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Large interindividual variations of *Plasmodium falciparum* growth in *in-vitro* cultures: Investigation of underlying factors especially related to red blood cells

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RESUMO

Fundamento: *Plasmodium falciparum* (*P.f.*) cresce a uma taxa variável entre indivíduos, resultando em diferentes parasitemias, cargas parasitárias e graus de gravidade da doença. Além da imunidade, outros fatores parecem influenciar a taxa de crescimento do parasita e o prognóstico da doença, como o grupo sanguíneo ou as hemoglobinopatias, embora os mecanismos ainda não estejam esclarecidos. As culturas *in vitro* são uma ferramenta essencial para estudar o *P.f.* e investigar melhores intervenções. É fundamental entender como estas variações afetam o crescimento do parasita em cultura e quais os fatores envolvidos.

Objetivos: Este trabalho tem como objetivo investigar possíveis diferenças no crescimento de *P.f.* em indivíduos não imunes à malária num sistema de cultura *in vitro* e estudar possíveis associações para as variações observadas.

Métodos: Realizaram-se culturas *in vitro* de *P.f.* com sangue de 69 indivíduos não imunes à malária. Numa segunda fase, 24 voluntários foram novamente chamados para dar outra amostra de sangue para repetir culturas de *P.f.* e realizar uma avaliação analítica. Em 14 voluntários, a suplementação a cultura com soro bovino foi substituída por soro humano.

Resultados: A média da razão do crescimento do *P.f.* após 96 horas foi de 10.0, com uma grande variabilidade entre indivíduos (intervalo:3.0–19.3, DP:3.8). Três grupos foram criados. O grupo de "baixo crescimento" é definido por uma razão 1DP abaixo da média (n=12, média:4.7, SD:1.2, intervalo:3.0–6.1). O grupo de "elevado crescimento" é definido por uma razão 1DP acima da média (n=13, média:16.0, DP:1.8, intervalo:13.8–19.3). Os dados sugerem que os indivíduos que apresentam menor crescimento do *P.f.* estão ligados a países onde a malária é endémica (p=0.03). Além disso, um menor crescimento parece estar relacionado à anemia (p=0.01), apontando para algum problema nos eritrócitos, embora este estudo não tenha permitido encontrar o mecanismo subjacente. A suplementação com soro humano não alterou este padrão de crescimento. Além dos níveis de hemoglobina (p=0.03), nenhum outro parâmetro analítico apresentou diferença significativa.

Conclusão: Existem indivíduos cujo sangue permite um crescimento do *P.f.* consistentemente alto ou baixo. A grande variabilidade do crescimento não é influenciada pelo soro, sugerindo que os eritrócitos parecem ser responsáveis por este padrão. Esta ideia é reforçada pelo facto de que indivíduos com uma história clínica de anemia ou níveis de hemoglobina baixos apresentam menores taxas de crescimento. Assim, a variabilidade no crescimento do parasita pode ser explicada por pequenas variações genéticas ao nível da hemoglobina.

Palavras-chave: Malaria; *Plasmodium falciparum*; taxa de crescimento; carga parasitária; gravidade de doença.

ABSTRACT

Background: *Plasmodium falciparum* (*P.f.*) grows at a variable rate between individuals, resulting in different parasitaemia, total body parasite burden and degrees of disease severity. In addition to immunity, other factors appear to influence the parasite growth rate and disease prognoses, such as blood group or haemoglobinopathies, although the mechanisms are not yet clear. *In-vitro* culture is an essential tool for studying *P.f.* and for research into better interventions. It is critical to understand how these variations affect parasite growth in culture and what factors may influence it.

Objectives: This work aims to investigate possible interindividual differences in the *P.f.* growth in malaria-naïve individuals in an *in-vitro* culture system and to study possible associations for the observed variation.

Methods: *In-vitro P.f.* cultures were performed with blood from 69 malaria-naïve individuals. In the second phase, 24 volunteers were called back to give another blood sample to repeat *P.f.* cultures and perform blood tests. In 14 volunteers, the culture supplementation with bovine serum was replaced by human serum.

Results: The mean fold-change of *P.f.* growth after 96 hours was 10.0, with a large inter-individual variability (range:3.0–19.3, SD:3.8). Three groups were created. The "lower growers" group is defined by a fold-change below 1SD from the mean (n=12, mean:4.7, SD:1.2, range:3.0–6.1). The "higher growers" group is defined by a fold-change above 1SD from the mean (n=13, mean:16.0, SD:1.8, range:13.8–19.3). Data suggest that inidviduals presenting with a lower parasite growth are connected to malaria-endemic countries (p=0.03). Furthermore it appeared to be related to anaemia (p=0.01), pointing to some problem in the RBC, although this study did not allow to find the underlying mechanism. Supplementation with human serum did not change the growth pattern. Besides haemoglobin levels (p=0.03), no other haematology-biochemical parameter showed a statistically significant difference.

Conclusion: There are individuals whose blood allows a growth of *P.f.* consistently high or low. The large variability of parasite growth is not changed by serum, suggesting that the RBC are responsible for this pattern. This idea is reinforced by the fact that

individuals with a clinical history of anaemia or low haemoglobin levels have the lowest growth rates. Thus, differences in the parasite growth could be explained by some minor genetic variations in haemoglobin.

Keywords: Malaria; *Plasmodium falciparum*; growth rate; parasite burden; disease severity.

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ACKNOWLEDGMENTS

My short journey through the world of research taught me two great lessons. The first was that, in science, our plans often do not go as expected. It is necessary to have the resilience and the creativity to overcome the obstacles that arise along the way. The second (and perhaps the most important) was that nothing is built with one person alone. Science is made up of collaborations and partnerships, and without them, advances are small. As someone once said:

"If you want to go fast, go alone; if you want to go far, go together."

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ABREVIATIONS

CAPNetz – Community-acquired pneumonia competence network

CHMI – Controlled Human Malaria Infections

COVID-19 – Coronavirus disease 19

CR1 – Complement receptor 1

Ct – Cycle threshold

DARC – Duffy antigen receptor for chemokines

FUT2 – Fucosyltransferase 2

G6PD – Glucose-6-phosphate-dehydrogenase

GPI – General paresis of the insane

HG – Higher growers

HIV - Human immunodeficiency virus

HPLC – Haemoglobin high-performance liquid chromatography

LG – Lower growers

MCM – Malaria Complete Parasite Medium

MERS – Middle East respiratory syndrome

PfEMP1 – Plasmodium falciparum-erythrocyte membrane protein-1

PfHRP2 – Plasmodium falciparum histidine-rich protein 2

PfRh5 – Reticulocyte-binding protein homologue 5

SARS – Severe acute respiratory syndrome coronavirus-1

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2

VFR – adult travellers of sub-Saharan origin living in France and visiting family or relatives in their country of origin

WHO – World Health Organization

INTRODUCTION

The impact of infectious diseases

The world is full of microbes. It has been estimated that the human body contains about 100 billion microorganisms and thus that the "human" genome may be 99% microbial.[1] Whatever the exact proportions, the balance and harmony between host and microbes are certainly of crucial importance. If this balance is disrupted or when external pathogens enter, it may lead to an infectious disease.

Infectious diseases have marked human history. They have had a profound effect on the migration of human populations and vice versa.[2–4] Politics and conflicts have also been affected by infectious diseases, which have profoundly altered civilisations and their subsequent history.[2–4] For example, in the fourteenth century, the Black Death, thought to have been bubonic plague caused by the bacteria *Yersinia pestis*, was one of the most devastating epidemics recorded in the history of humankind. During five years, between 1347 and 1351, it resulted in the death of three Europeans out of ten (a total of 24 million European deaths) and 40 million deaths worldwide, in a world population of 475 million.[2] The coronavirus disease 19 (COVID-19) pandemic, one of the greatest fights of the early twenty-first century, is directed against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It forced the world to almost stop and kept the majority of people locked at home.

Although malaria is not endemic in Europe anymore, areas of extensive swampy wetland in Portugal, France, and Italy suffered from malaria, often with large numbers of sick people and many deaths.[5] Kings, popes, and military leaders were struck by this disease. During the two World Wars, armies were deeply affected by malaria. At least 1.5 million soldiers were infected in World War I, with case fatality rates ranging from 0.2 to 5.0%.[6] Especially, the II World War and others, such as the Vietnam War, were a vital stimulus in search for better and more effective antimalarial drugs.[7]

In the past, most people died from infectious diseases, such as diarrhoea, tuberculosis, scarlet fever or complicated wound infections.[8] Only a minority lived long enough to die from degenerative diseases. However, the tendency has reverted. People live longer and die more often from non-communicable diseases, such as cardiovascular diseases and cancer.[8,9] The life expectancy at birth increased from an estimated 30

years in 1800 to 73 years in 2019.[3] In the late nineteenth century, 149 children out of one thousand died in the first year of life.[3] In 2019, the infant mortality rate ranged from as low as one per thousand in Finland, two in Japan, Singapore and Sweden, three in Portugal and four in the UK, to as high as 107 in Afghanistan.[10] In the past, more than half of the children died before the age of fifteen, primarily due to infections rather than hunger or war.[11,12] The long-term decrease in overall mortality and increase in life expectancy were achieved by better living conditions, improved hygiene, vaccines, antibiotics and biotechnology.[3]

Nevertheless, infectious diseases remain a significant cause of death and disability worldwide, even with the considerable progress in their treatment and prevention in the last decades. In 2019, nearly 8 million people died from preventable infectious diseases, representing 13% of the total deaths worldwide, whereby most deaths occurred in low-income countries, which are more vulnerable.[13]

Infection with the same pathogen – why do some individuals have an asymptomatic or light infection while others have a severe infection or die?

Most infections with the same pathogen can present a spectrum of manifestations ranging from asymptomatic infections, mild disease, severe disease, and eventually death as an outcome. One typical example is infection with *Plasmodium*, the parasite that causes malaria. *Plasmodium* infection may show no symptoms in individuals with asymptomatic parasitaemia but also range from mild (uncomplicated) disease to severe manifestations in patients with life-threatening anaemia, metabolic acidosis, cerebral malaria, and multiorgan system impairment.[14] This pattern of manifestations can be observed in many other infectious diseases. For example, the infection with *Mycobacterium tuberculosis* presents a broad spectrum of disease manifestations and infection outcomes.[15] Most infections do not manifest clinically (asymptomatic, contained state - termed latent tuberculosis infection), while a subset of infected individuals may present with symptomatic tuberculosis.[15]

Another current example is COVID-19 which has a clinical spectrum of disease from mild to moderate to severe illness. Most individuals are either asymptomatic or have a mild influenza-like illness that cannot be differentiated from a simple upper

respiratory tract infection.[16] The most common symptoms are related to the respiratory system, as cough, sputum, shortness of breath, and fever.[17] However, other systems may be involved, such as the musculoskeletal system (myalgia, joint pain, headache, and fatigue) and the gastrointestinal system (abdominal pain, vomiting, and diarrhoea).[17] A small group, around 5%,[17] evolves to severe disease that requires hospitalisation and intensive therapy,[16] which may lead to death.

This raises a crucial question: **why do some individuals infected with the same pathogen have such different manifestations and disease outcomes? Why do some have no disease at all (asymptomatic), some have mild manifestations, while others suffer from severe disease or may die?**

Indeed, the immune system is one of the keys to answer this question, although the intricacies of how this may happen point to a complex and multifactorial interaction between the host (immune-system and the pathogen). However, two distinct mechanisms could be described:

a) Inappropriate immune response: As has been shown nicely for COVID-19, the more severe forms seem to be caused by an overreactive immune system, where the damage to the host is rather caused by the immune defence instead than by damage caused by the pathogen.[18] Consequently, immunosuppression (dexamethasone, prednisolone) is used to correct the inappropriate immune response.[19] Similar concepts have been entertained for sepsis[20] but the use of immune-modulating drugs has been relatively unsuccessful so far,[21] possibly, because it is difficult to modulate the immune system to produce the optimal immune response against the infection, even more so, as no simple markers exist which could guide such an intervention.[21]

b) Pathogen burden (or load): One aspect, however, which seems to be rather well established is the association that more pathogens may lead to more damage to the host and thus to more disease[22–25] (see section Pathogen Load and Disease Severity, page 17). In fact, the central task of the immune system is to control pathogen growth and, eventually, to eliminate (reduce) the pathogen burden. Immunodeficiencies illustrate this relation well, where even pathogens with few virulence factors can replicate unchecked and cause disease.[26] Still, the immune system is only one part of this, as the capacity to replicate (virulence) of the pathogen is the other determinant of

replication capacity and thus disease outcomes.[27] Whatever the exact underlying nature, a rather simple mechanistic idea could be established and described in a simple expression: **(↑ pathogens = ↑ severe disease)**.

Pathogen Load and Disease Severity

It appears that one of the central factors that determine disease severity in most infectious diseases is the number of pathogenic organisms in the human body,[28] also called the pathogen burden or pathogen load. The underlying rationale is simple: the more pathogens a patient has in his body, the more damage they cause and thus, the more severe is the disease.

Malaria may be a good example because severity seems to be correlated to total body parasite load. It should be noted that in *Plasmodium falciparum* infections, most parasites may be sequestered in the capillary bed, and thus peripheral parasitaemia, determined by microscopy in blood smears, is usually a poor predictor of total parasite burden.[29,30] Contrary to this, *P. falciparum* histidine-rich protein 2 (PfHRP2) is released into the blood by parasites as they proliferate,[31] and thus, the antigenaemia can be correlated with disease severity. Patients with higher levels of PfHRP2 are more likely to have severe malaria, indicating a direct link between total parasite burden and disease severity[30,32,33] (Figure 1).

Moreover, this link can also be seen when treating patients with malaria. The most effective antimalarial drugs cause the quickest decline in parasite densities:[34,35] the more rapid the elimination of parasites is (and thus the total parasite load), the faster is the clinical improvement of the patient. This reduction in parasites (Figure 2) also explains the superiority of artemisinin, especially the better outcomes with intravenous artesunate in the treatment of severe malaria, compared with quinine.[36,37]

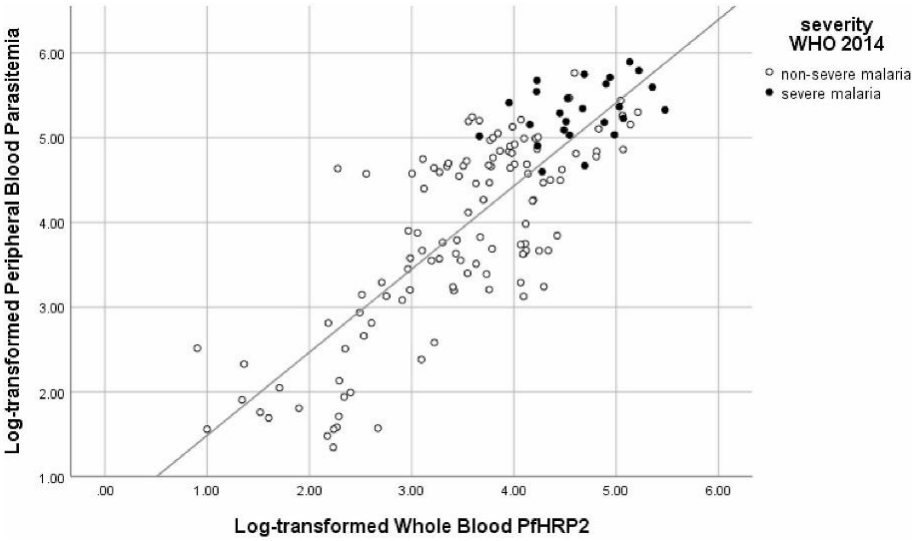

Figure 1 – Correlation between PfHRP2 and peripheral blood parasitaemia in severe and non-severe malaria patients

A strong, positive correlation between PfHRP2 and peripheral blood parasitaemia is shown. Levels of PfHRP2 are significantly higher in patients with severe malaria compared to patients with non-severe malaria.

Adapted from Kwak, J. D. et al. (2021). 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.* 42, 102076.

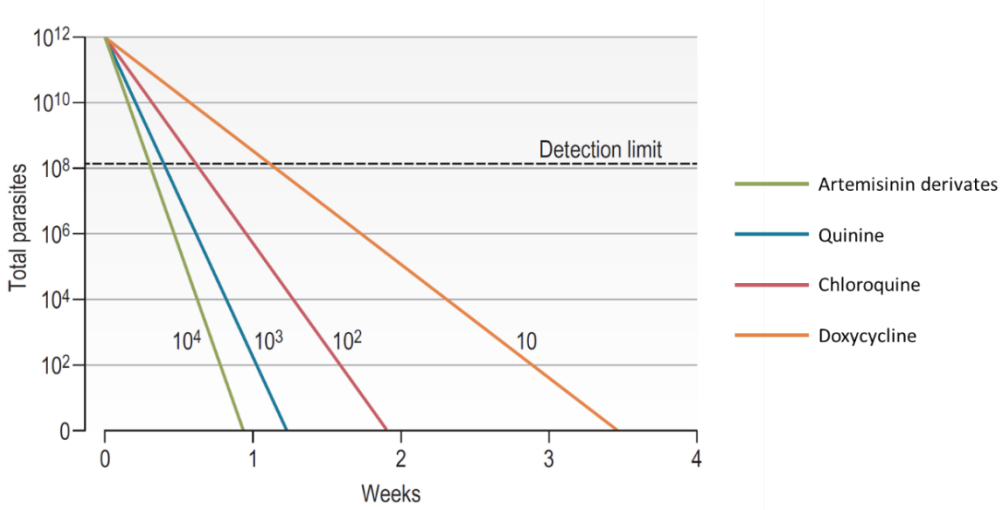

Figure 2 – The effects of antimalarial drugs on parasite killing with different rates on the elimination of the malaria infection.

The individual parasite biomass is given on the vertical axis. The artemisinin derivatives achieve the quickest parasite reduction ratios (PRR): 10^4 per asexual cycle – green line. Most of the other antimalarials achieve PRR values of 10^2 to 10^3 per cycle. Blue line represents quinine (PPR: 10^3

per asexual cycle) and the red line chloroquine (PPR: 10^2 per asexual cycle). The orange line represents antimalarial antibiotics, like doxycycline, which have PRR values around 10.
Adapted from White, N. J. (2014). Malaria. In *Manson's Tropical Diseases: Twenty-Third Edition* (pp. 532–600). Saunders Ltd.

The correlation between the pathogen load and the disease severity seems to occur in other diseases, such as COVID-19. Patients with severe disease compared to mild or moderate disease have higher viral RNA loads.[38–40] Cycle threshold (Ct) values for the severe disease were around 21 compared to 27 to mild.[41] Severe ill patients also had the virus for longer, up to forty days versus ten days after onset of disease.[42,43] Also, they have viral shedding in various tissues, while those with mild illness have viral shedding restricted to the respiratory tract.[40]

Another typical case can be made for the infection with the human immunodeficiency virus (HIV). The viral load is one of the relevant markers of the course of the infection and is predictive of progression to the acquired immunodeficiency syndrome (AIDS) and death.[44] According to the World Health Organization (WHO), routine viral load testing is the standard method for monitoring HIV-infected patients and treatment efficacy;[45] the lower the viral load, the more effective the treatment appears to be.

Another example is sepsis, usually caused by a wide range of bacteria, which remains a major cause of morbidity and mortality worldwide.[20] Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection.[46] According to the Surviving Sepsis Campaign guidelines, antibiotics should be administered to patients with sepsis within an hour of recognition, representing the major therapy's goal.[47] For every hour delay in administering antibiotics to patients with sepsis, there is a 7.6% increase in mortality over the first six hours.[48] The underlying principle could be summarized again as: early appropriate antibiotic therapy reduces the number of circulating pathogens, and thus, decreases the stimulus that led to the beginning of a harmful response for the patient himself, and this may lead to a faster clinical improvement of the patient **(↓ pathogens = ↑ disease outcome)**.

Factors which influence Pathogen Load

Assuming the correlation (**↑ pathogens = ↑ severe disease)** is correct, it becomes essential to understand what factors influence a larger or smaller number of pathogenic organisms in the human body. Multiple intrinsic and extrinsic factors could be causing this, which can be classified in different categories.

Infectious dose

For every infection to occur, the pathogen has to enter the human host at the appropriate site where it can establish itself, replicate and thus cause infection. Certainly, a crucial factor is the pathogen load to which the host is exposed, named the infectious dose. This concept may be more complex in cases where the organism is intrinsically present in some part of the body, but may cause disease when infecting another part, such as *Staphylococcus aureus* (example: anterior nares → skin infection) or Escherichia coli (example: intestine → urinary tract infection). However, in cases where the infection is from an external source, usually, there is a clear correlation between pathogen number and infection, including a minimal infectious dose.[49]

For example, the infectious dose of SARS-CoV-2 might be a determining factor in the outcome of COVID-19.[50,51] Data from the Italian National Institute of Health showed that healthcare workers in tracheal intubation might be exposed to higher plaque-forming units of SARS-CoV-2 and exhibit more severe forms of COVID-19.[52] Similarly, in the Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome coronavirus-1 (SARS), a correlation between the infectious dose and the severity of complications and disease progression was noted.[50] Moreover, in influenza infection, the infectious dose is positively correlated with disease severity.[53] Another example is tuberculosis. The infectious dose is critically important in determining the disease progression. A low infectious dose of *Mycobacterium tuberculosis* increases the probability of latent tuberculosis infection rather than the acute forms of the disease.[15]

Interestingly the reverse may also be true, as convincingly illustrated by the classical case of the original practice of variolation. The inoculation of a low dose of purulent material containing a relatively low dose of the poxvirus did usually not cause severe disease or death but was sufficient to confer protective immunity.[54]

The general picture seems to emerge: the number of pathogens at the time of infection, and thus the overall capacity of that pathogen to produce a significant pathogen burden, is predictive of the severity of the disease. Once again pointing to **(↑ pathogens = ↑ severe disease)**.

Intrinsic Pathogen Factors

It is helpful to recall the underlying evolutionary concepts before addressing intrinsic pathogen factors. In evolutionary terms, the goal of microbes (or any organism) could be described as the propagation of their genetic information through their offspring.[55,56] Microbes, especially pathogens, may achieve this through infection of a susceptible host, multiplication to achieve large biomass, which guarantees ideal transmission to a new host.[12,55,56] Certainly, pathogens do not necessarily have to cause disease or kill their host, which might even be to their disadvantage. Consequently pathogens that co-evolved with humans over long periods usually have adapted to cause minor disease while still efficiently transmitting themselves (geo-helminthic infections might be an example).[57] However, many, if not most bacterial and viral pathogens, have developed efficient transmission methods, where they produce large numbers of themselves and consequently may cause disease or death of their host. The underlying strategy is that enough hosts more efficiently transmit the pathogen, even if the pay-off may be the death of the host; may that be only occasional (like in the majority of diseases) rather frequent (like in the case of plague[58] or smallpox[59]), or even the general outcome (like in the case of rabies[60]).

Virulence is the term that describes the pathogenicity of an organism, referring to the extent of pathology caused by it.[61] Microbes possess virulence factors which have been defined as structures, molecules or systems which allow the microbe to achieve: (i) colonization and entry into the host and/or host cells; (ii) mechanisms to evade the immune system or even suppress it and, relevant here (iii) growth by obtaining nutrients from the host.[12]

The degree of virulence is related directly to the ability of the organism to cause disease despite host resistance mechanisms, and, importantly, it is usually correlated with the ability of the pathogen to multiply within the host.[27]

The higher the pathogen virulence, the higher the ability to multiply, leading to a higher pathogen load and, likely, a more severe disease, once again pointing to **(↑ pathogens = ↑ severe disease)**.

Host factors

Several predisposing host-related factors associated with disease severity could be described, some of which may be overlapping: age, gender, comorbidities or prior illness[12]. However, three aspects can be highlighted in their critical role for the pathogen load: **immune system**, **genetic susceptibility** and **nutritional status**.

Immune system

Undoubtedly, the immune system plays an essential role in establishing the infection and its symptoms. The importance of the host defence mechanisms becomes apparent when the immune system is impaired or even "lacking" due to congenital or acquired defects. For example, cancer patients undergoing myelosuppressive chemotherapy may develop severe neutropenia, making them more susceptible to infections caused by Gram-negative enteric bacilli, *Pseudomonas* spp., *Staphylococcus* spp., but even to rather less pathogenic microbes, such as fungal infections caused by *Candida* spp., or even *Aspergillus* spp.[12,62] Also, patients with primary immunodeficiency diseases, including many genetic disorders that affect the immune response, have an increased susceptibility to infectious diseases.[63] The more common agents depend on the part of the immune system that is affected. For instance, in X-linked agammaglobulinemia, common agents are *Streptococcus pneumoniae* and other streptococci.[12]

Therefore, the inflammatory response from the host is critical for interruption and resolution of the infection. However, the immune system also plays a vital role in the disease manifestations, being frequently responsible for the signs and symptoms of the disease. Two good examples, as described previous are sepsis and severe COVID-19 (see section Infection with the same pathogen – why do some individuals have an asymptomatic or light infection while others have a severe infection or die?, page 14), in which symptoms result mainly from the patient's overreactive inflammatory

response, often termed a cytokine storm.[18,20] Thus, while the immune system should mount the strongest possible response to reduce the total parasite burden and thus disease manifestations as quick as possible, it has to do so, with minimal or no damage to the host body. Unfortunately, this often precarious equilibrium is not attained and the overreaction of the immune system may cause, even serious or lethal damage to the host.[64]

Nevertheless, the immune system has the purpose of fighting against the invading organism, which can be described as reducing the pathogen load, and hence less severe disease, and after elimination it achieves the cure. Once again this could be described as **(↓pathogens = ↑ disease outcome)**.

Genetic Susceptibility

The role of genetics in infectious diseases has been widely researched, and it is now known that genetic factors play a crucial role in susceptibility to infectious diseases, mainly in immunodeficiency.

The first evidence emerged with observations from ethnic and family groups in which some rare and common infections, following a Mendelian pattern, seem to prevail.[65] Studies carried out where adopted children were followed up showed that the predisposition to infectious diseases was mainly inherited,[66] contrary to what seems to happen in diseases associated with environmental risk factors such as gastric cancer.[67] Other studies with twins have shown that the concordance rate of infectious diseases is higher in monozygotic twins than in dizygotic twins,[65] strengthening the theory of genetics' importance in susceptibility to infectious diseases. The initial evidence of genetics' role in susceptibility to infectious diseases was supported by primary immunodeficiencies, in which genetic changes lead to disturbances in the immune system, favouring certain infections depending on the affected part of the immune system.[26] Interestingly most of these findings established a correlation between impaired immune response and more frequent/severe disease. Contrary to this, inverse correlations were also observed.

Several associations between genetic variations and less susceptibility to certain infectious diseases were already found.[65,68] Interestingly, a significant part of genetic variations which seem to confer resistance to infectious diseases is based on the lack of

host receptors necessary for pathogen invasion[65] (Table 1). Consequently, the difficulty or impossibility of the pathogen trying to enter the cell translates into an overall difficulty to proliferate, and hence, a lower number of pathogenic organisms in the host, leading to an asymptomatic infection or mild disease. Once again, pointing to the association **(↓ pathogens = ↓ severe disease)**.

Table 1 – Some examples of associations between genetic variations and decreased susceptibility to infectious diseases

Pathogen	Gene Region	Main function of the protein encoded	Immunological phenotype	Clinical phenotype	References
HIV-1	CCR5	Chemokine receptor	Lack of coreceptor for pathogen	Natural resistance	[51], [55]
Parvovirus B19	B3GalNT1	Globoside receptor	Lack of receptor for productive infection	Natural resistance	[46], [47]
Norovirus	FUT2	ABH histoblood group antigen	Lack of coreceptor for pathogen	Natural resistance	[58], [49], [50]
Plasmodium vivax	DARC	Blood group antigen	Lack of coreceptor for pathogen	Natural resistance	[51], [52]
Plasmodium	HbS	Haemoglobin subunits		Natural resistance	[55], [56], [57]
Plasmodium	G6PD	Carbohydrate metabolism		Natural resistance	[56]

In HIV-1 infection, there appears to be some resistance in individuals who have mutations that affect the extracellular domain of the CCR5 receptor.[65] CCR5 is the principal coreceptor to HIV-1 to enter CD4+ T cells. Homozygous individuals for the most common deleterious CCR5 (CCR5-Δ32) mutation have shown strong protection against infection with CCR5-tropic HIV-1.[69] Heterozygotes exhibit delayed progression to AIDS.[69] Moreover, HIV patients carriers of CCR5-Δ32 mutation have lower viral loads,[70] favouring the expression **(↓ pathogens = ↑ disease outcome)**.

Parvovirus B19 causes erythema infectiosum and severe forms of acute erythroblastopenia.[65,71,72] The erythrocyte P antigen has an essential role at a post-entry step for productive infection.[72] Individuals whose red blood cells do not have this receptor are naturally resistant to parvovirus B19 infection,[71,72] as the virus multiplication seem to be impaired,[72] leading, once more to the idea **(↓ pathogens = ↓ severe disease)**.

Another example is norovirus, one of the leading causes of acute gastroenteritis.[73] The fucosyltransferase 2 (FUT2) gene encodes an α(1,2)-fucosyltransferase that regulates the expression of the ABH histoblood group antigens on the surface of epithelial cells and in mucosal secretions.[11,74,75] ABH antigens work as norovirus coreceptors. Several inactivating FUT2 mutations are responsible for the non-secretor phenotype characterised by a lack of expression of these coreceptors, conferring complete resistance to symptomatic norovirus infection[74,75] as it leads to lower viral load. Again the expression **(↓ pathogens = ↓ severe disease)** stands out.

Similar observations have been made in malaria. Protection against *Plasmodium vivax* is conferred by the absence of the Duffy blood group antigen, which is also a receptor for a family of proinflammatory cytokines and, of relevance here, one of the receptors for *P. vivax* entry into the red blood cell.[76,77] More about the Duffy blood group will be discussed below (see chapter The genetic protection to not develop malaria disease, page 37).

Since these genetic changes appear to have clear benefits for life-threatening infectious diseases, they are expected to be under strong positive selection pressure. The Duffy blood group may be a good example for this, as the negative phenotype seems to be more frequent in populations in which malaria is more common,[78,79] especially in Occidental Africa, where more than 95% of the population is Duffy negative.[76] Malaria appears to have been an important genetic selective force in this case.

Another important area where genetic variability influences infectious diseases is certain haematology disorder of red-blood-cells. Variants of red blood cell disease-causing genes confer a significant resistance against malaria caused by *Plasmodium* and are much more common in regions where the parasite is endemic.[65] It seems that under selective pressure, certain populations have acquired genetic “make-up”, which make them less susceptible to malaria. One of the best-known examples is sickle cell anaemia

and thalassaemia, prevalent in Mediterranean countries, Africa, and Asia. These diseases have a well-known relationship with resistance to malaria[80–82] and will be discussed below (see chapter The genetic protection to not develop malaria disease, page 35).

Genetic seems to play an important role in infectious disease severity. Certainly, a large part, perhaps the majority, can be linked to the immune system and its capacity to fight the invading microbe. However, another aspect of the genetically determined susceptibility are factors that allow the pathogen to invade (e.g. receptors) and multiply (nutrient availability). Moreover, independent of the mechanism, the expression **(↓ pathogens = ↑ disease outcome)** seems to be always present.

Nutritional Status

The relationship between nutrition and disease is complex.[83] The patient's nutritional status, state of nourishment, and food intake pattern may influence especially the immune system in all its aspects, from the physical barriers, such as skin and intestinal mucous membranes, to the intricate systems of the innate immune response and the adaptive immune response,[84] leading to impaired immune function. Studies in children with malnutrition showed that they are at higher risk for acute infections.[85,86] Of note, the immune system can also have an impact on nutrition metabolism.[84]

The patient's nutritional status also influences the gene expression of the pathogen.[87,88] Studies in rodent model of *P. berghei* infection showed that caloric restriction reduces body weight, blood glucose levels, lipids, and hormones, associated with enhanced health and survival.[88] Moreover, it also influences the growth of *Plasmodium* and the disease severity.[88] It reduced parasitaemia and total parasite load because the parasite senses the host nutritional status and adapts to it by adjusting its multiplication rate.[88] It appears again: **(↓pathogens = ↑ disease outcome)**.

Studies in rodents also showed that selenium, vitamin E and iron play an important role in disease severity, mainly through oxidative stress mechanisms.[87] In rodent models with selenium and vitamin E deficiency infected with Coxsackievirus B3, virus replication led to viral mutations, changing from an avirulent virus into a virulent

one.[87] Moreover, when these mutations occur, even hosts with normal nutritional status, who did not develop the disease before, become susceptible to the newly virulent strain.[87]

Coxsackievirus is thought to be related to Keshan disease, an endemic cardiomyopathy found in Keshan, north-east China, and first described in 1935.[89,90] Epidemiological surveys uncovered that selenium levels in highly endemic areas of the disease were lower than the level in control regions free of the disease. With the supplementation of those individuals with nutritional amounts of sodium selenite, disease prevention occurred.[87] Selenium is an essential mineral that plays a crucial role in protecting the body against oxidative stress.[90] Selenium host deficiency also seems to lead to increased viral mutations in the influenza virus genome, resulting in more virulent strains.[87]

Iron is crucial for both hosts and pathogens.[91] Because of its properties, iron is an indispensable cofactor of many essential enzymes but is also potentially toxic when present in excess.[92] Fundamental cellular operations, such as DNA synthesis and the generation of ATP, require iron.[93] During the infection process, the host and the pathogen compete for iron.[94] Pathogens have evolved specialized mechanisms for obtaining iron from the host.[93] The host had developed protection mechanisms associated with iron, as iron is shifted from the circulation into storage, resulting in hypoferremia and iron-deficient erythropoiesis, ultimately contributing to inflammation's anaemia.[94] As less iron is circulating, the pathogen multiplication is impaired, and less disease is observed, pointing out once more for the expression **(↓ pathogens = ↓ severe disease)**. In *Plasmodium* infection, it seems that iron deficiency may offer protection against the development of malaria.[95–97] Moreover, iron supplementation of young children living in malaria-endemic regions may increase the risk of malaria-related hospitalisation and mortality.[98] Also, in HIV-1 and hepatitis C virus infections, iron overload seems to be associated with a poor prognosis,[93,99] **(↑ pathogens = ↑ severe disease)**.The negative effect of iron in infectious diseases is also evident in programs of malnourishment refeeding. The iron supplementation is recommended to be started after the child starts gaining weight, usually on the second week of treatment, while the other multivitamins supplementation starts on day

one.[100] The iron supplementation is delayed as it may worsen the existing infection.[100,101]

However, not only undernutrition is associated with more severe infections. On the other side of the nutrition spectrum, overnutrition (overweight and obesity) seems to be associated with a more severe outcome of infectious diseases.[102] The infection risk curve seems to have a U-shape related to nutritional status, suggesting that both underweight and obesity predispose to infection.[103] Obesity has a negative effect on respiratory infections, such as H_1N_1 influenza infection[104] and COVID-19,[105] and increases the risk of postsurgical and nosocomial infections.[104]

Together with obesity, diabetes has increased globally,[9] and both of these conditions seem to augment susceptibility to common infections.[106] A retrospective study performed in Canada compared the rate of infection and death attributable to infectious disease between patients with and without diabetes, totalling more than 500 000 cases per group.[107] The data demonstrated that diabetes confers an increased risk of developing and dying from an infectious disease; the highest rates were observed for bacterial infections such as osteomyelitis, urinary tract infections, pneumonia, cellulitis, sepsis or peritonitis.[107] Diabetes is characterized by a high level of plasma glucose (hyperglycaemia).[108] Sepsis patients with hyperglycaemia seem to have a higher mortality rate than those with lower plasma glucose levels at admission (43.0% versus 27.2%).[109] The German CAPNetz (community-acquired pneumonia competence network) study group showed that hyperglycaemia at hospital admission is associated with worse outcomes in patient with community-acquired pneumonia, being a good marker to identify patients in need of intensified care.[110] Also, diabetes doubles the risk of urinary tract infections.[111] Although the mechanisms are not very well understood, one explanation is glycosuria (glucose in the urine), as treatment using sodium-glucose cotransporter 2 inhibitors, which leads to glycosuria, increases the risk of urinary tract infections.[111,112] More glucose seems to favour more pathogens, leading to disease and more severe forms of it, once more pointing to the expression **(↑ pathogens = ↑ severe disease)**.

Indeed, nutrition also seems to influence disease severity. Independently talking about nutrient shortage or excess, the expression **(↓ pathogens = ↓ severe disease)** appears to stand out.

In summary, the immune system plays an essential, if not the key role in disease progression, severity and outcome. A well-orchestrated and equilibrated immune response is necessary, as an overactive immune system may contribute significantly to pathology and disease progression, even independent of pathogen burden.

However, it appears clear that the total pathogen load, which is determined by the microbe's capacity to multiply in the host, is directly correlated to disease progression, severity and outcome, and that the general formulas **(↑pathogens = ↑ severe disease)** or **(↓ pathogens = ↓ severe disease)** or **(↓pathogens = ↑ disease outcome)** may be a good description for this association.

Infections with the parasite *Plasmodium falciparum* show a broad spectrum of manifestations, ranging from asymptomatic parasitaemia to mild/uncomplicated malaria to severe malaria, and eventually death. Interestingly, total parasite load also appears to be related to disease progression and disease severity,[113] which raises questions about the factors determining the capacity of the parasite to multiply in the host.

The biology, clinic aspects and disease susceptibility of Malaria

Malaria is a global vector-borne, parasitic disease caused by *Plasmodium* spp. Despite the efforts to eliminate it, malaria is still one of the common causes of fever in the tropics,[114] with significant morbidity and mortality. In 2019, there were an estimated 229 million malaria cases with 409 000 deaths, of which 67% were children aged under five years.[115]

Five species cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.[116] Of these, *P. falciparum* and *P. vivax* are the ones that cause most cases, and *P. falciparum* is the most virulent and responsible for the majority of deaths.[14] *P. knowlesi* is a zoonosis in South-East Asia, and it remains to be established if the human-to-human transmission does occur at all.[117]

Plasmodium life cycle

The parasite is transmitted by the bite of the female *Anopheles* mosquito at the time of feeding.[118] The mosquito injects sporozoites into the human host with its saliva.

Within minutes and up to an hour,[116] sporozoites migrate into the liver, where they bind to hepatocytes and invade them, beginning the phase of asexual reproduction. Inside the hepatocytes, sporozoites multiply over an asymptomatic replication cycle of five to fifteen days.[119] Usually, relatively few sporozoites are injected, approximated eight to fifteen.[116] However, a massive parasite amplification happens during this pre-erythrocytic phase, as each sporozoite produces up to 90 000 merozoites.[119] Since only a few liver cells are infected, this phase is entirely asymptomatic.

After this time, merozoites are released into the bloodstream, where they infect red blood cells.[116,120,121] The merozoite attachment to the red blood cell is mediated by a family of erythrocyte binding proteins. For *P. vivax* and *P. knowlesi*, the Duffy blood group antigen, and for *P. falciparum,* the reticulocyte-binding protein homologue 5 (PfRh5).[116] After binding to the red blood cell, the merozoite induces the invagination of the red blood cell membrane, creating a vacuole to promote its entry into the cell. The intracellular parasite, called trophozoite, initially has the appearance of a small ring. As it grows, it consumes the haemoglobin contained in the red blood cell. Haemoglobin proteolysis liberates the haem group, which is toxic, and the parasite detoxifies it by turning it into a crystalline substance called hemozoin.[122]

The intracellular parasite becomes more active and irregular in appearance. Nuclear division occurs, producing a multinucleated schizont. The cytoplasm condenses around each schizont nucleus to aggregate into six to thirty-six daughter cells, the merozoites.[123] The last step of the development inside the red blood cell culminates in the lysis of the cell membrane and the release of the merozoites into the bloodstream, in a cascade of tightly orchestrated steps. Released merozoites soon infect other blood cells, starting a new asexual cycle. All the pathological symptoms of human malaria disease are directly associated with the erythrocytic cycle of infection,[81,123] after the prepatent and incubation period.

Some merozoites are transformed into gametocytes (sexual form).[124] These are the stages that transmit the infection following ingestion within the blood meal of a biting mosquito. In the mosquito, fecundation occurs, forming a zygote (ookinete) that penetrates the mosquito midgut wall where it forms an oocyst. Eventually, the oocyst bursts liberating sporozoites that migrate into the mosquito salivary glands. There they

wait for inoculation into the next human host during mosquito feeding. Figure 3 represents the life cycle of *Plasmodium* spp.

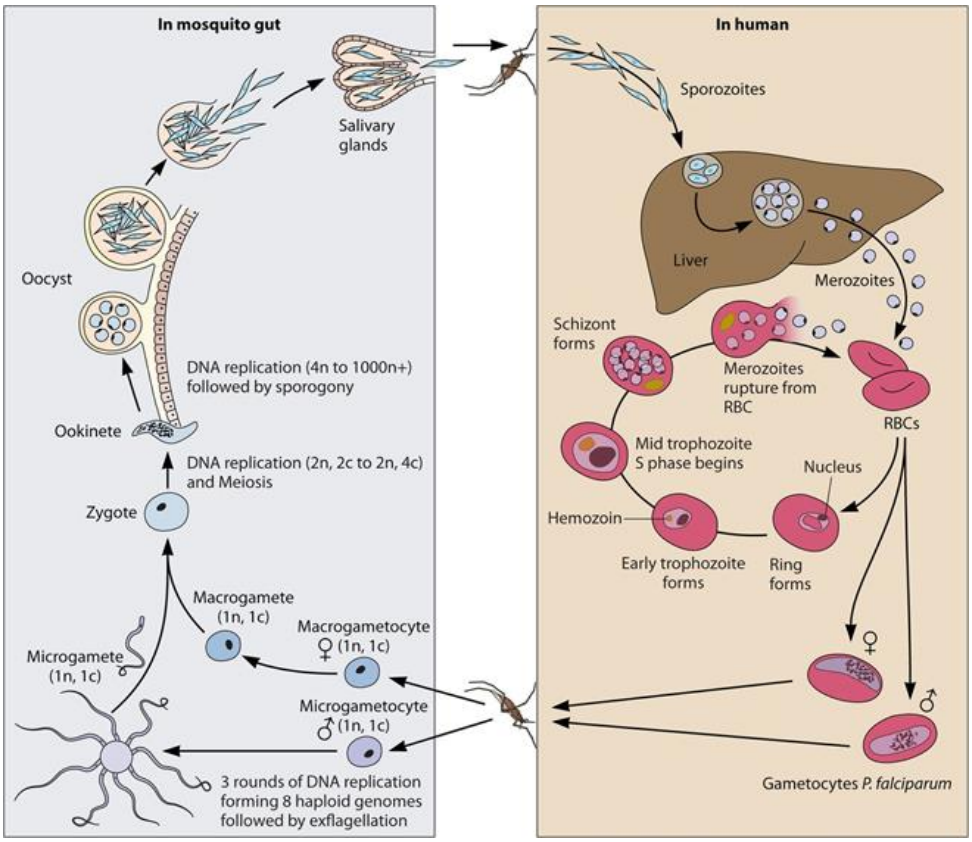

Figure 3 – The life cycle of the *Plasmodium* spp.

RBCs = red blood cells

Adapted from Lee, A. et al. (2014). DNA repair mechanisms and their biological roles in the malaria parasite *Plasmodium falciparum*. *Microbiol Mol Biol Rev, 78(3):469-486.*

Plasmodium infection manifestations

Classic definitions describe periodic fever spikes at intervals corresponding to the erythrocytic cycle length of the infecting species, such as 48 hours for *P. falciparum*, *P. vivax*, or *P. ovale* and 72 hours for *P. malariae*. This would result from the synchronisation of developmental stages, but such patterns are infrequent in *P. falciparum*.[116,118]

P. falciparum infection shows a broad spectrum of manifestations, ranging from asymptomatic parasitaemia (also called by WHO malaria infection) to manifestations of

symptoms and signs (also called by WHO malaria disease).[82] The clinical manifestations can be divided into two presentations: uncomplicated and severe.

Symptoms of uncomplicated malaria may include fever, chills, body aches, headache, cough, diarrhoea, anaemia, and splenomegaly.[116,118] As they are very non-specific manifestations, clinical diagnosis of malaria alone is not accurate.[125]

Common manifestations of severe malaria are cerebral malaria, acute lung injury, which can progress to acute respiratory distress syndrome, acute kidney injury, and acidosis[120,126,127] (Figure 4).

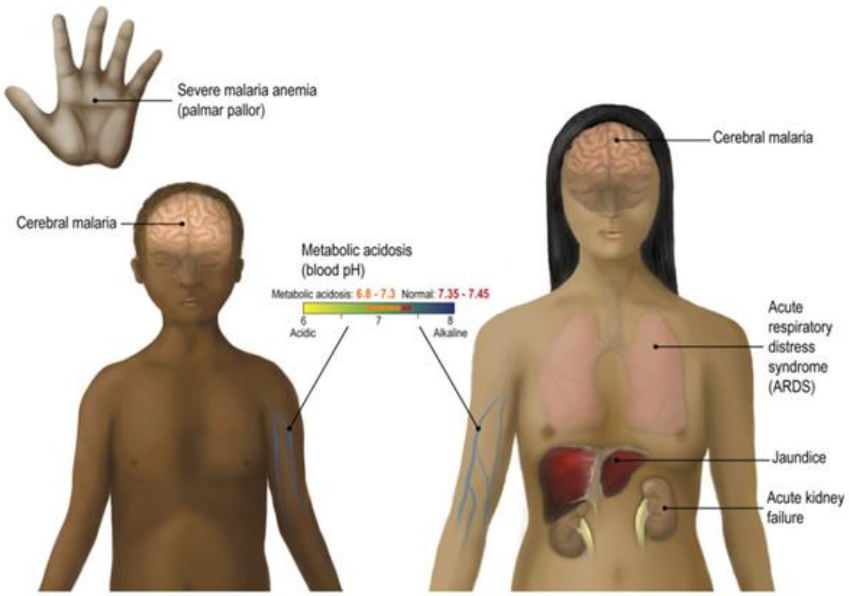

Figure 4 – Major clinical complications associated with severe malaria in adults or children.

Clinically severe malaria is a multisystem disorder that can affect different organs and differs in presentation between children and adults. The major clinical complications in children are cerebral malaria, severe malaria anaemia and metabolic acidosis. In adults, cerebral malaria is frequently accompanied by multiorgan system complications, including metabolic acidosis, acute kidney failure, jaundice, and acute respiratory distress.

Adapted from Wassmer, S. et al. (2015). Investigating the Pathogenesis of Severe Malaria: A Multidisciplinary and Cross-Geographical Approach. *Am. J. Trop. Med. Hyg, 93(Suppl 3): 42-56.*

The criteria to define severe malaria according to WHO are described in Table 2. The case fatality rate of treated cerebral malaria is usually 10 to 20% and can reach 50%

in pregnant women.[118]The development of severe malaria syndromes results from sequestration in small vessels, destruction of infected red blood cells, and the host immune reaction to the infection, with cytokines liberation.[116] *P. falciparum* is unique in that red blood cells containing mature parasites exhibit the protein *P. falciparum*-erythrocyte membrane protein-1 (pfEMP1)[120], responsible for cytoadherence and rosetting. These two processes are associated with severe malaria as they lead to the sequestration of the infected red blood cells (also see the section on The genetic protection to not develop malaria disease, page 38).

Table 2 – Diagnostic criteria for severe malaria

Clinical Criteria	
Impaired consciousness	A Glasgow Coma Score < 11 in adults or a Blantyre coma score < 3 in children.
Respiratory distress	Rapid, deep and laboured breathing.
Jaundice	Plasma or serum bilirubin > 3mg/dL together
Pulmonary oedema	Radiologically confirmed, or oxygen saturation < 92% on room air with a respiratory rate > 30/min, often with chest indrawing and crepitations on auscultation
Significant bleeding	Including recurrent or prolonged bleeding from nose gums or venipuncture sites; haematemesis or melaena
Shock	Compensated shock is defined as capillary refill time ≥ 3s or temperature gradient on the leg (mid to proximal limb), but no hypotension. Decompensated shock is defined as systolic blood pressure < 70mmHg in children or < 80mmHg in adults with evidence of impaired perfusion (cool peripheries or prolonged capillary refill time).
Laboratory criteria	
Severe anaemia	Haemoglobin < 5g/dL or a haematocrit of < 15% in children <12 years of age (< 7g/dL and < 20%, respectively, in adults)
Hypoglycaemia	Blood or plasma glucose < 40mg/dL
Acidosis	A base deficit of > 8mEq/L or a plasma bicarbonate of < 15mmol/L or venous plasma lactate > 5mmol/L.
Acute kidney injury	Plasma or serum creatinine > 3mg/dL or blood urea > 20mmol/L
Hyperparasitaemia	*P. falciparum* parasitaemia >10%

Reprinted from WHO (2014). Severe Malaria. *Tropical Medicine and International Health, 19*: 7-131.

Plasmodium infection: a broad spectrum of manifestations – Why?

Although only 0.2% of malaria cases are fatal, this still means that nearly half a million people die of malaria each year, mainly young children in Africa.[115] Research into why certain individuals develop severe malaria and might die is important to identify better interventions that could improve the outcome of the infection. The immune response, as described above (see the section on Immune system, page 24), while a crucial aspect, does not seem to explain everything with regards to the broad spectrum of diseases manifestations.

In the case of malaria, several other factors appear to influence the parasite load and, thus, disease severity, in particular hemoglobinopathies,[82] blood group,[128] and the host nutritional status[83], although the underlying mechanisms are not yet completely understood.

From *Plasmodium* infection to malaria disease – the importance of the immune system

The naturally acquired immunity generates a set of antibodies that act on specific components of the *Plasmodium* growth, such as merozoite neutralisation and clearance, killing or clearance of infected cells, and targeting cytoadherence processes and reducing disease severity.[129–131] In *Plasmodium* infection, most individuals do not develop sterilising immunity and remain vulnerable to ongoing blood-stage infection but do not develop severe malaria or any symptoms at all (asymptomatic parasitaemia).[64,132] This phenomenon of partial immunity is termed premunition, also known as an infection-immunity. Premunition limits the parasite load and thereby decreases malaria-associated morbidity and mortality without eliminating the infection.[133] In fact, it seems to fit with the overall idea that children may not control parasite replication well and thus suffer severe disease **(↑ pathogens = ↑ severe disease)**. At the same time, adults with premunition cannot only tolerate the presence of parasite without symptoms but seem to have a lower parasite burden **(↓ pathogens = ↓ severe disease)**. Controlled Human Malaria Infections (CHMI)[134,135] have shown that people from malaria-endemic areas, who have been previously exposed to the parasite, have lower parasite growth rates than malaria-naïve individuals suggesting that immunity reduces parasite multiplication.[136]

The importance of immunity can also be deduced in children, who have not yet developed protective immune mechanisms, being at greater risk of clinical malaria, severe disease and death than adults.[137,138] Furthermore, travellers from non-endemic areas have more commonly severe malaria and higher death rates.[137,139] A retrospective study from France compared clinical characteristics of imported *P. falciparum* malaria in adult travellers of sub-Saharan origin living in France and visiting family or relatives in their country of origin (VFR) and travellers of European origin. This study demonstrated that rates of severe malaria among all patients with *P. falciparum* malaria were 3% for the VFR group and 11% for the non-immune European group. Furthermore, initial parasitaemia above 2% was more common in the non-immune European group than in the VFR group (24% versus 16%).[140] Another prospective hospital-based study conducted in France compared clinical characteristics of *P. falciparum* malaria in non-immune European travellers and VFR who had been resident in Europe for at least four years. Comparing to the European travellers, the VFR group had lower mean parasite densities (0.8% versus 1.4%), less frequent severe disease (4.4% versus 15.2%), accelerated parasite clearance, and higher levels of antibodies against *P. falciparum*.[141] These observations lead, once more, to the expression **(↓pathogens = ↑ disease outcome)**. It would be interesting to make cultures with red blood cells from immune and non-immune malaria individuals to observe if the same differences between the two groups continue to exist in a scenario where the immune system is absent.[142,143]

Indeed, the immune system plays a crucial role in controlling the disease and, of course, the parasite burden in the body. However, is the immune system enough to explain all variation observed in individuals with different manifestations? Other host-related factors, often under genetic control, may significantly limit the parasite proliferation capacity and, hence, the disease manifestations.

<u>The genetic protection to not develop malaria disease</u>

It is known that the human host genetic background can determine the susceptibility to different clinical presentations of malaria,[64] independently of the immune system. The biological success of *P. falciparum* in the human host depends entirely on its ability to invade, grow and survive inside red blood cells.[81,144]

In endemic-malaria areas, the prevalence of certain haematological diseases that affect red blood cells, namely haemoglobinopathies and enzymopathies, led to the hypothesis that malaria deaths may have driven the selection of these genetic variations.[78,80,81,144] Some examples of apparent malaria protective gene variants are represented in Table 3.

Table 3 – Examples of apparent malaria protective gene variants and their mechanisms.

Gene	Encoded protein	Variant	Mechanistic hypotheses
HBB	β-globin	Heterozygous carriers of sickle haemoglobin (HbAS)	- Increased clearance of sickled infected red blood cells by the spleen; - Acquired host immunity and increased phagocytosis of ring-parasitised red blood cells; - Reduced cytoadherence and resetting; - Impaired trafficking of parasite proteins to red blood cell surface; - Inhibition of parasite growth due to oxygen-dependent polymerisation of sickle haemoglobin.
HBB	β-goblin	Heterozygous β-thalassaemia (absent or reduced β-globin)	- Enhanced antibody binding and subsequent clearance of infected red blood cells; - Increased phagocytosis of ring-parasitised red blood cells.
HBA	α-globin	α-thalassaemia (deletion or inactivation of one or more of the normal four α-globin genes)	- Increased phagocytosis of infected red blood cells by monocytes; - Enhanced antibody binding and subsequent clearance of infected red blood cells.
G6PD	G6PD	Female heterozygotes for G6PD deficiency	- Increased phagocytosis of ring-parasitised red blood cells due to enhanced oxidative stress.
CR1	Complement	Swain-Langley 2 (Sl2)	- Reduced binding of Sl2 red blood cells to the parasite rosetting ligand.

FY	Duffy antigen receptor for chemokines	FY*ES allele	- Inhibition of *P. vivax* invasion of Duffy negative red blood cells through impaired junction formation.
ABO	Glycosyltransferase enzyme	ABO single nucleotide deletion - Blood group O	- Reduced *P. falciparum* rosetting.
ATP2B4	PMCA4 calcium transporter	ATP2B4 single nucleotide polymorphisms	- Altered binding of transcription factors to ATP2B4 enhancer elements, leading to decreased gene expression and subsequent dysregulated intracellular calcium homeostasis.
GYP	Glycophorins	Duplicate GYPB-A hybrid genes encoding the Dantu blood group	- Inhibition of *P. falciparum* invasion due to increased membrane tension of Dantu red blood cells.
L23R, IL12-RB2	Interleukin 23 and Interleukin 12 receptor complex	IL23R-IL12RB2 single-nucleotide polymorphism clusters	- Immunoregulatory roles in protective immunity in malaria infections.

Reprinted from Kariuki, S. et al. (2020). Human genetics and malaria resistance. *Human Genetics, 139*: 801-811.

Adult haemoglobin A is usually composed of two α-globin chains and two β-globin chains. In haemoglobinopathies, haemoglobin A is altered by polymorphisms that lead to amino acid substitutions in beta-globin (haemoglobin S, C and E) or reduce the production of α-globin (α-thalassaemia) or β-globin (β-thalassaemia).[82,145]

Protection against clinical *falciparum* malaria varies between inherited red cell disorders, but in general, it is highest against severe malaria, moderate against uncomplicated malaria, and none against asymptomatic parasitaemia.[80–82] Sickle cell anaemia trait confers the most robust protection described against severe malaria, with an effect size superior to 80%, while α-thalassaemia confers a protective effect of around 40% in homozygous.[78,144] The mechanisms by which haemoglobinopathies affect parasite growth are not very clear yet, and some of them are described in Table 3. Several mechanisms of protection are hypothesised in sickle-cell trait, such as the sickling of the infected red blood cells,[146] increased splenic phagocytosis,[147] premature

haemolysis and parasite death,[148] impaired haemoglobin digestion,[148] and weakened cytoadherence.[149] However, these do not explain the differences observed in *in-vitro* cultures, where these mechanisms are absent. In this case, the low oxygen concentrations seem to be a more suitable explanation, as demonstrated in an *in-vitro* study.[148] The concentration of oxygen in the arterial blood is approximately 13%. However, when infected red blood cells are sequestered in the bone marrow, brain and liver vessels, the oxygen concentrations may be lower than 7.5%.[148,150] Low oxygen concentrations impair parasite growth in red blood cells containing the haemoglobin AS (sickle-cell trait),[148] which leads to a lower parasite burden and, consequently, to less severe disease, as in the expression **(↓ pathogens = ↓ severe disease)**.

What concerns enzymopathies, Glucose-6-phosphate-dehydrogenase (G6PD) deficiency is one of the most studied. G6PD deficiency is an X-linked disorder characterised by abnormally low levels of G6PD, being the most common enzymopathy in the world.[79,151] G6PD deficient red blood cells are more vulnerable to damages caused by oxidative stresses provoked by foods and drugs, leading to acute haemolytic anaemia.[151] It seems to be protective against *falciparum* malaria.[79,151] Although the protection mechanism is not completely clear, it is very likely related to the increased susceptibility to oxidative stress in these red blood cells.[79,151] A higher oxidative stress environment leads to parasite death which condition a lower parasite load and, consequently, less severe disease, pointing to the expression **(↓ pathogens = ↓ severe disease)**.

Other less-studied polymorphisms also confer some protection against malaria disease, most of them affecting red blood cells,[144] such as variants of the gene for complement receptor-1 (CR1) or 1ATP2B4 calcium transporters.[78,144] Even so, heritability studies imply that genetics account for only 25% of the total variation in malaria incidence and hospitalisation, with sickle cell status and α-thalassaemia, the two most relevant polymorphisms discovered so far in terms of their frequencies and effect sizes, accounting for only 2% of this variation.[78,144]

Another factor related to genetics and which may influence the multiplication of *P. falciparum* is the blood group. The clearest example of the influence of the blood group in the development of malaria is the Duffy blood group antigen. The importance of the Duffy blood group system in clinical medicine is related to transfusion

incompatibilities.[76] The system consists of four alleles, five phenotypes, and five antigens (Fy[a+b-], Fy[a+b+], Fy[a+b-], F[a-b+], Fy[a-b+], Fy[a-b-]).[76,77,152] The Duffy blood group antigen serves as a blood group antigen, a receptor for a family of proinflammatory cytokines (Duffy antigen receptor for chemokines [DARC]), and a receptor for *P. vivax* and *P. knowlesi*.[76,77,152] These parasites need interaction mainly with the Duffy antigen to enter the red blood cell. The negative phenotype seems to be more frequent in populations in which malaria is more common.[78,79] *P. vivax* is almost absent in Occidental Africa, where more than 95% of the population is Duffy negative.[76] As the parasite is not able to enter in the reticulocyte, it is not able to multiply and, therefore, no disease is manifest: **(↓pathogens = ↑ disease outcome)**.

What concerns the AB0 blood group system, studies show that individuals with blood group A, B and AB (i.e. non-0) appear to be more susceptible to severe *P. falciparum* infection and blood group 0 has a protective effect.[128] Despite an *in-vitro* study that developed an erythrocyte preference assay to distinguish invasion into four different AB0 blood group red blood cells populations co-incubated (in the same culture) revealed that *P. falciparum* parasites seem to invade preferentially group 0 over group A red blood cells.[153] However, the discrepant results could be explained by different mechanisms. While *in-vitro* growth may favour group 0, the variability in the severity of *P. falciparum* malaria in individuals with different AB0 blood groups may be due to cytoadherence and rosetting,[77,128] two processes associated with severe malaria. Rosetting is characterised by binding the infected red blood cells to uninfected red blood cells to form clusters,[77] facilitating the direct passage of merozoites from infected to uninfected cells. Cytoadherence is when infected red blood cells adhere to the endothelial and block the flow of small blood vessels leading to a limited oxygen supply and eventually ischaemia.[128] These two processes also affect the immune system clearance of infected red blood cells by reducing their circulation towards the spleen.[128] In non-0 blood groups, the rosettes are larger and stronger than those formed in group 0 red blood cells. [77,128] This is due to A and B antigens, absent in group 0, which are receptors to the parasite protein PfEMP1[116,120] expressed on the surface of infected red blood cells.[77,128] This way, rosettes formed in blood group 0 tend to be smaller, weaker and more unstable, and cytoadherence is reduced.

Although blood group 0 is more common in malaria-endemic countries, the frequency rates are 40-80%.[77] Notwithstanding, if group 0 protects against severe malaria, why is its frequency not higher in malaria-endemic countries? Probably because malaria is not the only life-threatening disease in these areas. Diseases such as diarrhoeal diseases, mainly cholera, seem to be more severe in group 0 individuals.[154] All this may translate in that other diseases also exert selective pressure on genetic characteristics, with *Plasmodium* playing an essential but not exclusive role.

Two more blood groups were found to have a possible relation with parasite multiplication: the Knops blood group and the MNS blood group. The Knops blood group antigens are located on the CR1 molecule.[77] The Knops system's null phenotype, called Helgeson red blood cells, showed significantly reduced rosetting[77] as described in blood group 0. However, the relation between this blood group and severe malaria is not very clear. The effect of CR1 expression level appears to vary depending on malaria endemicity: in populations with low malaria transmission, a significant correlation between low CR1 expression and severe malaria seems to exist, and in a higher transmission area, high CR1 levels seem to be associated with disease.[77] Regarding the MNS blood group, the Dantu blood group antigen results from a structural rearrangement in the glycophorin gene cluster.[155] This antigen seems to increase membrane tension of the Dantu variant red blood cells, inhibiting *P. falciparum* invasion,[144,155] which confers a strongly protective effect of 74% against all forms of severe *falciparum* malaria.[144]

According to the International Society of Blood Transfusion, there are 38 formally registered blood group systems codified in 45 different genes.[156] Thus, although advances in understanding interactions between malaria parasites and some blood groups, it is clear that many intriguing possibilities remain to be explored.

The described variants on the red blood cell that seems to confer protection against the *Plasmodium* are illustrated in Figure 5.

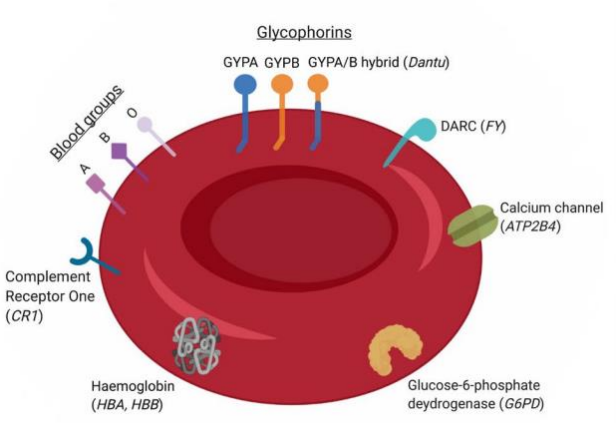

Figure 5 – Malaria protective variants that have an important role in the red blood cell.
The majority of malaria protective variants that have already been described have impact on the structure and function of the red blood cell.
Adapted from Kariuki, S. et al. (2020). Human genetics and malaria resistance. *Human Genetics, 139*: 801-811.

Nutrition role in Malaria

As described before (see the section on Nutritional Status, page 25), the patient's nutritional status may influence disease severity. Like all invading pathogens, also *Plasmodium* depends on the host nutrients' availability to multiply and survive.[83] Nutrients are a primary source of energy and act as regulators of gene expression, metabolism and growth. Parasites seem to have the capacity to sense and adjust to varying environmental conditions. *Plasmodium* parasites react to host dietetic calorie alterations by rearranging their transcriptome, leading to a considerable regulation of their multiplication rate, as shown in a rodent model.[88]

The relationship between malaria and nutrition appears complex. Indeed, while severe protein-energy-malnutrition seems to predispose to severe malaria, the association is not clear cut under all conditions, as pointed out by a recent review.[157] However, and though controversial, a study described raised *falciparum* parasitaemias and the development of malaria after a few days of refeeding famine patients during the Central African drought.[158] These clinical observations seemed consistent with studies in rodents,[88] where mice with a caloric restriction diet were switched to a regular diet during an ongoing infection. In addition to an increase in body weight, a rise

in parasitaemia was observed, which led to premature death. These observations seem to suggest that *Plasmodium* can rapidly adjust its multiplication rate based on nutrient availability, with fewer nutrients translate into lower parasitaemia and hence less disease severity. Once again, a case for **(↓ pathogens = ↓ severe disease)** could be made.

As mentioned before, not only undernutrition seems to increase disease severity but also overnutrition. Obesity and diabetes have increased globally, including in malaria-endemic countries.[9] These conditions seem to increase susceptibility to common infections,[106] including malaria. In a Swedish nationwide study with 937 adults with *P. falciparum* malaria, obesity and diabetes were identified as risk factors for severe malaria both in non-immune travellers and immigrants from sub-Saharan Africa.[159] Obese patients had higher parasitaemia compared with nonobese patients what was also describe in diabetes patients.[159] In another case-control study of 1466 adults in Ghana, patients with type 2 diabetes mellitus had a 46% increased risk for infection with *P. falciparum*.[160]

In brief, although nutrition and malaria seem to have a complex relation, the availability of the nutrients seem to influence the parasite load: fewer nutrients available may influence a lower parasite burden and less severe disease **(↓ pathogens = ↓ severe disease)**, as in the opposite side more nutrients available may lead to a higher parasite burden, and more severe disease **(↑ pathogens = ↑ severe disease)**.

Observations on parasite growth in-vivo and in-vitro

Growth of *Plasmodium* and consequently body parasite burden may be influenced by other, mostly unknown factors which are independent of the immune system. This raises the intriguing question if different parasite growth rates have been observed before in different individuals, especially in malaria-naïve individuals, that is without prior immune-system priming.

Indeed, a good example is the use of malaria to treat late-stage syphilis (malariotherapy) at the beginning of the twentieth century. Infection with *Plasmodium* induces high fever which was thought to promote the death of *Treponema pallidum*, the pathogen responsible for syphilis.[161] In the pre-penicillin era, the advanced infection

the spirochete could cause neurosyphilis, which affects neural performance and function, leading to general paresis of the insane (GPI), a severely debilitating mental disorder and almost universally fatal.[162] A lot of data was generated at that time, especially, as the parasitaemia of the patients was rigorously monitored.[161,163] A study with 245 patients from the South Carolina State Hospital in Columbia, and the South Carolina and the Georgia State Hospital in Georgia, revealed that the fold-change in interindividual parasite growth varied between 5.5 and 12.3.[164] Obviously, the discovery of penicillin ended malariotherapy.[161]

However, the controlled infection of individuals as a tool to evaluate candidate antimalarial drugs was recognised as useful and it was employed in healthy, non-immune males by inoculating *Plasmodium*-infected blood or by mosquito bite.[135] This practice, nowadays called CHMI already provided evidence in 1948 of a clear relation between *P. vivax* parasite burden and the manifestation of symptoms[165] (Figure 6).

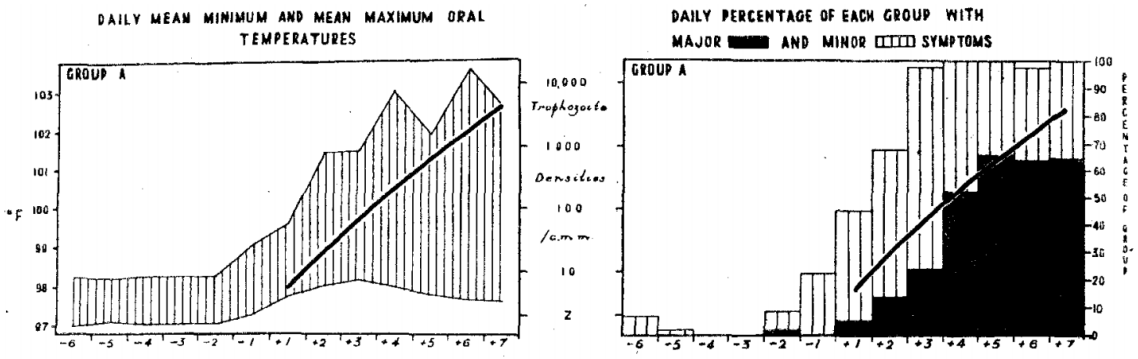

Figure 6 – Changes in oral temperatures and in the incidence of symptoms in volunteers with sporozoite induced malaria.

The x-axis represent the days before and after inoculation of sporozoites. The linear line represents the trophozoites density. On the right, the graph shows the daily mean medium and mean maximum oral temperatures of individuals infected with sporozoite induced malaria, and on the left, the daily percentage of major and minor symptoms of the volunteers. Both graphs show that parasite density is directly proportional to the manifestation of symptoms.

Adapted from Bickerton Blackburn, C. R. (1948). Observations on the development of resistance to vivax malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *42*(2), 117–162.

A recent CHMI study with malaria-naïve healthy individuals showed a large variability of parasite growth rates between participants with rather similar characteristics[166] (Figure 7).

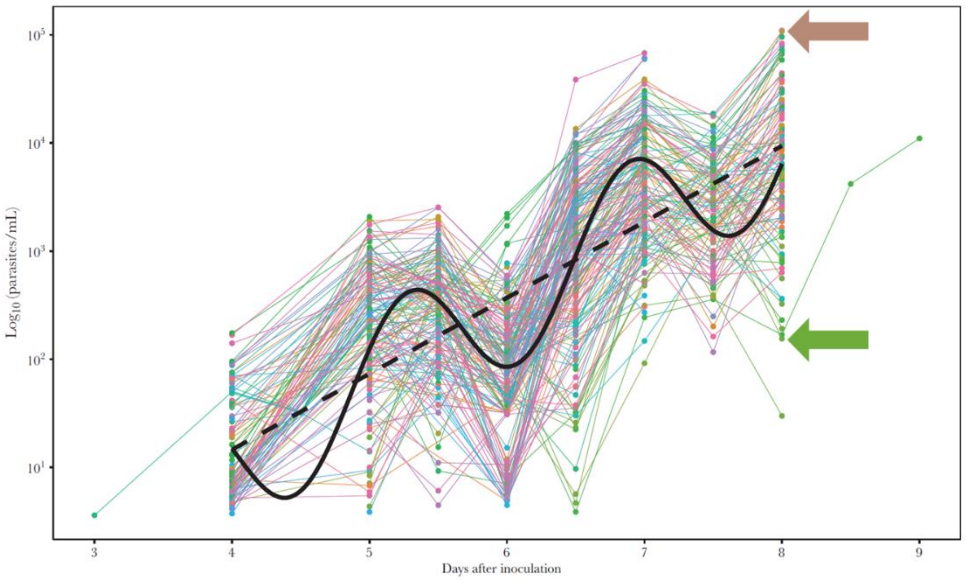

Figure 7 – Variability in individual parasitaemia profiles of non-immune volunteers infected with *Plasmodium falciparum*.

Each coloured lines represent the individual parasitaemia profile of one of 117 non immune volunteers. Note the large variability of parasite growth between different malaria-naïve individuals with two example marked on day eight: lowest growth ~10^2 (green arrow) – highest growth ~10^5 (brown arrow). Even assuming that the green one started at 10^1 (lowest) and the brown one at 10^2 (highest) on day 4, there would be a huge difference in the growth rate: green one from 10^1 to 10^2 = 10-fold; brown one from 10^2 to 10^5 = 1000-fold.

Adapted from Wockner L. et al. (2020). Growth Rate of Plasmodium falciparum: Analysis of Parasite Growth Data From Malaria Volunteer Infection Studies. *The Journal of Infectious Diseases, 221(6), 963–972.*

If the immune system was the only factor responsible for the variation of parasite growth and disease severity between infected individuals, it would be plausible to think these differences would be attenuated in *in-vitro* cultures of red blood cells, where the immune system component is absent.[143] However, large interindividual differences were also observed in *in-vitro* studies. For example, an *in-vitro* study that looked at the relationship between parasite invasion phenotypes and the red blood cell phenotypic

characteristics showed considerable variability in parasitaemia between red blood cells from different donors,[167] as shown in Figure 8.

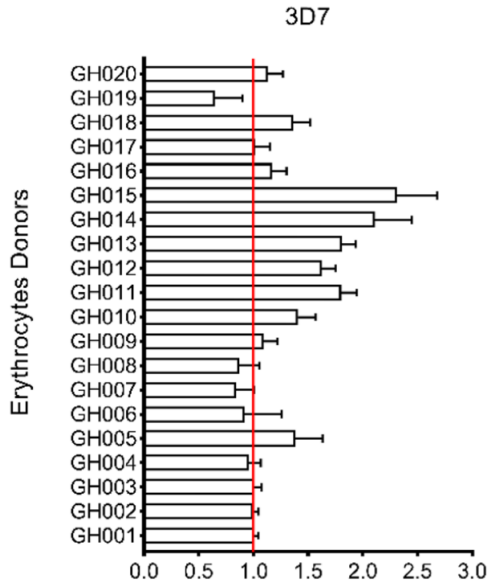

Figure 8 – Fold changes in parasitaemia (x-axis) in different red blood cell donors relative to a reference sample.

In an *in-vitro* study, parasitaemia from individual donors were normalised using a reference red blood cell sample. The graph shows a large variability in parasitaemia between individual. Adapted from Thiam L. G., et al. (2021). Blood donor variability is a modulatory factor for *P. falciparum* invasion phenotyping assays. *Nature*, 11:7129.

Yet, another example is an *in-vitro* study that looked at the influence of glucose on the growth of *P. falciparum* in *in-vitro* cultures.[168] In this study *P.* falciparum was cultured with red blood cells from thirty-nine malaria-naïve volunteers before and after a high-calorie high sugar meal. It was observed that, independently of glucose levels, cultures had discrepant rates of *P. falciparum* growth[168] (Figure 9).

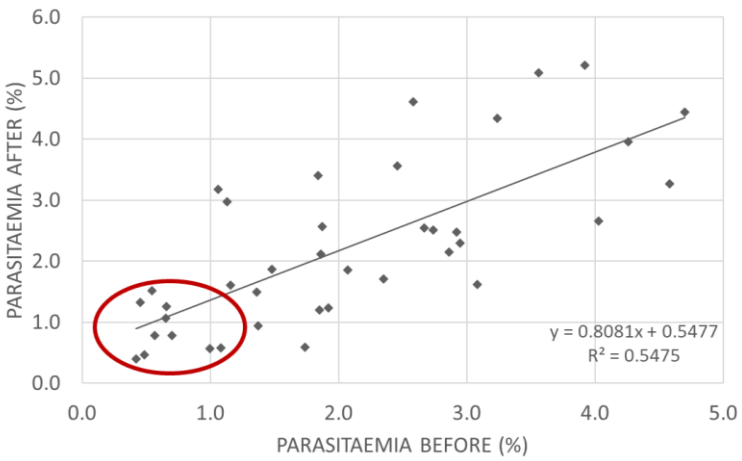

Figure 9 – Variability in parasitaemia of *Plasmodium falciparum* in *in-vitro* cultures of different individuals before and after a high-glucose meal.

Parasitaemia after 96 hours of *P. falciparum* cultures with blood from 39 healthy and non-immune volunteers before and after a high-calorie sugar meal. Note the large inter-individual variability of parasite growth that does not seem to be related to glycaemia neither glycaemia variation. The red circle points out 10 volunteers which presented consistently low final parasitaemia, both in the culture before and after the sugar-rich meal.

Based on data from Branco, A. (2018). Effect of underlying metabolic diseases, diet and metabolites on the growth of blood stage parasites: *Plasmodium falciparum*. Master Dissertation, Instituto Superior Técnico da Universidade de Lisboa. (Data provided by author and reanalysed).

All these observations raise the intriguing question what justifies that healthy non-immune individuals whose red blood cells are infected (*in-vivo* or *in-vitro*) with the same parasite strain and similar quantities have such different *P. falciparum* growth rates?

Hypothesis & Objectives

Of all the five known *Plasmodium* species that cause malaria in humans, *P. falciparum* is the only one that grows in continuous cultures,[169] a major important method to research success, leading to a greater understanding of the parasite.[170] *P. falciparum* culture protocol was proposed by Trager and Jansen[169] and requires a basic tissue culture medium to which red blood cells are added. Human red blood cells are

indispensable for *P. falciparum* growth as part of the parasite life cycle happens inside the red blood cells. Under *in-vitro* culture conditions, there is no immune pressure, and something related to the red blood cells appears the most likely explanation for the observed parasite growth variability.

This work hypothesises that the *P. falciparum* grows at different rates between individuals. Aside from immunity, other factors, specifically those related to the red blood cell, influence the growth of *P. falciparum* between individuals.

Objective: This work aims to investigate possible interindividual differences in the *P. falciparum* growth in malaria-naïve individuals in an *in-vitro* culture system and, if confirmed, to study possible associations and eventually causes for the observed variation.

MATERIAL AND METHODS

Volunteers

Malaria-naïve volunteers recruited were students, teachers, medical doctors and researchers from the Centro Académico de Medicina de Lisboa. All volunteers gave written informed consent before participation.

Reagents

Reagents for Malaria Complete Parasite Medium

The standard medium for *P. falciparum* culture comprises RPMI 1640, Hepes 1M, gentamicin (Life Technologies, Bleiswijk, Netherlands), L-glutamine (Sigma-Aldrich, Dorset, UK) and Albumax solution. The description of MCM components is described in Table 4. The proportions of the MCM components are described in Table 5.

The Albumax solution has in its composition RPMI, Albumax II (Life Technologies, Bleiswijk, Netherlands), gentamicin, Hepes, sodium bicarbonate and glucose (Aldrich, Dorset, UK). The proportions of the Albumax solution are detailed in Table 6.

Table 4 – Description of reagents used for Malaria Complete Parasite Medium

Malaria Complete Parasite Medium	
Reagent	**Description**
RPMI 1640 medium (no L-glutamine, with NaHCO3)	Roswell Park Memorial Institute (RPMI) is a medium used for cell culture and 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 has to be supplemented with serum for cell culture.
Hepes 1M	Hepes is a buffering agent commonly used in cell culture due to its ability to maintain the physiological pH despite CO_2 changes caused by cellular respiration.
Gentamicin (50mg/ml)	Gentamicin is a broad-spectrum antibiotic used in cell cultures because of its non-toxicity to the cell cultures, stability independent of both pH and temperature.
L-glutamine (200mM)	L-glutamine is the most abundant free amino acid in the human body, and it is necessary for the production of NAD+, very important in oxidative stress.
Albumax II solution	Albumax is a highly purified lipid-rich bovine serum albumin used as a supplement substitute for human serum.

Table 5 – Proportions of reagents used for Malaria Complete Parasite Medium

Malaria Complete Parasite Medium	
Reagent	**Quantity**
RPMI 1640 medium (no L-glutamine, with NaHCO3)	500 mL
Hepes 1M	12 mL
Gentamicin (50mg/ml)	500 µL
L-glutamine (200mM)	5 mL
Albumax II solution	50 mL

Table 6 – Reagents used for 500 mL of Albumax II solution*

Albumax II Solution	
Reagent	**Quantity**
RPMI (with L-glutamine, without NaHCO3)	5.2 g
Albumax II	25 g
Glucose	1 g
Gentamicin (50mg/ml)	10 µL
Hepes	2.98 g
Hypoxanthine	0.1 g
NaHCO3	1.67 g

* Filtered to sterilise and stored in 50 mL Falcon tubes at -20ºC

Reagents for parasitaemia assessment with microscopy

Smears from the cultures were fixed with absolute methanol (Merck, Darmstadt, Germany) and stained with a standard Giemsa solution (Merck, Darmstadt, Germany). The Giemsa was diluted in a proportion of 1:10 in PBS 1x and stored at a temperature room and protected from light.

Reagents for parasitaemia assessment with flow cytometry

For flow cytometry, *P. falciparum* cultures were marked with SYBR® Green Nucleic Acid Gel Stain (Invitrogen, Paisley, UK), stored at –4°C, protected from light. Solution of SYBR® Green I was prepared by diluting the stain in a proportion of 1:10 000 that was stored protected from the light at 0ºC.

Reagents for measurement of haemoglobin concentration

To determine the haemoglobin concentration, a Drabkin's reagent was used. The Drabkin's reagent is composed of potassium ferricyanide, potassium cyanide, dihydrogen potassium phosphate (anhydrous), non-ionic detergent (e.g. Sterox S.E. or Triton X-100) diluted in water.[171] The Drabkin's reagent used was kindly given by the Serviço de Patologia Clínica do Hospital Santa Maria.

Reagents for blood group testing

AB0 and Rhesus blood types were tested with Seraclone™ Anti-A, Anti-B, Anti-AB and Anti-Rh (Bio-Rad, Dreieich, Germany).

Materials

Equipment for maintenance of *Plasmodium falciparum* cultures

The *P. falciparum* cultures were incubate in a 5% carbon dioxide atmosphere at 37ºC in a Heracell™ incubator. To manipulate cultures a biological safety cabinet was used.

Equipment and programs for microscopy

Blood smears were examined by oil immersion with a Leica microscope using the 100x objective. Photographs of the blood smears were taken with Leica FireCam 3.4.1 (Leica Microsystems, Milton Keynes, UK) Afterwards, parasitaemia was determined using ImageJ (National Institutes of Health, USA).

Equipment and programs for flow cytometry

As for flow cytometry, *P. falciparum* cultures with volunteers' blood samples were analysed on a BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK) at the IMM Flow Cytometry Unit. Samples were analysed using a blue laser (488 nm) and a 535/45 nm bandpass filter. Parasitaemia analysis was performed using the program FlowJo (TreeStar, Ashland, Oregon).

Material for Blood collection

For the blood collection, the S-Monovette® system (Sarstedt, Nümbrecht, Germany) was used. Tubes with sodium citrate were used to collect anticoagulated blood destined to be transformed into plasma and red blood cells, while tubes with a clot inducer were used to collect serum. EDTA tubes were used for blood analyses, haemoglobin measurement and blood typing. To complement the system for blood collection, a Safety-Multifly® needle (Sarstedt, Nümbrecht, Germany) was used.

Equipment for glucose measurement

The device used to measure the volunteer's glycaemia was a home measuring device, OneTouch Select® Plus (LifeScan, Gubelstrasse, Switzerland). The test strips were the ones recommended to the glucometer.

Equipment for measurement of haemoglobin concentration

To measure haemoglobin concentration, the spectrophotometer Spectronic™ Helios™ Gamma (Thermo Electron, Massachusetts, EUA) was used.

Methods

Plasmodium falciparum cultures

P. falciparum laboratory strain 3D7 was routinely cultured in human red blood cells from the Portuguese Blood Institute (IPS – Instituto Português do Sangue e da Transplantação), according to Trager and Jensen's protocol.[169] Five per cent haematocrit was used in all cultures, and the medium was changed every day. Parasitaemia was assessed daily by microscopy. When it was superior to two per cent,

the culture was diluted with new red blood cells. The cultures were continuously incubated under controlled atmosphere conditions (37ºC and 5% CO_2).

Assessment of parasitaemia

The parasitaemia of main cultures was evaluated through microscopy. As for cultures performed with volunteers' blood, parasitaemia was assessed through microscopy and flow cytometry.

To assess the parasitaemia of *Plasmodium cultures* by microscopy, smears of the cultures were fixed with absolute methanol and stained for thirty minutes with the Giemsa-stained solution. A minimum of 1000 cells was counted for each smear. With microscopy, it was also possible to monitor the parasites' appearance (healthy, non-healthy).

To analyse parasitaemia by flow cytometry, *P. falciparum* cultures with volunteers' blood samples were marked with the solution of SYBR® Green I. Fifty microliters of this solution was added to five microliters of culture and incubated for thirty minutes at room temperature in the dark.[172]

Preparation of Red Blood Cells

For red blood cells separation, either from blood from the IPS or volunteers, the blood was centrifuged at 1800 rpm for 5 minutes. Then, the supernatant was removed, and the cells were washed three times with RPMI and centrifuged at 1800 rpm for 5 minutes. Afterwards, the red blood cells were stored at 4ºC for a maximum of 6 days.

Preparation of Serum

The serum was separated after the blood clot was completely formed in the clot inducer tube. Then the tube was centrifuged for 5 minutes at 4000 rpm. The serum obtained was immediately used to make MCM serum.

Optimisation of the Malaria Complete Medium protocol

As described before, the commonly used medium for *P. falciparum* cultures is composed, among other reagents, of Albumax solution, a bovine serum. To create an environment closer to the one found in the human host, the protocol was optimised by

replacing bovine serum with human serum, as described before.[168] The quantity of each of the constituents had to be adjusted, and glucose was added as the parasite is not able to grow without it. The proportions of MCM Serum used are described in Table 7. The optimisation of the protocol is represented in Figure 10.

Table 7 – Reagents used for the optimised Malaria Complete Parasite Medium with human serum

MCM 10% Serum	
Reagent	**Quantity**
RPMI 1640 medium (no L-glutamine, with NaHCO3)	87.5 mL
Hepes 1M	250 µL
Gentamicin (50mg/ml)	90 µL
L-glutamine (200mM)	900 µL
Solution 20% Glucose	900 µL
Serum	10 mL

*Values for 100 mL of Serum MCM

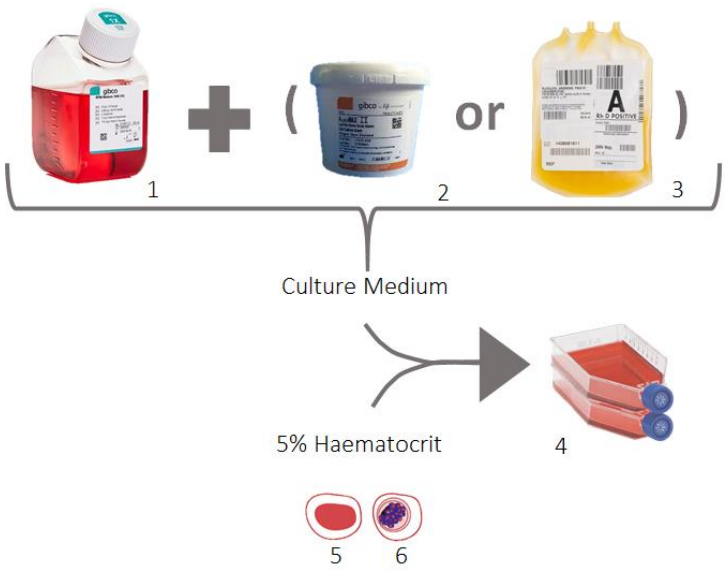

Figure 10 – Scheme of the Malaria Complete Parasite Medium optimisation protocol with human serum.

The culture medium is prepared with RPMI 1640 and supplemented by either serum or its substitute, such as Albumax, a bovine serum, and added to a culture flask. Then red blood cells (infected and uninfected) are added to the culture for a total of 5% haematocrit and a parasitaemia below 2%. Legend: 1 - RPMI 1640; 2 - Bovine Serum; 3 - Human Serum; 4 - Culture Flask; 5 - Uninfected Red Blood Cell; 6 - Infected Red Blood Cell

Study protocol

In the first phase, each volunteer filled a questionnaire to obtain volunteers' information, namely age, gender, birthplace, eating habits, and clinical and family history. Afterwards, blood was collected. At the time of blood collection, capillary blood glucose and haemoglobin concentration were measured, and AB0 and Rhesus blood groups were tested for each volunteer. Non synchronised *P. falciparum* cultures were made with volunteers' blood (Figure 11). After 96 hours, each culture parasitaemia was analysed using standard microscopic techniques and flow cytometry as described before.

In the second phase, volunteers who fell outside at least one standard deviation from the average, either with high or low parasite growth, were invited again to do another blood collection for blood screening and repetition of *P. falciparum* cultures to confirm the parasitaemia estimated in the first phase.

Questionnaire

Each volunteer filled a questionnaire, as mentioned before. The intention was to collect a set of information that may influence the *P. falciparum* growth and study possible correlations. The questionnaire was divided into six sections: personal data, blood donation, food, malaria history, history of anaemia or symptoms associated with it, and diseases and medication.

In section A, Demographic Characteristics, age, gender, blood group, nationality, place of birth and ancestries (parents, grandparents, great-grandparents) of non-Portuguese nationality were asked. With this section, we intended to find out if some genetic factors may influence parasite growth.

Figure 11 – Scheme of data obtained from each volunteer on the first phase of the study

Each volunteer filled a questionnaire on clinical and family history. At the time of blood collection, capillary blood glucose and haemoglobin concentration were measured, and AB0 and Rhesus blood groups were tested for each volunteer. Non-synchronised *P. falciparum* (*P.f.*) cultures were made with volunteers' blood.

In section B, Blood Donation, we asked if the volunteer was a blood donor and the last donation. As blood donation may influence certain characteristics in the donor's blood, including the age of circulating red blood cells, we intended to study any relationship between the age of red blood cells and any changes in the parasite growth.

In section C, Food, eating habits were asked, namely the composition of the last meal and how long it was, the eating pattern and any food intolerance. This way, we were able to study if food patterns could be related to parasite growth.

With section D, Malaria History, we intend to exclude volunteers that already had malaria.

In section E, a history of anaemia were asked, including any specific treatment and if any close family member had an anaemia diagnosis.

Finally, in section F, we asked about diseases, medication taken either daily or punctually in the last week before blood collection, and close family members' diseases.

Therefore, we could detect factors that may affect the parasite growth and check if there may be any correlation.

Measurement of haemoglobin concentration

Twenty microliters of volunteers' blood from the EDTA tube was added to five millilitres of a detergent Drabkin's reagent, mixed well, and allowed to stand at room temperature for ten minutes. The absorbance was measured, against the reagent blank, in the spectrophotometer at 540 nm. The spectrophotometer was previously calibrated, and a standard curve (y=0,0284x) was created to relate the absorbance to the haemoglobin concentration in g/dL.[171]

Blood group testing

For the detection of AB0 and Rhesus blood types, a rapid test was done according to the Seraclone™ leaflet. First, a drop of the reagent was placed into the slide, a small drop of whole blood was added and mixed. After two minutes, the agglutination was read according to Table 8.

Table 8 – Interpretation of reaction patterns characteristics and isoagglutinins in blood testing with Seraclone™ Anti-A, Anti-B, Anti-AB and Anti-Rh

- A	- B	- AB	AB0 Blood Group
+	-	+	A
-	+	+	B
+	+	+	AB
-	-	-	0

- Rh	Rhesus Blood Group
+	Rh +
-	Rh -

Legend: + agglutination, - no agglutination

Plasmodium falciparum cultures with volunteers' blood

For the *P. falciparum* cultures, both red blood cells and serum from healthy and non-immune volunteers were used. According to Trager and Jensen's methodology, the red blood cells were infected with non-synchronised *P. falciparum*, as described before.

The culture medium was supplemented with both bovine serum and human serum medium. Cultures were performed in 6 well plates, with 3.8 mL of medium and 5% haematocrit (20 µL of infected red blood cells and 180 µL of red blood cells from the volunteer). For each volunteer, triplicates were done for both culture medium. The cultures were incubated for 96 hours at 37ºC in an atmosphere of 5% CO_2. The medium was changed daily. The parasitaemia was measured at 0h and 96h either through microscopy and flow cytometry, as explained above. The protocol is represented in Figure 12.

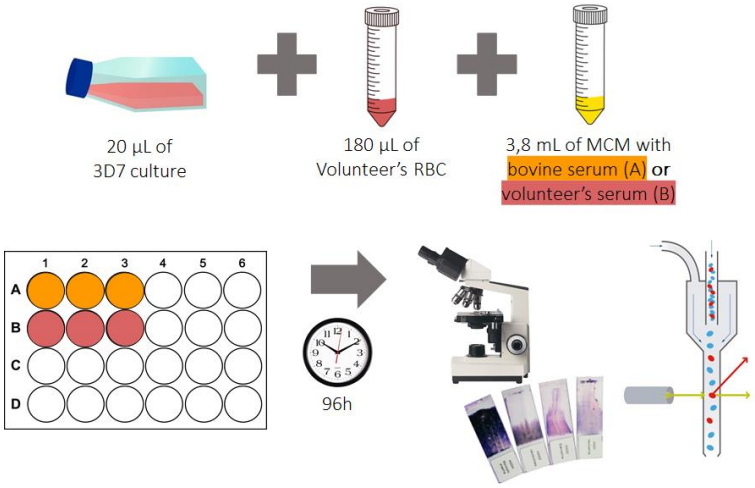

Figure 12 – Protocol scheme for Plasmodium falciparum cultures with volunteers' blood.

The volunteer's red blood cells (RBC), after being isolated, are infected with 20 µL of infected red blood cells. This culture is incubated with medium with both bovine serum and volunteer serum in a 5% haematocrit. The final parasitaemia was evaluated by microscopy and flow cytometry.

Tests on haematological and biochemical parameters in the blood

The volunteers who were called again for the second phase gave a new blood donation to repeat the *P. falciparum* cultures and to carry out tests on haematological and biochemical parameters in the blood. The blood tests were performed in a certified laboratory inserted in routine lab analyses. The blood tests performed were as follows: complete blood count, reticulocytes count, iron study (iron and ferritin), vitamin B12, folic acid, haemoglobin high-performance liquid chromatography (HPLC), α-

thalassaemia genetic tests, total bilirubin, lactate dehydrogenase, C-reactive protein, sedimentation rate, lipid profile (HDL cholesterol, total cholesterol and triglycerides), glucose and glycated haemoglobin and zinc assay.

Statistical analysis

Data were collected and analysed using Graphpad Prism for Windows (GraphPad Software, San Diego, USA).

In the descriptive statistics, categorical variables were given as frequencies, whereas numerical variables were expressed as median, standard deviation and range.

Numerical variables that presented a normal distribution and similar variances were compared using the t-student test (if comparing two variables) or the one-way ANOVA test (if comparing three or more variables). For numerical variables that did not follow a normal distribution, a Mann-Whitney test was used to compare to variables. Finally, categorical variables were compared using Fisher's exact test.

Ethics

This study was submitted and approved by the Ethics Committee from the Centro Académico de Medicina de Lisboa.

RESULTS

Volunteers

In total, 100 participants were recruited, and results for analysis could be obtained from 69. Two were excluded because they already had malaria, one was excluded because the volunteer had been in a malaria-endemic country in the previous six months of blood collection, and twenty-eight were excluded due to technical problems during the *in-vitro* culture. Therefore, results were available for 69 malaria-naïve volunteers. Table 9 summarises the demographic data of the volunteers.

Table 9 – Demographic description of the 69 volunteers of the study.

Demographic characteristics	**Total study volunteers n = 69**
Average age (range) in years	26 (18 – 56)
Sex (n)	
Female	55 (79.7%)
Male	14 (20.3%)
Nationality (n)	
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%)

Plasmodium falciparum growth after 96h hours of culture

The growth of *P. falciparum* after 96 hours of culture with blood from the 69 volunteers is represented as a fold-change calculated from final parasitaemia at 96 hours divided by the initial parasitaemia when the culture was initiated (Figure 13). The data is normally distributed, with a minimum of 3.0, a maximum of 19.3, a mean of 10.0 and a standard deviation (SD) of 3.8.

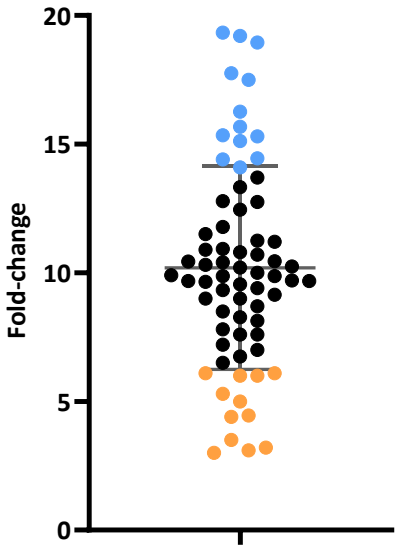

Figure 13 – Fold-change of *Plasmodium falciparum* after 96 hours of cultures with blood from 69 volunteers.

Mean and +/- 1 SD are shown. The orange dots represent volunteers with a consistently low parasite growth below one standard deviation from the mean (n=12). The blue dots represent volunteers with a consistently high parasite growth above one standard deviation from the mean (n=13).

Three groups were created: 1) "lower", 2) "normal", and 3) "higher" growers (Figure 13 and Figure 14). The "lower growers" (LG) group is defined by a fold-change below one standard deviation from the mean, and it is composed of twelve volunteers with a fold-change below 6.2 (mean: 4.7, SD: 1.2, range: 3.0 – 6.1). The "higher growers" (HG) group is defined by a fold-change above one standard deviation from the mean, comprising thirteen volunteers with a fold-change above 13.8 (mean: 16.0, SD: 1.8, range: 13.8 – 19.3). Finally, the "normal growers" group lies (within +/- 1 SD from the mean) between the two other groups and is constituted by 44 volunteers (mean: 9.7, SD: 1.7, range: 6.5 – 13.3) (Figure 14).

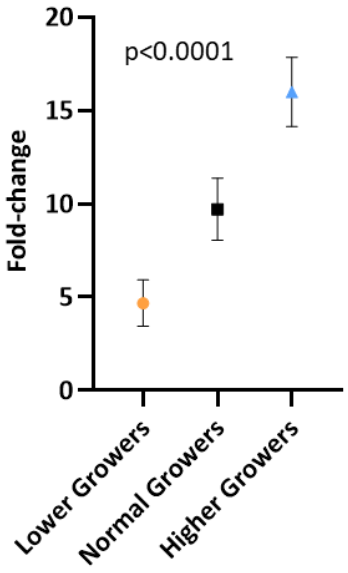

Figure 14 – Three groups distinguished by their *Plasmodium falciparum* growth pattern.

Each column mean represents one of the three groups distinguished by *P. falciparum* growth. The orange bar represents the "lower growers" group, the black bar the "normal growers" group and the blue on the "higher growers" group. A one-way ANOVA test was used to compare the means of the three groups and a Tukey's multiple comparisons test was used to compare each of the groups.

Reproducibility of the observed interindividual variability of Plasmodium falciparum growth

Twenty four volunteers agreed to give a second blood sample. Unfortunately, due to technical problems, results of *P. falciparum* cultures were available only for thirteen of these volunteers; four were from the LG group and seven from the HG group (Figure 15). A Wilcoxon test was performed to compare the fold-change from the first phase and the fold-change in the second phase. No statistically significant differences were found (p=0.99). Of note, *P. falciparum* cultures were performed many times over the past three years with blood from one volunteer from each group. The growth pattern of these volunteers was always consistently low or high (results not shown); that is, it was consistently low in the LG grower volunteer and consistently high in the HG volunteer.

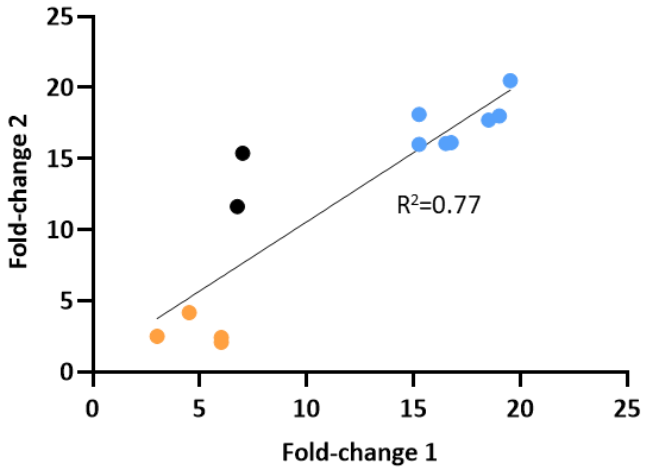

Figure 15 – Fold-change of *Plasmodium falciparum* in the first and second phases.

The orange dots represent the volunteers from the LG group, and the blue dots the volunteers from the HG group. The graph shows a correlation between the growth of *P. falciparum* in the cultures performed with blood from the same volunteer in the first (fold-change 1) and second phases (fold-change 2). "Lower" growers maintained their "lower" grower" pattern, and "higher" growers continue to be "higher" growers.

Variables that might influence the Plasmodium falciparum growth

In the first phase of this study, variables obtained with the questionnaire and haematology- biochemical laboratory tests were analysed for any relation between them and the found differences in *P. falciparum* growth between the LG and HG groups (Table 10).

Five sections of the questionnaire were analysed: A (demographic characteristics), B (blood donation), C (food), E (previous history of anaemia) and F (diseases and medication). Section F was used to exclude volunteers that were already exposed to the parasite.

Table 10 – Studied variables with possible influence on the *Plasmodium falciparum* growth.

Variables	Lower Growers n = 12	Higher Growers n = 13	P-value
Age, years – mean (range)	28 (21 – 48)	29 (19 – 56)	0.72
Sex			
Female	10 (83.3%)	9 (69.2%)	0.64
Male	2 (16.7%)	4 (30.8)	
Nationality			
European*	10 (83.3%)	13 (100.0%)	0.22
Endemic-malaria country**	2 (16.7%)	0 (0.0%)	
Ancestries native country* **			
European*	6 (50.0%)	12 (92.3%)	0.03
Endemic-malaria country**	6 (50.0%)	1 (7.7%)	
AB0 blood group			
Blood type A	5 (41.6%)	4 (30.7%)	0.71
Blood type B	2 (16.7%)	2 (15.4%)	
Blood type AB	0 (0.0%)	2 (15.4%)	
Blood type 0	5 (41.6%)	5 (38.5%)	
Haemoglobin concentration, g/dL – mean (range)	12.3 (10.6 – 15.1)	14.8 (13.0 – 19.0)	0.03
Symptoms of anaemia◊ in the previous 2 years	2 (16.7%)	2 (15.4%)	0.99
Previous history of anaemia	5 (41.7%)	0 (0.0%)	0.01
Family history of anaemia	8 (66.7%)	3 (23.1%)	0.04
Blood Donors	5 (41.7%)	4 (30.8%)	0.99
Food pattern			
Omnivorous	11 (91.7%)	13 (100.0%)	0.48
Pesco-vegetarian	1 (8.3%)	0 (0.0%)	
Food intolerance	3 (25.0%)	3 (23.1%)	0.99
Time since last meal before blood collection			
<1h	7 (58.3%)	2 (15.4%)	0.04
> 1h	5 (41.7%)	11 (84.6%)	
Glycaemia, mg/dL – mean (range)	103.3 (81 – 131)	99.3 (84 – 123)	0.52
Concomitant underlying medical conditions▪	7 (58.3%)	4 (30.7%)	0.24
Regular medication▪▪	8 (66.7%)	5 (38.5%)	0.24

European nationality:** Portuguese, Germain. Only one volunteer is Germain (higher growers group), and the others are Portuguese. ****Endemic malaria countries:** Angola, Brazil, India, and Mozambique. ** **Ancestries native country:** country where parents, grandparents and great-grandparents are from. ◊ Symptoms of anaemia: brittle nails, difficulty concentrating, dizziness, fatigue, feeling faint, hair loss, headaches, loss of appetite, low blood pressure, palpitations, and sleep alterations. ▪ **Concomitant underlying medical conditions:** allergic rhinitis, asthma, arterial hypertension, and dyslipidaemia. ▪▪ **Regular medication:** combined hormonal contraceptive, antihistamine, antidepressant, antihypertensive and statins.

Different statistical tests were used as described in the Methods (see section Statistical analysis, page 57).

The demographic characteristics of the two groups were found to be similar. Age, sex and nationality are not statistically significantly different between LG and HG groups (Table 10). However, the ancestries native country is statistically significant between the two groups (p=0.03). The ancestries native country is the country where parents, grandparents and great-grandparents are from. More volunteers of the LG group present ancestries from endemic-malaria countries, namely Angola, Brazil, India, and Mozambique, compared to the HG group (Table 10).

The food pattern (p=0.48) and food intolerance (p=0.99) show no statistically significant difference between the two groups (Table 10). Of note, the time since the last meal shows a statistically significant difference between groups. Volunteers in the LG group had the last meal closer to the blood collection than the HG group (p=0.04) (Table 10). All meals were similar, with most being a main meal, like lunch, including carbohydrates. Nevertheless, although the LG group had a mean glycaemia higher than the HG group, there are no statistically significant differences between the two groups (LG – 103.3 mg/dL vs HG – 99.3 mg/dL, p=0.52) (Table 10).

Regarding the previous history of anaemia, two volunteers in both groups report symptoms associated with anaemia in the previous two years of this study. The symptoms described were mainly fatigue accompanied by brittle nails, difficulty concentrating, dizziness, fatigue, feeling faint, hair loss, headaches, loss of appetite, low blood pressure, palpitations, or sleep alterations. This result shows no statistically significant difference between the LG and HG groups (p=0.99) (Table 10). However, a previous history of proper anaemia diagnosis is more likely in the LG group (p=0.01) (Table 10). Moreover, having a family history of anaemia is also more likely in the LG group (p=0.04) (Table 10). The previous history of anaemia described is usually related to microcytic anaemia, with haemoglobin levels close to the lowest reference value not always justified by lower iron and with no further evaluation for haemoglobinopathies. Other known diseases related to the red blood cell were asked, but no more were reported besides anaemia. Furthermore, the haemoglobin concentration of the volunteers of the LG group is statistically significantly lower than the HG group (LG – 12.3 g/dL *vs* HG – 14.8 g/dL, p=0.03) (Table 10).

The blood group also shows no statistically significant difference between the LG and HG groups (p=0.71) (Table 10). The more frequently observed blood types between the groups were blood type A (LG – 5 *vs* HG – 4) and blood type 0 (LG – 5 *vs* HG – 5) (Table 10).

Section B and F show no statistically significant differences between the LG and HG groups (Table 10).

In summary, there is found some evidence for presenting a lower growth of the *P. falciparum* in *in-vitro* culture for:

- Individuals connected to malaria-endemic countries;
- Something that cannot be further explained with this study but is related to anaemia, pointing to some problem in the red blood cells.

Tests on haematological and biochemical parameters of the blood and the Plasmodium falciparum growth

The twenty-four volunteers that agreed to give a second blood sample had tests on the haematological and biochemical parameters of the blood. Of these, six volunteers were from the LG group and nine from the HG group.

The parameters assessed were analysed for any relation in the differences in *P. falciparum* growth between the LG and HG groups. Except for the haemoglobin concentration (p=0.04), no statistical significance difference between the *P. falciparum* growth and the parameters assessed was observed (Table 11).

In particular, haematological and biochemical parameters associated with red blood cells such as haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, iron, vitamin B12 and folic acid were studied for a correlation. No correlation between the referred parameters and the parasite growth was observed (Figure 16).

Table 11 – Tests on haematological and biochemical parameters of the blood and the *Plasmodium falciparum* growth

Parameter	Lower Growers n=6 mean (range)	Higher Growers n=9 mean (range)	P-value
Red blood cells count RR: 3.8 – 5.1 x10^{12}/L	4.65 (4.10 – 5.26)	4.70 (4.11 – 5.46)	0.83
Haemoglobin concentration RR: 12.0 – 15.3 g/dL	12.7 (11.9 – 14.3)	13.9 (12.7 – 15.6)	0.04
Haematocrit RR: 36.0 – 15.3 %	39.4 (35.6 – 45.0)	40.1 (33.8 – 46)	0.77
Mean corpuscular volume RR: 80.0 – 97.0 fL	83.8 (78.7 – 86.9)	86.3 (80.0 – 91.4)	0.08
Mean corpuscular haemoglobin RR: 27.0 – 33.0 pg	27.4 (21.8 – 29.3)	29.4 (27.1 – 30.8)	0.08
Mean corpuscular haemoglobin concentration RR: 31.5 – 35.5 g/dL	33.2 (30.8 – 34.2)	33.8 (32.7 – 34.5)	0.44
Red Cell Distribution Width RR: 11.5 – 14.5 CV%	14.0 (13.1 – 17.4)	13.2 (12.2 – 17.4)	0.43
Reticulocytes RR: 0.5 – 1.5 %	0.9 (0.6 – 1.2)	1.2 (0.7 – 1.6)	0.07
Leucocytes RR: 4.0 – 11.0 x10^{9}/L	7.08 (4.20 – 8.80)	7.71 (5.60 – 10.1)	0.53
Platelets RR: 150 – 450 x10^{9}/L	277 (172 – 396)	267 (205 – 409)	0.90
Sedimentation rate RR: ≤12 mm	13.8 (2.0 – 30.0)	12.0 (4.0 – 23.0)	0.70
C-reactive protein RR: <0.5 mg/dL	0.23 (0.03 – 0.66)	0.14 (0.03 – 0.45)	0.77
Zinc RR: 10.7 – 18.3 µmol/L	10.5 (8.5 – 13.1)	9.7 (8.5 – 12.8)	0.88
Glucose RR: 70 – 110 mg/dL	70 (64 – 76)	89 (60 – 140)	0.11
Glycated haemoglobin (HbA1C) RR: 4.0 – 6.0 %	5.1 (4.7 – 5.3)	5.1 (4.9 – 5.6)	0.82
Total billirubin RR: <1.2 mg/dL	0.29 (0.15 – 0.46)	0.63 (0.21 – 0.73)	0.12
Iron RR: 33 – 193 µg/dL	75.5 (51.1 – 121.4)	103.1 (68.7 – 138.3)	0.07
Ferritin RR: 13 – 150 ng/mL	64.6 (5.7 – 237)	66.8 (12.9 – 258)	0.61
Vitamin B12 RR: 195 – 770 pg/mL	424 (153 – 574)	586 (164 – 1056)	0.26
Folic acid RR: 4.6 – 18.7 ng/mL	9.5 (7.2 – 12.4)	8.7 (4.1 – 20.0)	0.36
Lactate dehydrogenase RR: 100 – 250 U/L	180 (164 – 204)	176 (126 – 285)	0.85
Total cholesterol RR: <190 mg/dL	174 (135 – 237)	176 (108 – 271)	0.66

RR = Reference range

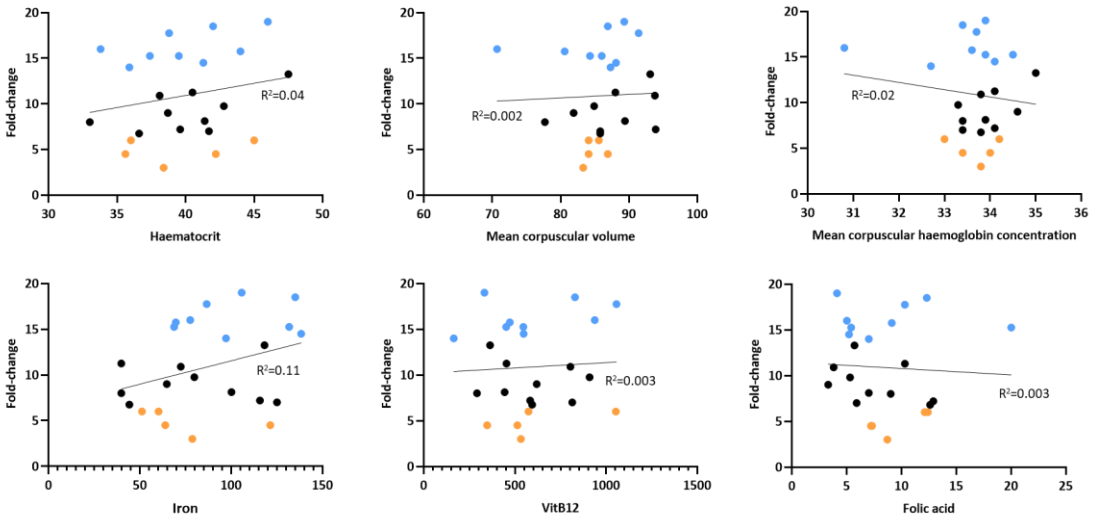

Figure 16 – Correlation between blood tests related to red blood cells and the *Plasmodium falciparum* growth.

The blue dots correspond to the volunteers from the HG group (n=9) and the orange dots to the volunteers from the LG group (n=5).

Haemoglobin types were investigated by high-performance liquid chromatography (HPLC) (Table 12). The results were compared between the LG and HG groups using a Student-t test. Both percentages of haemoglobin F (p=0.53) and haemoglobin A2 (p=0.21) show no statistically significant differences between the two groups. Furthermore, the observation on HPLC pattern curves shows overlapping curves of the volunteers from the LG and HG groups. For example, Figure 17 shows two HPLC curves from one volunteer of each group representing the others volunteers of the same group.

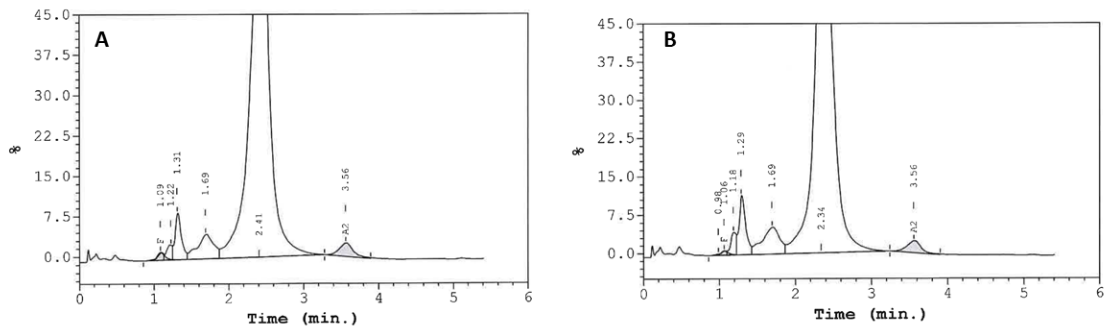

Figure 17 – Two representative haemoglobin curves of two volunteers from different growth patter group

Haemoglobin curves were obtained through an high-performance liquid chromatography. The curve A is from a "lower grower" volunteer and the curve from a "higher grower" volunteer. There are no other apparent peaks apart from the expected ones.

Table 12 – Haemoglobins, separation and assay through high-performance liquid chromatography

Volunteer	Growth Group	Haemoglobin F assay (%)	Haemoglobin A2 assay (%)	Variants (%)
1002	HG	0.3	2.6	0
1003	HG	0.4	2.6	0
1005	HG	0.3	2.6	0
1007	NG	0.4	2.1	0
1009	HG	0.6	2.3	0
1010	NG	0.3	2.5	0
1012	NG	0.7	2.6	0
1017	LG	0.2	2.4	0
1018	LG	0.3	2.4	0
1020	NG	0.7	2.6	0
1021	HG	0.5	2.5	0
1024	HG	0.5	2.8	0
1027	LG	0.7	2.5	0
1028	LG	0.8	2.8	0
1029	LG	0.2	3.1	0
1033	HG	0.4	2.6	0
1035	NG	0.5	2.7	0
1039	NG	0.6	2.9	0
1040	HG	0.4	2.3	0
1042	NG	1.0	2.4	0
1043	NG	0.6	2.3	0
1045	HG	0.2	2.3	0
1052	NG	0.6	2.5	0
1075	LG	0.2	2.8	0

Haemoglobin F reference range: «1%
Haemoglobin A2 reference range: 1.8 – 3.5%
Growth group: HG – “higher growers”; LG – “lower growers”; NG – “normal growers”

Concerning further characterisations of the haemoglobin, so far, it was only possible to perform an α-thalassaemia genetic test on two volunteers (one volunteer from the LG group and the other from the HG group). The volunteer from the LG group tested positive for heterozygosity for the deletion 3.7 kb. This volunteer has haemoglobin levels permanently closed to the lower reference value and microcytosis (mean corpuscular volume < 80 fL), which this α-thalassaemia trait could explain. On the other hand, the volunteer from the LG group tested negative.

Plasmodium falciparum growth in culture supplemented with bovine serum (Albumax) versus human serum

Fourteen cultures were performed with autologous human serum. *P. falciparum* growth after 96 hours in cultures supplemented with human serum was compared with the parasite growth in cultures supplemented with bovine serum. The mean fold-change in the bovine medium was 10.0 (SD: 4), and the mean fold-change in the human serum supplemented medium was 8.2 (SD: 4) (Figure 18). A paired Student-t test showed statistically significant differences between the two groups (p=0.001). Moreover, when observed under the optic microscope, the parasites have an less viable appearance in the smears of cultures supplemented with human serum than in the ones supplemented with bovine serum (Figure 19).

However, cultures supplemented with bovine serum and human serum from the same volunteer appeared to maintain the pattern of the parasite. Hence, an LG culture supplemented with human serum continued to show a pattern of an LG, and the same applies to the HG volunteers (Figure 20).

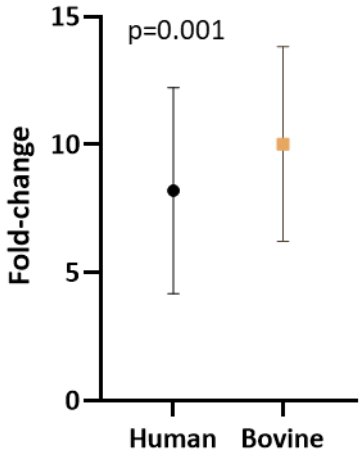

Figure 18 – Representation of fold-change of *Plasmodium falciparum* growth after 96 hours of culture supplemented with bovine serum versus human serum.

Fold-change of *P. falciparum* growth in cultures supplemented with bovine serum (n=14) and human serum (n=14). A paired Student-t test shows statistically significant differences between groups (p=0.001).

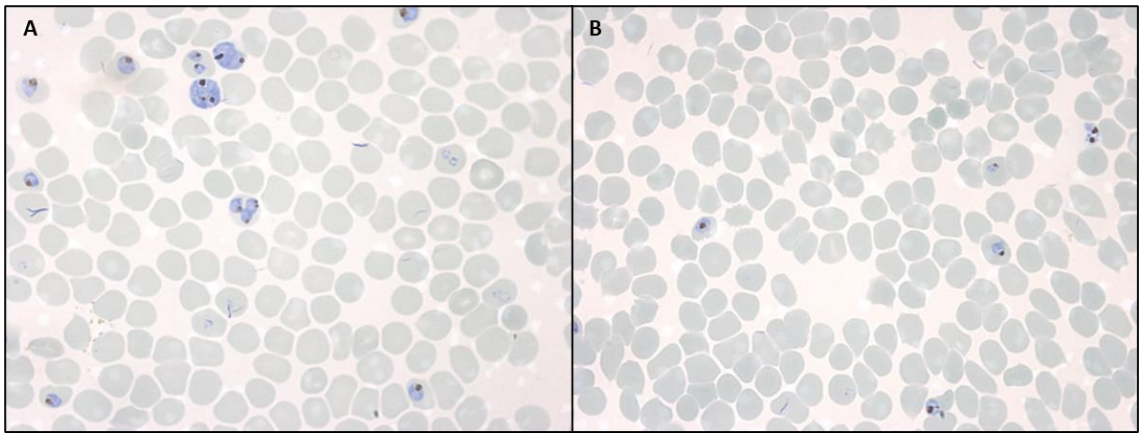

Figure 19 – Representative smears of *Plasmodium falciparum* cultures supplemented with bovine serum versus human serum.

A representative microscopic field of a culture with albumax (A) and human serum (B) are shown. The parasites of the culture supplemented with bovine serum (A) are larger than the parasites of culture supplemented with human serum (B). Both cultures were performed with blood from the same volunteers and autologous serum was used. (Giemsa stained culture, 1000x magnification)

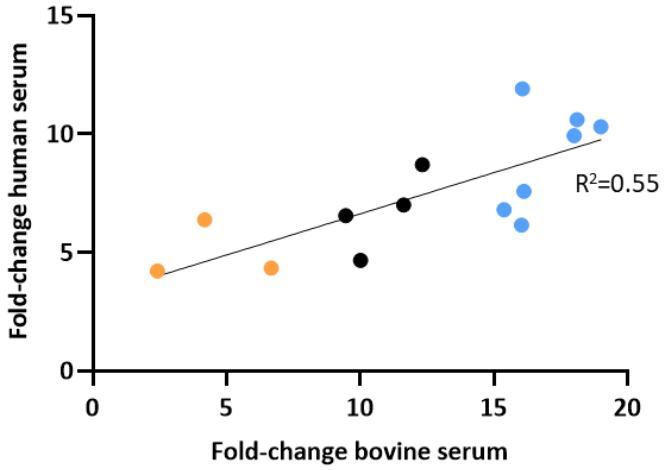

Figure 20 – Relations between fold-change of *Plasmodium falciparum* growth after 96 hours in culture supplemented with bovine serum versus human serum.

The orange dots represent the “lower growers”, the black dots the “normal growers”, and the blue dots the “higher growers”.

DISCUSSION

The mean fold-change in the *P. falciparum* growth observed in this study coincides with the replication rates described in the literature, which can vary between 2.3 and 6.0 fold per 48 hours.[166] Nevertheless, this *in-vitro* study demonstrates that **the parasite grows at different rates (reproducibly) in culture with red blood cells from different donors, showing a considerable variability between individuals**.

Although it was possible to recruit 100 volunteers, results were not available for all due to technical problems, representing a limitation to this study. The COVID-19 pandemic made it difficult for the volunteers to come to the laboratory for blood collection due to the circulation restrictions, which delayed the execution of the present work and added limitations to the second phase, with only 24 volunteers. Notwithstanding, 69 volunteers constituted the present study, a considerable sample compared to other recent studies in the field.[167,173,174]

It was possible to identify clearly individuals who have lower growth and those with higher growth. Of note, these observations were made in a fairly homogenous population, a sample composed mainly through medical students who may not be representative of the general population, as they are younger (mean: 26 years; median: 23 years; mode: 20 years), mainly female (80%), with healthy lifestyles and no major comorbidities. This raises the question: If it was possible to find these differences in this sample, would it be possible that the interindividual difference is even more prominent in an unselected population? In future work, it would be interesting to extend this study and try to include a more unselected population, especially focusing on immigrant populations from Africa.

Certainly, the implications of these growth patterns *in-vivo* have to be studied further as this study used *in-vitro* cultures and thus may not predict how the observations may impact infections *in-vivo*, as so other factors may be involved, namely the immune system.

P. falciparum cultures were supplemented with human serum, and the fold-change growth was compared with cultures from the same volunteer supplemented

with bovine serum. The fold-change in parasite growth was, in general, lower in cultures supplemented with autologous human serum. The strain used was a laboratory strain (3D7), and it is conceivable that it has adapted to medium supplemented with bovine serum, which could explain why the parasite grew less well when the supplement is switched to human serum. In fact, when observed under the optic microscope, the parasites have a "less healthy" appearance (smaller) in the smears of cultures supplemented with human serum than in the ones supplemented with bovine serum (Figure 19). However, even in the culture supplemented with autologous human serum, the interindividual parasite growth pattern was maintained, even though the fold-change was generally lower. A culture from an LG continues to show a pattern of an LG and an HG culture a pattern of an HG (Figure 20).

As the supplementation with autologous human serum did not change the *P. falciparum* growth pattern, the only other component from the culture that could explain the observed differences is something within the red blood cells. Although the cultures were only repeated with the blood from thirteen volunteers, the fold-change between cultures performed at different time points were not statistically significantly different, and volunteers maintained the same parasite growth pattern (Figure 15). A further argument to an origin within the erythrocytes comes from the observation that the growth pattern is reproducible, which seem to exclude temporary influences, such as nutrients. In fact, two volunteers (one from the LG group and the other from the HG group) provided blood regularly for culture maintenance and as controls (results not shown), and consistently kept their growth pattern as HG and LG, another strong indicator that the origin of this growth is a rather immutable property of the red blood cells. All these raise the question: What could cause the observed inter-individual variability in *P. falciparum* growth?

There was no statistically significant effect observed for sex, nationality, food pattern, and concomitant underlying medical conditions. Interestingly, there was no evidence for impaired parasite growth and blood group, which contradicts some previous observational studies showing a relation between a reduction in disease severity and blood type 0[128] (see section The genetic protection to not develop malaria disease, page 39). However, it should be noted that the relation between blood groups

and *P. falciparum* growth is not consensual. For example, an Ethiopian study with 1065 febrile patients suspected of having malaria did not show a significant difference between non-severe *P. falciparum* malaria and individuals of blood group A versus O or B versus O or AB versus O.[175]

Two mechanisms that have been proposed as an explanation for the susceptibility to *P. falciparum* infection of non-0 blood types are cytoadherence and rosetting.[176] These processes tend to be stronger in non-0 blood groups, and favour sequestration in small vessels and affect the immune system clearance of infected red blood cells, leading to more severe malaria[77,120,128,176] (see section The genetic protection to not develop malaria disease, page 38). However, in *in-vitro* cultures, these events do not have the same (if any) expression as *in-vivo*.[153,167] This could at least explain the observed contradictions between the blood group and *P. falciparum* growth, although it would not explain the Ethiopian study. Moreover, a recent *in-vitro* study also reported no relation between *P. falciparum* growth and blood group in culture.[167]

Although the food pattern shows no relation to the parasite growth between the LG and HG groups, the time from the last meal until the blood collection showed a statistically significant relationship with the parasite growth. In the LG group, the volunteers had eaten less time before the blood collection than the volunteers from the HG group. Thus, if food or nutrients had positively influenced the growth in the in-vitro cultures, one would have expected a more pronounced growth in the LG group – thus, if the food was rigorously controlled before blood collection, the observed effects could even be more prominent.

As far as the questionnaire could establish, all meals were similar, with most being main meals, like lunch, and all meals included carbohydrates. In nondiabetic individuals, which is the case of the volunteers of this study, glycaemia peaks about one hour after the start of a meal and returns to pre-prandial levels within two to three hours.[177] Interestingly, although there was no statistically significant association between the growth of the parasite and glycaemia, the volunteers of the HG group had lower blood glucose levels than the volunteers from the LG group. Nevertheless, another factor that should have reduced the observed difference between LG and HG, as it should have negatively influenced the growth in the HG group.

According to the literature, studies that seek to relate hyperglycaemia with the severity of infectious diseases, as discussed above (see section Nutritional Status, page 27), seem to show that higher blood glucose levels seem to be related to disease severity. Interestingly, a recent *in-vitro* study performed with cultures supplemented with human serum showed evidence between glucose and the *P. falciparum* growth.[173] To conclude this, *P. falciparum* was cultured with red blood cells from diabetic and nondiabetic individuals and medium supplemented with 10% autologous serum.[173] Glucose supplementation was changed between cultures to study its influence on parasite growth. Curiously, a more pronounced correlation was noticed when no glucose supplementation was added to the culture, and the parasite growth showed a higher fold increase in the cultures from diabetic individuals.[173] As the glucose supplementation of the culture was increased, there was a loss of correlation between the parasite growth and blood glucose.[173] The authors presented this as evidence that parasite growth might be higher in diabetic patients due to the blood glucose levels in the serum.

This conclusion is in stark contrast to the present work. When optimising the MCM protocol with human serum, it was noticed that the *P. falciparum* could not grow without glucose supplementation. This observation raises the question of how it was possible to compare the parasite growth between cultures supplemented with serum if the total glucose concentration may have been below the minimum requirement of the parasite. In fact, at closer observation,[173] the graph that correlates fold-increase with blood glucose in a non-supplemented glucose medium (Figure 21) shows that the fold-increase in cultures from nondiabetic individuals is lower than one, indicating that the parasite is not growing but dying, likely because it is starvation. On the other hand, in cultures from diabetic individuals, the parasite is able to grow because these individuals have higher blood glucose levels, and 10% serum may be enough to provide the minimum glucose to the culture needed by the parasite to be able to thrive. However, when glucose supplementation is added above the minimal requirement, the parasite is able to grow well, and the correlation is lost.

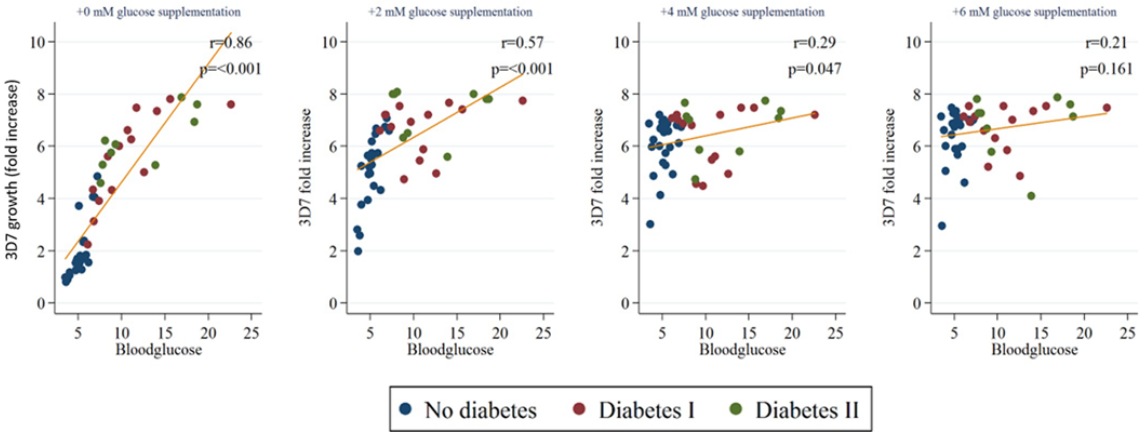

Figure 21 – Correlation between blood glucose and fold-increase of *Plasmodium falciparum* growth.

P. falciparum cultures were performed with red blood cells from diabetic and nondiabetic individuals and medium supplemented with 10% autologous serum was used. Each graph represents the correlation between blood glucose and fold-increase of *P. falciparum* growth with different glucose supplementation of the culture.

Adapted from Ch'ng, J. H., et al. (2021). Enhanced virulence of *Plasmodium falciparum* in blood of diabetic patients. *PLOS ONE*, *16*(6), e0249666.

The present study shows a relation between people with origins (and thus likely genetic "connections") to malaria-endemic areas and who seem to have a history of anaemia, either a personal history and/or family history, especially of borderline anaemia (haemoglobin value just below the cut-off).

One example of a volunteer in the LG group is a young female with genetic origins from India who started with fatigue accompanied by brittle nails, difficulty concentrating, dizziness, feeling faint, hair loss, headaches, and palpitations. Tests on haematologic-biochemical parameters of the blood showed borderline, microcytic anaemia, with haemoglobin levels just below the lower reference value. The remaining parameters were within reference values (low iron load did not seem to explain the haemoglobin concentration). The symptoms were even treated with iron what did not improve haemoglobin concentration and the mean corpuscular volume. Seven in twelve of the LG volunteers would fit in this example.

On the other hand, a typical example of an HG volunteer is a middle-aged male with origins from North Europe with haemoglobin levels consistently in the top percentile of the reference range.

Further characterisations of the haemoglobin types of these two volunteers revealed an α-thalassaemia trait in the LG volunteer and no alterations in the HG volunteer. Although these are very preliminary observations and may not be the causal explanation, they may indicate the importance of haemoglobin types on *P. falciparum* growth. In fact, several studies point out that individuals from malaria-endemic countries have more probability of having haemoglobinopathies since they confer protection against malaria[78,80,81,144] (see section The genetic protection to not develop malaria disease, page 36). Moreover, affected haemoglobin synthesis and function are widespread worldwide,[178] favouring that this could be easily found in any study sample. Of note, in this study, no common haemoglobinopathy or any atypical curves in the HPLC were observed in the LG or HG groups.

This observation reinforces the importance of haemoglobin, the primary substrate to the *Plasmodium* to thrive, and the fact that the variations of the red blood cells that confer protection against malaria may not manifest as a proper disease. Furthermore, other variables related to the red blood cells, such as haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, iron, vitamin B12 and folic acid, show no correlation with the parasite growth (Table 11 and Figure 16).

It should be noted that the study of the metabolism of the red blood cells was not performed. Defects in red blood cell enzymes are also connected to protection against *P. falciparum* infection, namely in G6PD[79,151] (see section The genetic protection to not develop malaria disease, page 37). G6PD deficiency is a hereditary condition in which red blood cells haemolysis when the body is exposed to certain triggers.[179] Symptoms during a haemolytic episode may include dark urine, fatigue, paleness, tachycardia, shortness of breath, and jaundice, followed by a rise in LHD levels and bilirubin levels,[179] which was not the case of the volunteers of this study. Although the protection mechanism is not completely clear, it is very likely related to the increased susceptibility to oxidative stress in these red blood cells,[79,151] which could be present in *in-vitro* cultures, contrary to rosetting and cytoadherence.

The observations of this presented study provide more evidence for the importance of red blood cell factors for parasite growth, likely genetically determined and as such likely hereditary. It is possible that some genetic variations in the red blood cells, possibly the haemoglobin “make-up”, may somehow inhibit rapid parasite growth and may, in fact, have some protective effects against malaria; still, these haemoglobin variations may be to minor to manifest as a “full-blown“ disease.

Unfortunately, so far, this study could pinpoint the exact nature of this variation. Besides the AB0 blood group, other membrane receptors were not tested. Could some minor variations in the membrane receptors justify the observed differences? A recent *in-vitro* study undertaken to investigate the effect of blood donor variability in *P. falciparum* invasion phenotyping assays revealed that, even after treating the red blood cell with enzyme treatment, the differences between *P. falciparum* growth were evident.[167] These observations suggest that the observed differences do not correlate with red blood cell receptors such as the blood group.[167] Another explanation might be variations in the red blood cell enzymes, which were also not investigated in the present study.

However, a more likely explanation seems to be minor changes in the haemoglobin, as this kind of change may more likely cause borderline anaemias. These are mainly missense mutations that destabilise haemoglobin, alter the affinity to oxygen, or most commonly alter the haemoglobin function minimally, and are rarely life-threatening or health-compromising.[178]

CONCLUSION

There is evident, reproducible variability in the growth rates of *P. falciparum* in *in-vitro* cultures with blood from different donors. An impaired *P. falciparum* growth is related to individuals with a genetic "connection" to malaria-endemic countries and who have a history, either personal and familiar, of borderline, microcytic anaemia, not explained by further studies on haematological and biochemical blood parameters. Thus, differences in the parasite growth could be explained by some minor genetic variations in haemoglobin.

In future work, it is intended to expand the number of volunteers to a more generic population, including migrants from endemic-malaria areas. Also, using flow cytometry techniques, it is intended to study which step of *P. falciparum* infection is impaired or increased, leading to the differences observed between individuals.

Apart from possible implications for malaria infections *in-vivo*, *in-vitro* cultures are an essential tool for studying the parasite and researching better medications, vaccines, and interventions: Thus, it is critical to define criteria to choose the red blood cell donors used in culture to minimise this variability and have more reliable results.

In addition, it would be interesting to understand how these observations in *in-vitro* cultures reflect in in-vivo studies to (a) investigate if the differences observed *in-vitro* between individuals happen in-vivo, and (b) to understand what criteria should be used to choose volunteers for CHMI studies, also a key tool for *Plasmodium* research and to explore more routes to fight the infection and have improved outcomes.

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