.
[262] L. G. Thiam *et al.*, 'Cell trace far-red is a suitable erythrocyte dye for multi-color *Plasmodium falciparum* invasion phenotyping assays', *Exp Biol Med (Maywood)*, vol. 245, no. 1, pp. 11–20, Jan. 2020, doi: 10.1177/1535370219897393.
[263] N. I. Atuh, A. D. Nota, F. J. Cho, F. Bojang, and A. Amambua-Ngwa, 'A modified two-color flow cytometry assay to quantify in-vitro reinvasion and determine invasion phenotypes at low Plasmodium falciparum parasitemia'.
[264] H. Ali, T. Ahsan, T. Mahmood, S. F. Bakht, M. U. Farooq, and N. Ahmed, 'Parasite Density and The Spectrum of Clinical Illness in Falciparum Malaria', vol. 18, 2008.
[265] B. E. Barber *et al.*, 'Parasite Biomass-Related Inflammation, Endothelial Activation, Microvascular Dysfunction and Disease Severity in Vivax Malaria', *PLoS Pathog*, vol. 11, no. 1, p. e1004558, Jan. 2015, doi: 10.1371/journal.ppat.1004558.
[266] M. J. Mackinnon and A. F. Read, 'Virulence in malaria: an evolutionary viewpoint', *Phil. Trans. R. Soc. Lond. B*, vol. 359, no. 1446, pp. 965–986, Jun. 2004, doi: 10.1098/rstb.2003.1414.

[267] N. Rasti, M. Wahlgren, and Q. Chen, ‘Molecular aspects of malaria pathogenesis’, *FEMS Immunology & Medical Microbiology*, vol. 41, no. 1, pp. 9–26, May 2004, doi: 10.1016/j.femsim.2004.01.010.
[268] N. J. White, D. Chapman, and G. Watt, ‘The effects of multiplication and synchronicity on the vascular distribution of parasites in falciparum malaria’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 86, no. 6, pp. 590–597, Nov. 1992, doi: 10.1016/0035-9203(92)90141-X.
[269] L. B. Stewart *et al.*, ‘Intrinsic multiplication rate variation and plasticity of human blood stage malaria parasites’, *Commun Biol*, vol. 3, no. 1, p. 624, Oct. 2020, doi: 10.1038/s42003-020-01349-7.
[270] I. S. Walker and S. J. Rogerson, ‘Pathogenicity and virulence of malaria: Sticky problems and tricky solutions’, *Virulence*, vol. 14, no. 1, p. 2150456, Dec. 2023, doi: 10.1080/21505594.2022.2150456.
[271] P. Ringwald *et al.*, ‘Parasite virulence factors during falciparum malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines’, *Infect Immun*, vol. 61, no. 12, pp. 5198–5204, Dec. 1993, doi: 10.1128/iai.61.12.5198-5204.1993.
[272] N. D. Pasternak and R. Dzikowski, ‘PfEMP1: An antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum’, *The International Journal of Biochemistry & Cell Biology*, vol. 41, no. 7, pp. 1463–1466, Jul. 2009, doi: 10.1016/j.biocel.2008.12.012.
[273] E. B. Belachew, ‘Immune Response and Evasion Mechanisms of *Plasmodium falciparum* Parasites’, *Journal of Immunology Research*, vol. 2018, pp. 1–6, 2018, doi: 10.1155/2018/6529681.
[274] F. Y. Cai *et al.*, ‘Accounting for red blood cell accessibility reveals distinct invasion strategies in Plasmodium falciparum strains’, *PLoS Comput Biol*, vol. 16, no. 4, p. e1007702, Apr. 2020, doi: 10.1371/journal.pcbi.1007702.
[275] A. J. Tatem, P. W. Gething, D. L. Smith, and S. I. Hay, ‘Urbanization and the global malaria recession’, *Malar J*, vol. 12, no. 1, p. 133, Dec. 2013, doi: 10.1186/1475-2875-12-133.
[276] Malaria Genomic Epidemiology Network, ‘Reappraisal of known malaria resistance loci in a large multicenter study’, *Nat Genet*, vol. 46, no. 11, pp. 1197–1204, Nov. 2014, doi: 10.1038/ng.3107.
[277] S. Parikh and P. J. Rosenthal, ‘Human Genetics and Malaria: Relevance for the Design of Clinical Trials’, *J INFECT DIS*, vol. 198, no. 9, pp. 1255–1257, Nov. 2008, doi: 10.1086/592223.
[278] A. H. Shankar, ‘Nutritional Modulation of Malaria Morbidity and Mortality’, *J INFECT DIS*, vol. 182, no. s1, pp. S37–S53, Sep. 2000, doi: 10.1086/315906.
[279] A. E. Frosch and C. C. John, ‘Immunomodulation in *Plasmodium falciparum* malaria: experiments in nature and their conflicting implications for potential therapeutic agents’, *Expert Review of Anti-infective Therapy*, vol. 10, no. 11, pp. 1343–1356, Nov. 2012, doi: 10.1586/eri.12.118.
[280] J. Mischlinger *et al.*, ‘Imported Malaria in Countries where Malaria Is Not Endemic: a Comparison of Semi-immune and Nonimmune Travelers’, *Clin Microbiol Rev*, vol. 33, no. 2, pp. e00104-19, Mar. 2020, doi: 10.1128/CMR.00104-19.
[281] A. Géry *et al.*, ‘Long-Term In vitro Cultivation of Plasmodium falciparum in a Novel Cell Culture Device’, *The American Journal of Tropical Medicine*

and Hygiene, vol. 100, no. 4, pp. 822–827, Apr. 2019, doi: 10.4269/ajtmh.18-0527.
[282] L. K. Basco, 'Cultivation of Asexual Intraerythrocytic Stages of Plasmodium falciparum', *Pathogens*, vol. 12, no. 7, p. 900, Jul. 2023, doi: 10.3390/pathogens12070900.
[283] K. Srivastava, S. Singh, P. Singh, and S. K. Puri, 'In vitro cultivation of Plasmodium falciparum: Studies with modified medium supplemented with ALBUMAX II and various animal sera', *Experimental Parasitology*, vol. 116, no. 2, pp. 171–174, Jun. 2007, doi: 10.1016/j.exppara.2006.12.003.
[284] S. H. Hodgson *et al.*, 'Evaluating controlled human malaria infection in Kenyan adults with varying degrees of prior exposure to Plasmodium falciparum using sporozoites administered by intramuscular injection', *Front. Microbiol.*, vol. 5, Dec. 2014, doi: 10.3389/fmicb.2014.00686.
[285] L. G. Thiam, P. B. Nyarko, K. A. Kusi, M. Niang, Y. Aniweh, and G. A. Awandare, 'Blood donor variability is a modulatory factor for P. falciparum invasion phenotyping assays', *Sci Rep*, vol. 11, no. 1, p. 7129, Mar. 2021, doi: 10.1038/s41598-021-86438-1.
[286] S. Kumar *et al.*, 'A Malaria Parasite Cross Reveals Genetic Determinants of Plasmodium falciparum Growth in Different Culture Media', *Front. Cell. Infect. Microbiol.*, vol. 12, p. 878496, May 2022, doi: 10.3389/fcimb.2022.878496.
[287] C. Dohutia, P. K. Mohapatra, D. R. Bhattacharyya, K. Gogoi, K. Bora, and B. K. Goswami, 'In vitro adaptability of Plasmodium falciparum to different fresh serum alternatives', *J Parasit Dis*, vol. 41, no. 2, pp. 371–374, Jun. 2017, doi: 10.1007/s12639-016-0808-z.
[288] J.-H. Ch'ng *et al.*, 'Enhanced virulence of Plasmodium falciparum in blood of diabetic patients', *PLoS ONE*, vol. 16, no. 6, p. e0249666, Jun. 2021, doi: 10.1371/journal.pone.0249666.
[289] A. G. Maier and C. Van Ooij, 'The role of cholesterol in invasion and growth of malaria parasites', *Front. Cell. Infect. Microbiol.*, vol. 12, p. 984049, Sep. 2022, doi: 10.3389/fcimb.2022.984049.
[290] G. Min-Oo and P. Gros, 'Erythrocyte variants and the nature of their malaria protective effect', *Cell Microbiol*, vol. 7, no. 6, pp. 753–763, Jun. 2005, doi: 10.1111/j.1462-5822.2005.00524.x.
[291] V. R. R. De Mendonça, M. S. Goncalves, and M. Barral-Netto, 'The Host Genetic Diversity in Malaria Infection', *Journal of Tropical Medicine*, vol. 2012, pp. 1–17, 2012, doi: 10.1155/2012/940616.
[292] M. M. Goheen, S. Campino, and C. Cerami, 'The role of the red blood cell in host defence against falciparum malaria: an expanding repertoire of evolutionary alterations', *Br J Haematol*, vol. 179, no. 4, pp. 543–556, Nov. 2017, doi: 10.1111/bjh.14886.
[293] S. Schulman *et al.*, 'Growth of Plasmodium falciparum in human erythrocytes containing abnormal membrane proteins.', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, no. 18, pp. 7339–7343, Sep. 1990, doi: 10.1073/pnas.87.18.7339.
[294] M. Cappadoro *et al.*, 'Early Phagocytosis of Glucose-6-Phosphate Dehydrogenase (G6PD)-Deficient Erythrocytes Parasitized by Plasmodium falciparum May Explain Malaria Protection in G6PD Deficiency'.

[295] V. D. Mangano *et al.*, ‘Novel Insights Into the Protective Role of Hemoglobin S and C Against *Plasmodium falciparum* Parasitemia’, *J Infect Dis.*, vol. 212, no. 4, pp. 626–634, Aug. 2015, doi: 10.1093/infdis/jiv098.
[296] H. Lamptey *et al.*, ‘Association between alpha-thalassaemia trait, Plasmodium falciparum asexual parasites and gametocyte carriage in a malaria endemic area in Southern Ghana’, *BMC Res Notes*, vol. 12, no. 1, p. 134, Dec. 2019, doi: 10.1186/s13104-019-4181-8.
[297] S. N. Kariuki and T. N. Williams, ‘Human genetics and malaria resistance’, *Hum Genet*, vol. 139, no. 6–7, pp. 801–811, Jun. 2020, doi: 10.1007/s00439-020-02142-6.
[298] A. Driss, J. M. Hibbert, N. O. Wilson, S. A. Iqbal, T. V. Adamkiewicz, and J. K. Stiles, ‘Genetic polymorphisms linked to susceptibility to malaria’, *Malar J*, vol. 10, no. 1, p. 271, Dec. 2011, doi: 10.1186/1475-2875-10-271.
[299] A. Barragan, P. G. Kremsner, M. Wahlgren, and J. Carlson, ‘Blood Group A Antigen Is a Coreceptor in *Plasmodium falciparum* Rosetting’, *Infect Immun*, vol. 68, no. 5, pp. 2971–2975, May 2000, doi: 10.1128/IAI.68.5.2971-2975.2000.
[300] C. J. Uneke, ‘Plasmodium falciparum malaria and ABO blood group: is there any relationship?’, *Parasitol Res*, vol. 100, no. 4, pp. 759–765, Mar. 2007, doi: 10.1007/s00436-006-0342-5.
[301] J. A. Rowe, D. H. Opi, and T. N. Williams, ‘Blood groups and malaria: fresh insights into pathogenesis and identification of targets for intervention’:, *Current Opinion in Hematology*, vol. 16, no. 6, pp. 480–487, Nov. 2009, doi: 10.1097/MOH.0b013e3283313de0.
[302] A. Degarege, G. Medhin, A. Animut, M. Legess, and B. Erko, ‘Association of ABO blood group and P. falciparum malaria related outcomes: A cross-sectional study in Ethiopia’, *Acta Tropica*, vol. 123, no. 3, pp. 164–169, Sep. 2012, doi: 10.1016/j.actatropica.2012.04.012.
[303] P. M. Lelliott, B. J. McMorran, S. J. Foote, and G. Burgio, ‘The influence of host genetics on erythrocytes and malaria infection: is there therapeutic potential?’, *Malar J*, vol. 14, no. 1, p. 289, Dec. 2015, doi: 10.1186/s12936-015-0809-x.
[304] S. Perrotta, P. G. Gallagher, and N. Mohandas, ‘Hereditary spherocytosis’, vol. 372, 2008.
[305] S. Rocha *et al.*, ‘Erythrocyte membrane protein destabilization *versus* clinical outcome in 160 Portuguese Hereditary Spherocytosis patients’, *Br J Haematol*, vol. 149, no. 5, pp. 785–794, Jun. 2010, doi: 10.1111/j.1365-2141.2010.08166.x.
[306] P. Sharmila and M. Paul, ‘Hereditary Elliptocytosis’, *The Journal of Medical Sciences*.
[307] ‘Class I glucose-6-phosphate dehydrogenase deficiency - About the Disease - Genetic and Rare Diseases Information Center’. Accessed: Feb. 09, 2024. [Online]. Available: https://rarediseases.info.nih.gov/diseases/6520/class-i-glucose-6-phosphate-dehydrogenase-deficiency
[308] O. A. Enegela and F. Anjum, ‘Pyruvate Kinase Deficiency’, in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2024. Accessed: Feb. 08, 2024. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK560581/
[309] P. Mungkalasut, P. Kiatamornrak, W. Jugnam-Ang, S. Krudsood, P. Cheepsunthorn, and C. L. Cheepsunthorn, ‘Haematological profile of

malaria patients with G6PD and PKLR variants (erythrocytic enzymopathies): a cross-sectional study in Thailand', *Malar J*, vol. 21, no. 1, p. 250, Aug. 2022, doi: 10.1186/s12936-022-04267-7.
[310] T. Khammanee, N. Sawangjaroen, H. Buncherd, A. W. Tun, and S. Thanapongpichat, 'Prevalence of Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency among Malaria Patients in Southern Thailand: 8 Years Retrospective Study', *Korean J Parasitol*, vol. 60, no. 1, pp. 15–23, Feb. 2022, doi: 10.3347/kjp.2022.60.1.15.
[311] M.-O. Rodrigues, A. P. Freire, G. Martins, J. Pereira, M.-C. Martins, and C. Monteiro, 'Glucose-6-phosphate Dehydrogenase Deficiency in Portugal: Biochemical and Mutational Profiles, Heterogeneity, and Haplotype Association', *Blood Cells, Molecules, and Diseases*, vol. 28, no. 2, pp. 249–259, Mar. 2002, doi: 10.1006/bcmd.2002.0505.
[312] L. Manco and A. Abade, 'Pyruvate kinase deficiency: prevalence of the 1456C→T mutation in the Portuguese population', *Clinical Genetics*, vol. 60, no. 6, pp. 472–473, Dec. 2001, doi: 10.1034/j.1399-0004.2001.600612.x.
[313] M. A. Clark, M. M. Goheen, and C. Cerami, 'Influence of host iron status on Plasmodium falciparum infection', *Front. Pharmacol.*, vol. 5, May 2014, doi: 10.3389/fphar.2014.00084.
[314] N. Spottiswoode, P. E. Duffy, and H. Drakesmith, 'Iron, anemia and hepcidin in malaria', *Front. Pharmacol.*, vol. 5, May 2014, doi: 10.3389/fphar.2014.00125.
[315] M. M. Goheen *et al.*, 'Anemia Offers Stronger Protection Than Sickle Cell Trait Against the Erythrocytic Stage of Falciparum Malaria and This Protection Is Reversed by Iron Supplementation', *EBioMedicine*, vol. 14, pp. 123–130, Dec. 2016, doi: 10.1016/j.ebiom.2016.11.011.
[316] E. Prus and E. Fibach, 'Flow cytometry measurement of the labile iron pool in human hematopoietic cells', *Cytometry Pt A*, vol. 73A, no. 1, pp. 22–27, Jan. 2008, doi: 10.1002/cyto.a.20491.
[317] A. Soliman, V. De Sanctis, and S. Kalra, 'Anemia and growth', *Indian J Endocr Metab*, vol. 18, no. 7, p. 1, 2014, doi: 10.4103/2230-8210.145038.
[318] L. P. W. Goh, E. T. J. Chong, and P.-C. Lee, 'Prevalence of Alpha(α)-Thalassemia in Southeast Asia (2010–2020): A Meta-Analysis Involving 83,674 Subjects', *IJERPH*, vol. 17, no. 20, p. 7354, Oct. 2020, doi: 10.3390/ijerph17207354.