TÉCNICO LISBOA

Effect of underlying metabolic diseases, diet and metabolites on the growth of blood stage parasites: *Plasmodium falciparum*

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I hope this work meets all of your expectations.

Abstract

Malaria is still an important public health problem, currently with an increasing incidence. The causative parasite, *Plasmodium,* lives in the blood, sharing the host's nutrients. Thus, host nutrition must play an important part in parasite's development. Little information is available on this subject; restricted to malnutrition (due to its prevalence in the past) or to iron (due to anaemia). The effect of macronutrients, especially carbohydrates, is hardly explored. Furthermore, existing studies have inherent limitations, such as rodent models (importance for humans? or complex field studies, often with recall bias).

We studied the host's nutrition, especially carbohydrates (table sugar) influences the growth of *Plasmodium*, using a novel (*ex-vivo*) *in-vitro* based approach with a protocol to mimic *in-vivo* conditions. Parasites were cultures in erythrocytes from 50 volunteers, in 90% of their homologous serum without added glucose for 96 hours. Parasitaemia was compared in each volunteer before and after ingestion of a high-carbohydrate meal.

In 71% of the cultures there was an increase in parasitaemia observed after ingestion. The increased growth was largely proportional to an increased glycaemia, although the association was not linear. The reason for this is unclear, although other factors, such as fructose might have influenced this.

The ingestion of high-carbohydrate seems to promote parasite growth, in some cases markedly so. A consequence may be an increased risk for (severe) malaria, especially in diabetics or individuals on a high-carbohydrates diet, such as children.

Keywords: Malaria, Plasmodium falciparum, Nutrition, Glucose, Culture conditions.

Resumo

A malária continua a ser um problema de saúde pública importante, com a incidência atualmente a aumentar. O agente causador da doença, *Plasmodium*, vive no sangue, partilhando dos nutrientes do hospedeiro. Assim, a nutrição do hospedeiro deverá ter um papel importante no desenvolvimento do parasita. Existe pouca informação sobre o assunto, restrita a malnutrição (pela sua prevalência no passado) ou ferro (devido a anemia). O efeito dos macronutrientes, especialmente hidratos de carbono, é pouco explorado. Os estudos existentes têm algumas limitações associadas, como modelos animais ou complexos estudos no terreno, frequentemente enviesados.

Estudamos a influência da nutrição do hospedeiro, especialmente hidratos de carbono, no crescimento do *Plasmodium*, utilizando uma nova abordagem (*ex-vivo*) baseada em condições *in-vitro*. Eritrócitos de 50 voluntários, infetados com o parasita, foram colocados em cultura com 90% do soro homólogo e sem glucose adicionada, por 96horas. A parasitemia foi comparada, em cada voluntário, foi comparada antes e depois da ingestão de uma refeição rica em hidratos-de-carbono.

Em 71% das culturas houve um aumento da parasitemia observada após a ingestão. O aumento do crescimento foi proporcional com o aumento da glicemia, contudo a associação não é linear. A justificação não é clara, contudo outros fatores como a frutose poderão influenciar este efeito.

A ingestão de elevadas quantidades de hidratos de carbono aparenta promover o crescimento do parasita. Uma consequência será o risco aumentado de malária, especialmente em diabéticos ou indivíduos com uma dieta rica neste nutriente, como as crianças.

Palavras-chave: Malária, *Plasmodium falciparum*, Nutrição, Condições de Cultura.

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Abbreviations

ACT	Artimisinin-based Combination Therapy
AGE's	Advanced Glycation End-products
AOPP	Advanced Oxidative Protein Products
BAC	Blood After Consume of the meal
BBC	Blood Before Consume of the meal
BF	Before Fructose
BG	Before Glucose
BMI	Body Mass Index
BMJ	British Medical Journal
CFR	Case Fatality Rate
CPD	Citrate Phosphate Dextrose
CR	Caloric Restriction
DM	Diabetes *Mellitus*
GALT	Gut Associated Lymphoid Tissue
HCS meal	High Calorie High Sugar Meal
HDL	High-Density Lipoproteins
HIV	Human Immunodeficiency Virus
Hz	Haemozoin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
iRBC	Infected Red Blood Cells
LDL	Low-Density Lipoproteins
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium hydroxide
NBB	National Blood Bank
ND	Not Determined
PEM	Protein Energy Malnutrition
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
T2DM	Type 2 Diabetes *Mellitus*
Th1/Th2	T-helper cells type 1 /2
US$	United States' Dollars
VLDL	Very Low-Density Lipoproteins

I. Introduction

Problem

Malaria is still a serious disease worldwide which affected 216 million people in 2016 alone (1), almost twenty times the population of a country like Portugal. In the last report from the World Health Organization (WHO), malaria was responsible for 445 000 deaths worldwide in 2016, 16 000 more than the previous year(1), an alarming increase when the objective is the elimination of the disease. This increase seems to hint that progress towards elimination may have stalled. It almost appears somewhat paradoxical but despite the large numbers of morbidity and mortality, there is a lot of knowledge about malaria and the parasite that causes it, *Plasmodium*. In humans, malaria is caused by five species (2). In general, the most common is *Plasmodium falciparum*, followed by *P. vivax* and less common are *P. malariae*, *P. ovale* and *P. knowlesi*. *P knowlesi* is a zoonotic disease, mostly responsible for infection in monkeys, but was observed in humans too(3).

This parasite has a complex life cycle as shown in figure one, which has been thouroughly described in many sources, elsewhere(2). Briefly, after being bitten by the mosquito, the host is infected by the sporozoites. These will travel through the blood stream to the liver, where they will develop for about one to two weeks, depending on the species. This is known as the liver stage, where the sporozoites invade the hepatocytes and develop into a more mature stage, the schizont which contains ten-thousands of merozoites. At the end of the maturation, the schizonts burst and releases merozoites into the bloodstream. Potentially, each merozoite can infect one Red Blood Cell (RBC). Here starts the blood stage where the parasite grows in an asexual cycle of 48 hous in the case of *P. falciparum*. In the blood stream, 16-32 merozoites are released and each merozoite might infect a new RBC leading to a exponential growth. With this high increase in the numer of parasites in the host's body (parasite load), the symptoms and thus the disease start to manifest (malaria is a term which only applies ot the symptomatic disease). Certainly the nutritional requirements of the parasite come to mind in this scenario.

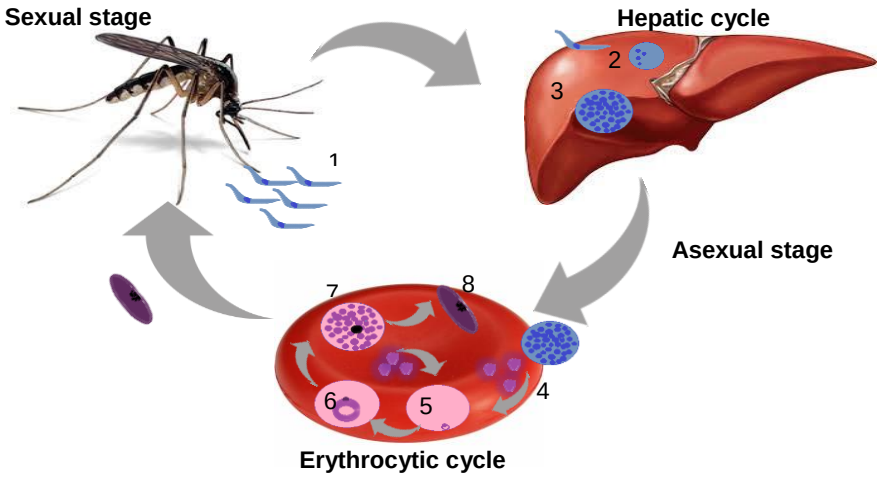


Figure 1 Schematic representation of the lifecycle of *Plasmodium falciparum*
1 – Sporozoites inoculated by the mosquito in the human host; Hepatic cycle - where the parasite matures until it reaches the schizont form and releases the merozoites into the blood stream; 4 -- Merozoites that will infect the RBC; 5 – Ring form; 2, 6 - Trophozoite;3, 7– Schizont; 8 – Gametocyte that will infect the vector and develop the sexual stage. See text for detailed description.

After a mature schizont ruptures, new uninfected RBC will be infected by the merozoites. In the case of *P. falciparum*, each erythrocytic cycle takes 48 h. Although uncompletely understood, it appears that if the parasite "senses stress", which could be lack of nutrients, it also develops into sexual forms, the gametocytes. These are taken up during feeding by the anopheline mosquito, infecting it. They reproduce sexually, forming an ookinete and later an oocyst in the mosquito gut. The oocyst will burst, which will end in salivary gland sporozoites. After migration to the salivary gland, the mosquito will inoculate them into a new host when it feeds.

Based on the previously mentioned incidence rates, it would seem that something is hampering the control of malaria, lowering the efficacy of measures that have been fairly, if not highly efficacious in the fight against malaria so far. Although highly efficient ways to fight this disease exist, such as diagnostics, highly effective treatments and vector control(1), further interventions could be of additional help. Perhaps, additional measures, could improve the current approach to control malaria.

To address this issue a close look at the current research efforts in diverse fields within the subject of malaria might be useful.

Research areas

One aspect that is often not considered and probably has some impact on malaria is nutrition. Because *Plasmodium* is an intracellular parasite which lives in the blood stream, it shares the pathway of all nutrients in the host's system. Thus, nutrition is very likely affecting the disease, either by improving parasite growth (thus, the severity of the disease) or by having an impact on the host's nutritional status. In fact, as reported in different reviews, some of the symptoms shown by patients suffering from malaria are related to their nutritional status and might

thus be linked to some pathway in the parasite development and metabolism(2). Although, when comparing the extent of research on nutrition in malaria with other malaria-related subjects (table one) it seems evident that nutrition is rather an overlooked subject in the scientific community.

Table 1 Relevant areas of research in malaria.

Major area of research	Relevance
Diagnosis	The gold standard for the diagnosis of malaria is still a stained blood film. Usually they are performed both as a thick and a thin smear so it is possible to determine the species of *Plasmodium* and the parasitaemia. Other methods include Polymerase Chain Reaction, as well as rapid tests for the diagnostic of malaria which are already used in some places(4).
Drugs	According to the World Malaria Report from 2016, the treatment for malaria is usually a combination of two antimalarials, the Artimisinin-based Combination Therapy (ACT), for effective treatment and to avoid resistance. However, reduced sensitivity to many antimalarials has appeared, such as aminoquinolines, mefloquine and even artemisinins(4).
Vaccine	Vaccines are a focus of the scientific community due to its potential in the control of malaria. One vaccine has already been licenced (RTS/S), however the efficacy is rather low (26%) (5). The parasite is a complex pathogen and the immune response to it not completely understood. Vaccines tend to target specific proteins which easily evolve, leading to the parasite's escape(6).
Vector control	The resistance of the mosquito to insecticides has been increasing(1), so the investigation of new ways to fight the vector has been an important focus for research. Some areas of interventions include: • Widespread use of insecticides and larvicides(7) • Destruction of breeding grounds(7) • Indoor residual spraying(1, 7) • Insecticide treated nets(1, 7)

Analysing the financed subjects in malaria research provides additional information. As can be seen in figure two the most financed subject is antimalarial drugs, followed suit by basic research and vaccines. In fact, while the effect of nutrition on malaria might be hidden in the basic research topic, it does not show up as a single subject, begging the question how much (or even if at all) it is being investigated.

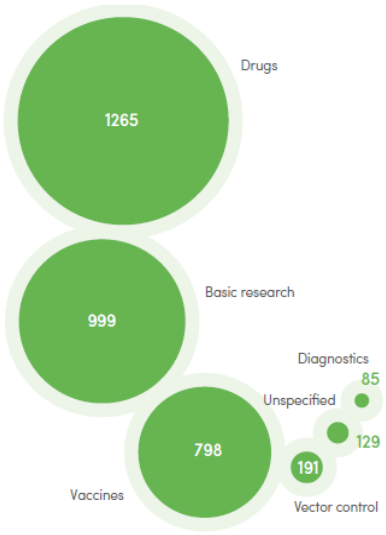


Figure 2 Funds according to research area
Distribution of the funds given to research according to research areas (in million US$), between 2010-2015. (Source: World Malaria Report, World Health Organization based on G-FINDER Public Search Tool Policy Cures. https://gfinder.policycuresresearch.org/PublicSearchTool, accessed on 20/12/2017)

When searching the more recent literature in PubMed, it appears that nutrition is the subject with the lowest number of publications when compared to vaccines, vector control, diagnosis and antimalarial drugs (figure three-A). Of note, most results obtained with the search terms “Nutrition AND Malaria”, were related to iron and malnutrition (figure three-B); while other subjects were very scarce.

Understandably, malnutrition was the main focus in the past, likely because of wars and prevalent poverty, especially in Africa. Malnutrition was the nutrition status of most of the world population. Concerning iron, it is the most common micronutrient deficiency worldwide, a condition that makes it an important subject to study. Moreover, it often accompanies the malnutrition status. Mozaffarian and Forouhi, in their analysis about nutrition sciences(8), describe pointedly, that in the past there was a tendency to attribute a single nutrient to a single disease, such as vitamin C is the answer to scurvy. However, this trend has recently been discarded. Nevertheless, it appears that in malaria this reasoning is still prevalent, because publications regarding the effect of iron on malaria are the most frequent in PubMed. It seems that iron was considered as the most important aspect regarding the host nutrition and malaria. In the last ten years most of the publications on the matter were either about the importance of iron and anaemia on malaria or the impact of malnutrition in the said disease. This lack of diversity concerning the role that nutrition may play on the course of malaria makes it difficult to really understand the extend of its true importance. Overall, figure three suggests that there is still a lot to learn about the effect that nutrition has in the course of malaria and what its role may be, both in the prevention and management of the disease. Furthermore, some of the symptoms presented by patients with malaria seem to be related to their nutritional status, such as acidosis, hypoglycaemia or anaemia as has been meticulously reviewed by White *et al*(9). In particular,

acidosis and hypoglycaemia seem to be associated with the glucose metabolism of the parasite, whereas anaemia is thought to be related with the iron intake by the parasite.(10)

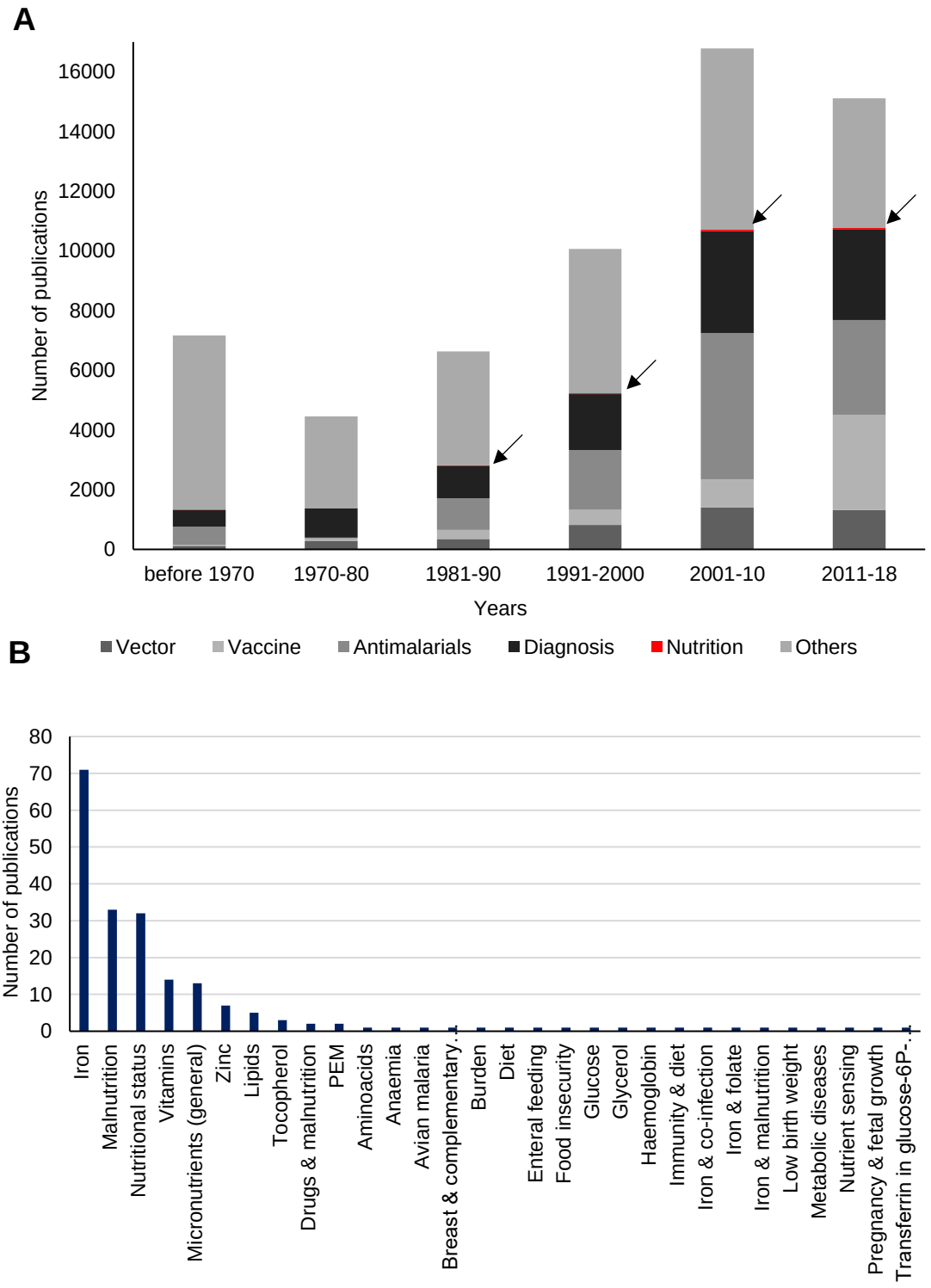


Figure 3 PubMed listed studies per research area in malaria
A - Results of a PubMed search with the MeSH terms "Malaria AND Vaccines", "Malaria AND Disease vector", "Malaria AND Antimalarials", "Malaria AND Nutritional sciences OR Nutritional status" for publications since 1970. Nutrition is in red and marked with the arrows. **B** – Subset of publication on the search on "Malaria AND Nutritional sciences OR Nutritional status" subdivided in more specific subjects (n = 148 publications). (Search in 16th January 2018)

In order to better understand how nutrition can influence the course of malaria it is useful to look into the role of nutrition on infection in general. That way it may be possible to understand which the most relevant nutrients in infection are, and how they may interact with microorganisms and influence disease. Because underlying mechanisms may very well be similar in other infections, this may provide some clues how nutrition affects the progression of malaria disease.

Interactions between Nutrition and Infection

Without a doubt, a relationship between nutrition and infection exists, as described in a recent textbook(11). Basically, one simple relationship is that nutrition is essential for a good immune response and thus the control of the disease. However, other infections, especially more severe ones, may affect the nutritional status of the patients, increasing their nutritional requirements (12). Nutrition provides all the building blocks for the immune system to work as well as the necessary energy to fight the disease. Consequently, both, the amount of nutrients some individual or population are consuming as well as the type of nutrients they are ingesting are relevant to generate the best possible immune response. Both the general nutritional status and some specific micronutrient deficiencies appear to have some effect on the course of infection(12).

Malnutrition

Table 2 Effects of malnutrition on infection (definitions).

Concepts	Comment
Malnutrition	Malnutrition refers to deficiency, excess or even imbalance of individual intake of energy and/or nutrients. It includes undernutrition and micronutrient deficiencies but also overweight, obesity and non-communicable diseases (13, 14).
Undernutrition	Undernutrition includes wasting, stunting, micronutrient deficiency and underweight. Wasting, also known as low weight for height, indicates recent and severe weight loss due to either insufficient intake of food or infection. Stunting, on the other hand, is also known as low height for age and is usually associated with continuous undernutrition. It is often related to poor socioeconomic conditions, maternal health and nutrition, frequent illness, and negligence early in life (such feeding and care). (13)
Protein-Energy-Malnutrition (PEM)	Protein-Energy Malnutrition is a nutritional deficiency caused by the insufficient intake of energy or protein. It can be manifested by either marasmus or kwashiorkor(15).
Nutritional Immunity	The mechanism of reducing the concentration of a determined nutrient, usually iron, to prevent its use by the invading pathogens(16).
Impact	↑Immunodeficiency ↑Susceptibility to infection ↓T-cells ↓Type I cytokines

↑ - Increase; ↓- Decrease.

The nutritional status represents the overall nutritional condition of the individual and, therefore, can give some insight about the problems this individual might have. Undernutrition is also associated with a poor health condition(13). When conducting a search in PubMed, it is possible to realise that it has been extensively reviewed(17). It seems to be one, if not the most studied factor, influencing infection. Probably, because undernutrition was/is more prevalent, and it was/is overlapping with those areas with a higher incidence of infectious diseases(18). It makes sense to infer that it could be a risk factor for infection. Undernutrition was found to be the most common cause of immunodeficiency because it affects immune homeostasis(19). In agreement with this hypothesis, undernourished people were observed to be more prone to have more severe infections as opposed to well-nourished people(17, 20, 21). This happens because neither the building blocks are present, nor the energy is being replenished for the immune system to work. Both adaptive and innate immune responses are known to be affected by undernutrition and mechanisms like phagocytosis and complement cascade are impaired(22, 23). A more recent term to describe this concept is Protein-Energy-Malnutrition (PEM)(15). Perhaps, an appealing illustration is the complement cascade where the lack of protein as building blocks might cause an impaired functioning, as represented in figure four. Another example would be antigen presenting cells which are found in lower amounts in malnourished people. PEM is also known to cause thymus atrophy and thus, reduced numbers of T-cells, a part of the acquired immunity(24, 25). Also, type 1 cytokines, a main mediator of immunity, are lower in undernourished people(26). Without all these parts of immunity, the immune system cannot develop a proper immune response to infection and the malnourished individual is more susceptible to infection.

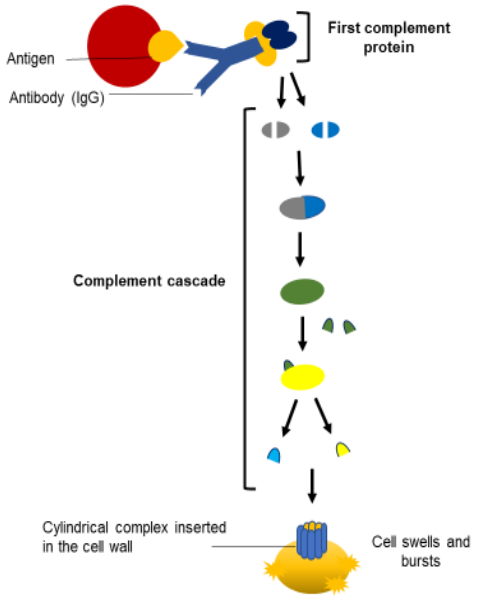


Figure 4 Complement cascade as an example of where PEM would have an effect
This part of the immune response is composed by proteins which circulate in the blood. These proteases cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. As a consequence, the complement cascade is activated and thus the phagocytes are stimulated to clear foreign and damaged. (Adapted from: NIH - Understanding the Immune System. How It Works)

Although additional evidence supports the notion that malnutrition is a risk factor for infection, some findings have suggested the opposite. As discussed above, most arguments in favour of malnutrition being a risk for infection are based on the immune response.

Contrary to this, arguments in favour of a protective role of malnourishment associate the host's lack of nutrients with a consequent lack of nutrients for the invading microorganisms. This concept is usually named as "nutritional immunity" and was reviewed by Weinberg in 1975(16). In support of this argument is the fact that the excess of iron seems to lead to a worse prognosis in a number of infectious diseases such HIV(27), malaria(28) and tuberculosis(29). Moreover, the supplementation of this nutrient appears to exacerbate the case fatality rate (CFR) of these diseases. As reviewed by Soares *et al*, the hypothesis that appears to explain this are the mechanisms created by the microorganisms to scavenge the iron of the host. It has been reported that the deletion of genes involved in the expression of siderophores has a detrimental effect on the virulence of the pathogen (30).

However, on the whole, the balance tips well in favour of the notion that undernourishment is harmful for the individual. Still, the picture is quite apparently not a black-and-white one. Some studies with children suggest that undernourished children are more prone to diarrhoea as well as bacteraemia and severe infection than their well-nourished peers. It was suggested that the gut, being the largest immune organ and a barrier against invading pathogens might be compromised in these people(31). This barrier is called Gut Associated Lymphoid Tissue (GALT) and it is a defence mechanism that can be modulated through diet(32). The authors propose that increased permeability of the intestinal barrier leads to a dissemination of the pathogens throughout the bloodstream and, thus, to a systemic inflammation(31). Moreover, low levels of serum albumin were found to be linked with hospital deaths caused by tuberculosis(33).

Assuming that undernutrition has indeed a harmful effect on infection, what about the inverse? What about obesity?

Obesity

Table 3 Effects of obesity on infection (defintions)

Concept	Comment
Definition Obesity	Obesity or overweight describes when a person weighs too much for his/her height. It is usually accompanied with excessive fat accumulation. It usually is classified according to BMI (Body Mass Index) scale, in which overweight and obesity correspond to a BMI above 25 and 30 respectively. It results from an imbalance between the energy intake and expenditure and to the consumption of high density foods, high in sugars and fats.(13)
Impact on infection	↑Phagocytosis ↑Oxidation ↓Adiponectin (anti-inflammatory hormone) ↑Leptin (pro-inflammatory hormone) ↑Insulin resistance ↑Susceptibility to infection

↑ - *Increase;* ↓- *Decrease.*

Over-nutrition is a problem that appears to become more common nowadays(18). It might have a negative influence, predisposing to infection as well. In keeping with the argument above that lack of nutrients deprives the pathogens from thriving, if the host has more nutrients in circulation, the pathogens will have more nutrients available for their growth. Furthermore, obesity may also have detrimental effects on the immune system, especially a type of pro-inflammatory state, including abnormal values of immune cells as well as higher numbers of phagocytic cells and oxidation. Obesity is characterized by diverse changes beyond the levels of circulating nutrients: for example changes in adipokines(34). Obese individuals frequently present lower levels of the anti-inflammatory hormone adiponectin but increased levels of the pro-inflammatory hormone leptin(35). Another interesting observation is the fact that insulin resistance, often associated with obesity, appears to be related to a pro-inflammatory response(36). This may be related to the pre-adipocytes' ability to differentiate into macrophages(37). The underlying idea is, that preadipocytes can differentiate into adipocytes or macrophages. Of course, the immune response would be smaller if less macrophages were produced.

Furthermore, remodelling of the adipose tissue is commonly accompanied with some inflammation, in particular oxidative stress triggered by obesity(38). The immune response depends on the communication between cells based on membrane bound receptors. Oxidative stress can damage the membrane, rich in phospholipids, trough peroxidation, then disrupting the

immune response (39). Likewise, the ingestion of glucose and fat, which is usually increased in the diets of obese individuals, seems to be related to some alterations in the immune response, like production of advanced glycation end-products (AGEs), reactive oxygen species (ROS) and lipid peroxidation. The former was shown to harm the innate immune system by activating receptors, which in turn, let to increased oxidative stress, ROS and advanced oxidative protein products (AOPP) and a consequent imbalance on Th1/Th2 response as Traoré *et al* describe in their review(40). In summary, many observational studies have observed that obese people appear to be more susceptible to infection and/or have aggravated infections(34-36). However, in a meta-analysis conducted by Wang *et al*(41), discrepant results were observed. In their study, overweight individuals seemed to be protected regarding infection whereas in the obese group no increased protection was reported, when compared with normal weight individuals.

With the dietary patterns changing worldwide, especially the increase of a westernized diet high in sugar and fat, the figures for the prevalence of obesity and BMI are also changing. BMI values are increasing as well as the prevalence of obesity, not only in high-income countries but also in middle and low-income countries(18). Aurino *et al*, in a two cohort comparison study, observed that adolescents, from four middle and low-income countries, were consuming more added sugars then before(42, 43). Moreover, a systematic assessment on dietary quality between 1990 and 2010 observed an increasing consumption of unhealthy foods such as sugar and fat(44). It goes without saying, that the dietary changes that occurred in the United States affected in some extent the dietary patterns worldwide, namely the consumption of sugary drinks and fast food. This is associated with the increase in the numbers of already prepared meals and snacks, which are linked with an urban environment(45). When comparing these results with the ones from BMI assessment(18) it is possible to notice the convergence of these trends.

Host nutrition and the effect on the invading pathogen

The effect that undernutrition or obesity play in infection might not only be because of their repercussions on the immune response and its components but, perhaps as importantly, due to some changes in the availability of particular nutrients to the pathogen. Thus, there may be a closer association of nutrition of the host and the capacity of the pathogen to grow inside the host. Certainly, this may be less evident in some cases, like during a common cold, but may have a large impact in other infections, such as tuberculosis or malaria.

While the interaction of nutrition with the immune system has been more extensively explored, the question of how nutrition and nutritional status may affect the growth of a certain pathogen is unknown. The underlying hypothesis is that nutrition may influence directly the development of pathogens. On a basic level, the idea might be fairly simple: more available nutrients may enhance the growth of an invading microorganism. However, it is also possible, if not likely, that the increase in the serum, of some other nutrients (or nutrient associated substances) may compromise the survival of the pathogens and consequently improve the prognosis. A rather interesting study by Collins *et al*(46) made some observations that point in this direction. The

authors observed that two ribotypes of *Clostridium difficile* (RT027 and RT078) appear to have an enhanced growth when incubated with trehalose, leading to an increased virulence in mice. Since trehalose is frequently used by the food industry and is therefore present in a lot of products (47), it may lead to a worse prognosis in the case of infection by these ribotypes of *C. difficile*. Then, is the modern diet changing the development of pathogens, hence their pathogenicity?

Micronutrients

Table 4 Effects of Micronutrient deficiencies on infection (definitions).

Concept	**Insufficient consumption of both vitamins and minerals, also known as micronutrients.(13)**	
Impact on infection	Iron	↓T-cells ↓Thymic activity
	Zinc	↓Mortality due to infection ↓Th1 cytokines ↑ Oxidative stress
	Vitamin A	↓ Protection of the mucosa ↓ Differentiation of immune cells

↑ - Increase; ↓- Decrease.

Some micronutrients showed a major effect on the inflammatory response, such as iron, vitamin A and zinc(48). Iron is the most common micronutrient deficiency globally and consequently many supplementation programmes were developed and implemented to alleviate its deficit (49). This nutrient was found to be essential for the survival of almost any pathogen and thus has a great impact on virulence of the pathogens as evidenced by many reviews like the one from Soares *et al*(30).

However, during the immune response, both the host and the pathogen are deprived of iron(16, 30). First, the immune system deprives the host from iron and consequently the invading pathogen is also affected in a process called “nutritional immunity”(16). The regulation of iron status is under the control of hepcidin, a protein that also mediates the immune response (figure five)(50). Studies have shown that iron-deficient individuals have lower amounts of T-cells and thymic activity(51). This might happen because several enzymes, which are part of the immune system, are dependent on iron to work(52). Iron deficiency also affects the production of specific cytokines by T-cells, a process that is not even reversible with iron supplementation(53). This suggests that iron deficiency may not be the only factor contributing to this effect.

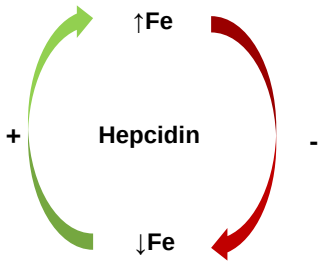


Figure 5 Regulation of Iron by hepcidin
Negative regulation mechanism of iron by hepcidin. A high concentration of iron leads to upregulation of hepcidin which in its turn downregulates the absorption of iron. Low concentrations of iron have the opposite effect. ↑- increase; ↓- decrease; Fe – Iron; **+** - upregulation of uptake; **-** - downregulation of uptake.

Zinc also appears to play an important role in immunity since its deficiency seems to increase mortality due to infection(54). It participates in the production of Th1 but not Th2 cytokines (55), leading to an imbalance in zinc-deficient individuals(55). Zinc also acts as an antioxidant protecting lymphocytes against oxidative stress of immune activation(56) . Finally, Vitamin A also plays a key role in immunity, although its mechanisms are not yet clear. Studies established this relationship when giving vitamin A to young children caused a reduction of all-cause mortality, namely by infection (reviewed in (11)). This vitamin also participates in the protection of the epithelium, in the production of mucin, IgA and lactoferrin(57). Consequently, vitamin A depletion leads to a break in the barrier of protection against pathogens in the mucosa. Another role vitamin A plays in immunity is in the terminal differentiation of myeloid cells like neutrophils, macrophages and dendritic cells(57). The relationship between all these nutrition-related factors are also believed to have an effect in the case of malaria, although the impact might be somewhat different from those observed in other infections.

In conclusion, it appears that nutrition plays an important role in the course of infection, even if all aspects are not yet fully understood and is still the focus of ongoing research efforts. Of note, several other diseases exist which are extensively affected by nutrition and nutrition status. HIV infection, for instance, often causes decreased body weight on infected patients, which is recovered when the patient is being treated(58). Many parasitic diseases are also responsible for weight loss, with different mechanisms according with the parasite mode of action(59). An interesting speculation might be that the weight loss could be a host reaction to fight off the parasite, or perhaps the “price to pay” for controlling or clearing the infection.

It also seems very likely that nutrition affects malaria. Regarding the effects that nutrition might have on malaria, the focus will be mainly on the impact that macronutrients might have, given the main focus of the present study.

Malaria

Nutritional status

Undernutrition, often associated with PEM, is the most studied nutrition-related subject affecting patients with malaria, mostly because it was a common denominator for most malaria patients, apart from being the easiest nutrition deficiency to spot(18). Interestingly, in the past, undernutrition was thought to protect against malaria, a finding that was reported in several studies, although mainly in older ones(60, 61). Murray *et al*, for example, found that the episodes of malaria were more common after a famine, when the population's nutritional stocks were replenished. Interestingly, Charchuk *et al*, reached the same conclusion in a more recent study from 2015(61). However, the analysis of such results must be made carefully, especially in older studies, because the definition of malnutrition was based merely on anthropometrics in the past (14). Contrary to this, nowadays it also takes into account growth, development, chronicity, cause of malnutrition, and the impact of malnutrition (undernutrition) on functional status(14, 62). With anthropometrics, only the physical aspect can be assessed. For example, an individual presenting with PEM would be classified as having malnutrition in the same way as a wasted individual, although their actual nutritional status is not the same. Nevertheless, recent studies observed that undernutrition was in fact a risk factor for malaria(63, 64). This disparities in the classification of the nutritional status might explain why the results on the effect of malnutrition in malaria are not completely consistent.

Contrary to the protective effect, many others reported detrimental effect. Some authors suggested that this detrimental effect, which malnutrition appears to have during malaria, might be due to lowered amounts of specific antimalarial IgG antibodies, as a consequence of undernutrition(65). Others observed that undernourished malaria patients were more likely to die or have some neurologic damage then their well-nourished peers. Shikur *et al* observed similar results in wasted children under five years old in Ethiopia. The authors suggested that this results might be a consequence of a poor immune response such as lymphoid tissue atrophy (the thymus) caused by severe wasting(66). Another study from Congo found that PEM was a risk factor for malaria. Furthermore, Caulfield *et al* observed that undernutrition was responsible for more than 50% of the deaths caused by malaria(67). However, to complicate things, some studies did not find a relationship between undernourishment and malaria(68). Once again, these discrepant results might be related to the different concepts of malnutrition over time. Although the role that undernutrition plays in malaria is not clear, it is still subject of controversy. It appears that the most agreed line of thought in the scientific community has been that undernutrition is a risk factor for malaria.

On the other hand, obesity's impact on malaria is not yet extensively studied. Likely, this reflects the lower prevalence of obesity in most malaria endemic countries in the past. However, this number is growing, mainly due to changes in lifestyle and dietary patterns, which clearly are not only seen in high-income countries anymore but also in middle and low-income countries(18).

Therefore, the subject is likely to be the focus of more research in the future. Robert *et al* observed that obese mice were able to resist better to increased parasitaemia and did not develop cerebral malaria, whereas their normal weight counterparts developed rapid neurologic symptoms even without high parasitaemia(69). However, the metabolism of rodents is different compared to humans, and results from rodent models might not be easy to extrapolate to humans as highlighted by Visser *et al* in a report on serum lipids and lipoproteins during uncomplicated malaria in humans(70).

In fact, Wyss *et al* published one of the few reports investigating the relationship between obesity and malaria in a Swedish national wide study(71) which included non-immune immigrants and travellers from Sub-Saharan Africa. Obesity seemed to be related to more severe cases of malaria disease. This result might be related to the fact that obese people were also found to suffer from other chronic diseases, because obesity is an etiological factor for some non-communicable diseases which were also associated with severe malaria(71). However, the results and data in this subject are not only scarce but also appear somewhat contradictory. Thus, further work is needed in order to understand and clarify this interaction.

Perhaps, to better understand the whole picture of nutritional interactions with malaria, it is necessary to look into the effect of more specific nutritional components, like glucose, lipids and protein (already mentioned along with malnutrition) as well as the effect of the micronutrients. These macronutrients may also further the knowledge about the relationship between obesity and malaria. This is of particular concern, because the western diet, high in sugar and fat, is entering many societies in malaria endemic countries.

Glucose

Glucose is the main energy source for the human body and can be obtained from three different sources. One is intestinal absorption and consequent carbohydrate digestion, that can be followed by either glycogenolysis for storage as glycogen or gluconeogenesis to retrieve the glucose from storage. It can either be stored as glycogen, undergo glycolysis or be released into circulation(72). Also of crucial importance, it is the main source of energy of the *Plasmodium* parasites, through glycolysis (figure six), when the parasite converts pyruvate into lactate(73). Because the parasites metabolize glucose in their erythrocytic cycle, changes in serum glucose levels are possibly influencing the growth of the parasite(74). Several studies demonstrate how the usage of glucose in infected RBC is more than double that of the uninfected ones(10, 74). This illustrates well how malaria causes a greater demand on glucose in the host. Since the parasite cannot store energy in the form of glycogen, it depends entirely on the hosts blood glucose to survive(75). Perhaps, not too surprisingly, hypoglycaemia is a commonly observed symptom of malaria, mainly in children, which may be caused by the excessive utilization of the host's glucose (76). Another possible link of the parasites anaerobic oxidation of glucose is the observation that the excess of production of lactate might contribute to the acidosis seen in malaria, in fact, one of the severe malaria symptoms(77).

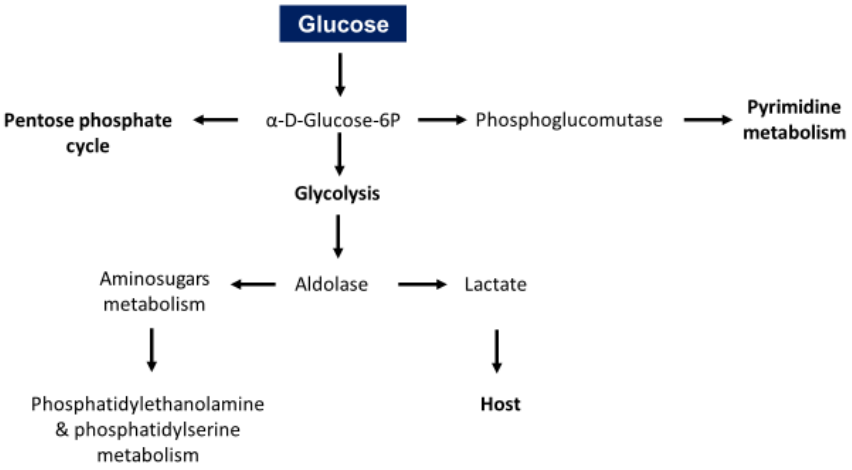


Figure 6 Glucose metabolism in *Plasmodium* species.
(adapted from Malaria Parasite Metabolic Pathways - *http://mpmp.huji.ac.il/* - accessed in 27/12/2018)

The effect of glucose on the parasite growth was studied by Humeida *et al,* who demonstrated in an *in-vitro* study that glucose was a limiting factor for parasite growth(74). A possible interpretation of all this information is, that higher blood glucose levels may allow higher parasitaemias while lower blood levels of glucose would be associated with lower parasitaemias. Support for this idea comes from a recent paper about caloric restriction (CR) in mice(70). In this study, the CR-fed mice appeared to be protected from more severe cases of malaria, as well as higher parasite counts as opposed to the *ad libidum*-fed mice. While the authors focused on the protective effects of CR in their discussion, one could argue another interpretation. A*d libidum*-fed mice have a higher risk of developing malaria then CR-fed ones. This argument would acknowledge the fact that *ad libidum* was associated with increased weight(78). It should also be noted that the CR regimen let to more changes, such as decreased body weight, changes in lipids and hormones(78). Moreover, studies on sympatric populations in Niger observed some protection against malaria and its more serious forms in a population which has a diet almost uniquely based on milk as opposed to a population with a diet based on grains(79). These results suggest that either a diet with high carbohydrate content might exacerbate malaria infection or that milk has a protective effect(79). Of course, other factors may be important, for example milk may have some intrinsic antimalarial effects(80). In conclusion, it seems reasonable to speculate that higher serum glucose may increase the susceptibility to malaria or severe malaria, and thus people with different metabolic sets, such as diabetes, should be more vulnerable to malaria. This is particularly alarming in a period when sugar consumption in developing countries is growing and already represents three quarters of the global sugar consumption(81), a number that could have been exacerbated by the higher proportion in terms of population, compared with developed countries..

Diabetes mellitus (DM) and malaria

Because a constant high blood glucose level is what defines diabetes mellitus(82) and because the prevalence of this disease is growing worldwide(83), it might well be a significant risk

factor for malaria. Diabetes has two common types, Diabetes mellitus type 1 and type 2. Type 1 is not as common as type 2 and is caused by the lack of insulin production in β cells in the pancreas, while type 2 is frequently associated with lifestyle, characterized by resistance to insulin. This implies that patients with type 2 Diabetes *mellitus* (T2DM) need more insulin per gram of glucose in the blood, so that they metabolise it(82). The possible effect on malaria might have grown because the prevalence of DM has been growing worldwide, including in malaria endemic countries(83). This is of some concern as it indicates associated lifestyle changes, such as sedentarism and the consumption of high fat and sugar diet(42).

The possible mechanism which T2DM might have on the course of malaria is thought to be glucose itself because insulin levels do not seem to have an effect on *Plasmodium* growth(74). In a population-based study from Ghana, T2DM patients were associated with increased susceptibility to infection with *P. falciparum* malaria than controls. It was also shown that a 1 mg/dl increase in glucose levels meant a 5% increased risk of malaria(84). Considering that malaria appears to be more common in T2DM patients(85), it seems that T2DM could possibly be considered a risk factor for *P. falciparum* malaria. However, an increased risk is not the only impact of diabetes on malaria. The outcomes of the infection in diabetic patients with malaria can be more severe than in patients without diabetes, even at lower parasite load (86). Another aspect is, that T2DM often leads to decreased zinc levels(87). The importance of zinc in the immune response has been thoroughly reviewed(56). Furthermore, studies suggest that it has protective effects against more severe cases of malaria(88).

Certainly, T2DM is considered a risk factor for malaria by some authors. Interestingly though, it might have some mitigating effects caused by some anti-diabetic drugs. Metformin, for example, is one of the most known and widespread oral glucose lowering drugs and it appears to have some properties with detrimental effects for the parasite(89, 90). A case-control study in Ghana found that T2DM patients treated with metformin had a lower incidence of malaria then those not taking this drug(90). Furthermore, an improved outcome in malaria treatment was observed when metformin-like drugs were taken together with others(91). Although the mechanism by which metformin affects parasite growth is not yet fully understood in humans, a study in rodents showed that the infected mice treated with metformin had less parasite replication(92). The simple effect of lowering the glucose levels in the blood might have a deleterious effect on parasite growth. Therefore, other oral glucose lowering drugs, such as sulfonylureas, might have the same outcome as observed for metformin. This might happen due to a change on the signalling cascade, part of a nutrient-sensing pathway of the parasite(78). Because there are no specific guidelines for the management of glycemia in malaria as an approach to reduce the parasitaemia, this subject must be studied more deeply in order to reach enough knowledge to assemble a set of guidelines for the nutritional and clinical approach to malaria.

Lipids

The effect lipids may have concerning malaria are not yet investigated thoroughly, however, the metabolic changes triggered by malaria might give some clues on the matter. Lipids are known to be important building blocks, for a number of cell functions such as cell membranes(93). They also play some role in infection (93, 94). After the consumption of a high fat meal, the free fatty acids activate the cell surface receptors which, in turn, lead to the release of a regulator of fat-induced inflammation and ROS and are directly linked with the immune response, as was nicely explained by Dias *et al* in a review on postprandial lipemia(95). This appears to be one reason why most infectious diseases lead to changes in serum lipid values(96). Furthermore, several studies noticed that the changes in serum lipid profiles was higher in malaria than in other infectious diseases(70, 96-98).

It is known that, when the human host ingests a fatty meal, the lipids are absorbed in the small intestine and are transferred in the form of chylomicrons to the blood. This is accompanied by an increase of very low-density lipoproteins (VLDL) produced in the liver(99, 100). However, when the individual is infected with malaria, a reduction of cholesterol levels is observed, both high-density lipoprotein (HDL) and low-density lipoprotein (LDL). However, triglycerides values were shown to be higher during malaria infection. All these values returned to normal during the convalescent phase(96, 101). Nevertheless, other studies have reported conflicting results when compared with these ones. Mohanty *et al* observed an increase in plasma lipid values except for triglycerides(102), whereas in a study in São Tome no correlation between lipid profile and malaria was observed(103). Sengupta *et al* results(10), based on a metabolomic analysis, were in concordance with the hypothesis that lipids were increased during malaria, and they suggest that the discrepant results might be due to specific parasite-host interactions, dependent on the geography of the cases. This might happen due to differences regarding diet. Still, despite these contradictions, the effects of malaria on serum lipid levels were deemed to be so significant, as compared to other infections, that it was suggested as an auxiliary diagnostic tool for malaria(104, 105).

Box 1 Acute-phase reaction

An acute phase reaction is a systemic reaction of the organism to systemic disturbances to its normal function. It can occur either because of infection, tissue injury, trauma, surgery, neoplastic growth or immunological disorders.(reviewed in (106))

The mechanism behind the observed changes in the lipid profile of malaria patients is not yet clear, though there are some possible explanations. The changes in lipid profile seem to be related to acute-phase reaction (Box 1), when capillary permeability increases and hence there is some plasma outflow(101). Another explanation could be related with the inability of the parasite to produce its own nutrients, and thus the need to scavenge them from the hosts to survive, as reported in several publications on the matter cited by Labaied *et al*(107) . Regarding

lipids, the parasite has both endogenous fatty acid production and scavenge mechanisms (figure seven). However, it cannot produce cholesterol itself, although the parasitophorous vacuole of parasite's liver form does contain sterols(107).

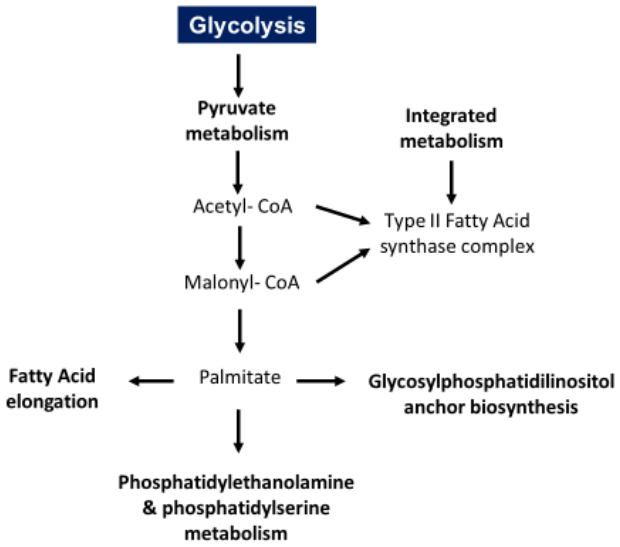


Figure 7 Fatty acid synthesis in the *Plasmodium*.
The fatty acid elongation occurs in the endoplasmic reticulum and the integrated metabolism in the apicoplast. (adapted from Malaria Parasite Metabolic Pathways - *http://mpmp.huji.ac.il/* - accessed in 27/12/2018)

One parasite function which depends on cholesterol is cellular division during schizogony(107). Each merozoite will then produce sixteen to thirty-two new merozoites. Consequently, lipids are needed to build all the necessary membranes of these new cells. The parasite diverts cholesterol from the hepatocytes until the release of the new generation of merozoites into the blood stream(107). Imrie *et al*(108), in support of this argument, added HDL to *Plasmodium* cultures and observed some improvements regarding growth and re-invasion. The conclusion seems to be that the parasite needs to scavenge host's lipids to improve growth and re-invasion(108).

Lipid metabolism by the *Plasmodium* species seems also to be linked with haemozoin (Hz) production, the product of the detoxification of free haem remaining from haemoglobin degradation. The parasite needs this pathway in order to detoxify after haemoglobin degradation. This happens either in lipid particles or in close association to the phospholipid membrane(109). It is not only Hz production that requires lipids, but also the inhibition of hosts monocyte functions, carried on by Hz, which is mediated by hydroxyl fatty acids generated by the parasite in the human host in large amounts(110).

Hyperlipidaemia and malaria

Assuming that the lipid demand is higher in patients with malaria, it would make sense to infer that the parasite would develop better in patients with higher serum lipid levels, like hypercholesterolemia and hyperlipidaemia. The prevalence of both conditions has been growing worldwide and affects up to 39% of the world population(111). One might think that this is a problem characteristic of high-income countries. However more than 20% of the population of low and middle-income countries may have hypercholesterolemia(111). Interestingly, the authors speculate that the higher utilization of lipids by the parasites might be contributing to lower the LDL and total cholesterol levels on a population level, where malaria is endemic.(112)

Finally, drugs that are usually used to decrease serum lipids, such as statins, were shown to have some effect against *P. falciparum*. In a rodent model, statins reduced the parasitaemia when compared with untreated mice(113). Moreover, Taoufiq *et al* observed that statins prevented *P. falciparum* cytoadherence and thus might have an antimalarial effect(114). Other trials with statins showed inhibition on *P. falciparum* growth(115, 116). However, it would add weight if epidemiological studies would show an association of statin use and lower parasitaemia or protection against malaria in humans.

Micronutrients

Malaria appears to also be affected by the intake of some micronutrients. Both Vitamin A and Zinc play, as mentioned before, an important role in the immune response. These micronutrients have been shown to increase resistance to malaria as well as reduce the parasite load in malaria patients(88, 117). Studies in rodents revealed inverse associations between zinc and oxidative stress and mortality. The supplementation with zinc was shown to improve the prognosis of malaria in a trial with supplemented children in Papua New Guinea, whereas deficiency in vitamin A could lead to ocular pathology during cerebral malaria(118). Shankar *et al* meticulously describe this effect in a review, precisely about nutrition modulation of malaria morbidity and mortality(48).

The effect of iron on malaria is more controversial. While some studies have observed an increased risk of malaria with iron supplementation as well as higher parasitaemia(119), others found no effect of supplementation either on infection or mortality(120, 121). As iron is the most common micronutrient deficiency worldwide, the supplementation programs usually include this nutrient. However, in a randomized placebo trial in Pemba Island it was observed that the children supplemented with iron and folate were more susceptible to severe illness and dead, which was thought to be in part because of malaria(28). Folate plays a particular role, because folate metabolism is a target for antimalarial drugs(122). Therefore a supplementation of folate might antagonize the effect of the drug as observed in a review by Verhoef *et al(123)*. However, megaloblastic anaemia patients were found to have higher infection rates, while malaria itself could lead to folate deficiency or even megaloblastic anaemia(120).

Table five presents a summary of some of the interactions between micronutrients and malaria. The literature clearly shows that several nutrients and nutrition-related conditions play some role in the course of malaria. However, many issues are controversial and further work is necessary to clarify the impact of nutrition on *Plasmodium* infection in humans. Although micronutrients are of great importance, this study will focus on the broader picture, such as nutritional state and macronutrients, and their impact on malaria.

Table 5 Associations between nutrition on malaria infection.

Nutrition Factors	**Effect on Malaria**
Malnutrition	↑CFR* ↑Severe Malaria ↑Neurologic damage
Overnutrition	↑Severe malaria *
Glucose	↑Demand in malaria ↑Severe Malaria ↑Parasitaemia
Diabetes	↑Susceptibility to malaria ↑Severe Malaria
Lipids	↑Cerebral Malaria**
Iron	↑Susceptibility to malaria*
Zinc	↑Resistance to malaria
Vitamin A	↑Resistance to malaria
Folate	↓Infection Rates

*Contradictory results between studies. **Results from rodent models.
↑ - Increase; ↓- Decrease.

The Idea

It appears clear that nutrition plays an important role in malaria. However, the information on the mechanisms are still sparse and often contradictory. Furthermore, considering the significant changes in dietary habits in endemic countries, such as obesity and other non-communicable diseases that are becoming more common in the last decades, many study results are difficult to interpret, especially older studies. Another, yet crucial aspect is that many studies which reach more convincing conclusions were *in vivo* studies performed in animal models, often in rodents which have a different metabolism and, obviously, “lifestyle” than humans. While animal models

are very important to investigate the mechanisms that trigger an effect, they are not as effective when studying an effect to then translate it to the human.

In order to analyse the effect of nutrition on the growth and development of *P. falciparum*, several approaches can be used. These include studies of *in vivo* rodent models, observational studies in humans, eventually interventional studies in humans, but also, of course *in-vitro* work.

- Despite many advantages, the *in vivo* approach in the rodent model has the disadvantage that the metabolism between mice and humans may be different and thus, results may be difficult to apply to humans. A recent editorial from the British Medical Journal (BMJ) mentions the lack of reliability of data from animal studies for application in humans(124). It inclusively mentions some works on this subject which substantiate this statement.
- Observational studies in humans are more common. However, they are also not the ideal because they can be biased by a lot of confounding factors; apart from their organisational complexity. This type of studies cannot establish causality, only associations. Such studies usually need a large number of volunteers.
- To appropriately study effects *in vivo* in the human host would be very difficult, as the only convincing way would be some type of randomised controlled intervention trial. These are extremely costly, entail ethical problems, apart from the usual difficulties, such as finding volunteers willing to participate in such a trial. Certainly, the best possible way might be Controlled Human Malaria Infections to investigate nutrition. However, it seems doubtful that putting the health of volunteers at risk would be justified (yet) to address nutritional questions.

Box 2 *Ex-vivo* based on *in-vitro* study

One arrives at the option to study nutritional effects in humans is *ex-vivo*; that is using an assay based on *in-vitro* methodology. The underlying idea is to mimic the growth of the parasites in the blood by using a blood culture with RBC and serum from human volunteers.

The *in-vitro* culture of the malaria parasite was not possible until the beginning of the 20th century. The first report of a *Plasmodium* culture dates back to 1912 when Bass and Johns accomplished the *in-vitro* culture of the parasite directly through samples from infected patients. Their technique consisted in incubating infected blood, without white cells, with serum in a column at 40ºC. However, with this method it was not possible to keep the culture growing for more than three cycles, because the parasites started to die after the second segmentation(125) (see figure seven – *Plasmodium falciparum* lifecycle). Since then, some attempts were made to improve this technique, although only in 1976 Trager and Jensen were able to maintain continuous cultures of *Plasmodium falciparum* using their “candle jar method”. This method involved the culture of the parasites from an infected monkey in a nutritive medium (RPMI 1640, supplemented with Hepes buffer and $NaHCO_3$) with 10% human serum and RBC that were mixed together with the blood

from the monkey(126). The culture was incubated in a plastic petri dish in a candle jar (in a high CO_2 and low O_2 atmosphere), with the medium being changed once a day (126).

Still today, continuous *in-vitro* cultures of *Plasmodium falciparum* are performed using adaptations of this method. However, some attempts were made to reduce the amount of serum needed or more correctly, substituting it. It was found that serum from another animal could be used instead of human serum. The most widely used is commercial Albumax solution, although it is readily available it is not as effective as human serum for parasite growth(127).

Because the idea of this study is to mimic what happens in the human body, it is apparent that the cultures should be performed with the maximum amount of blood constituents from a volunteer and the minimum number of artificial supplements and medium. A key issue here is, that it would be difficult to detect any changes of parasite growth when only using 10% of human serum, if the idea was to study the effect of nutrients in the serum of volunteers.

Consequently, the basic idea was to develop a new protocol, with a maximum amount of serum and a minimum concentration of added glucose. In this way, the effect of nutrition on constituents in the serum of the volunteer donors could be more reliably detected. The idea was to set up experiments where the serum is collected in different nutritional states (before meal, after meal, etc.) so that the consequent changes of donor serum constituents could be investigated with regards to their effect on *Plasmodium* growth *ex-vivo* / *in-vitro*. A simple underlying assumption was, that parasite replication and thus, parasitaemia will be the result of the presence (or absence) of nutrients in the donor's serum.

Objective

The objective of this study was to investigate the effects which different kinds of food and its nutrients may have on the development and growth of the malaria parasite in an *ex-vivo* / *in-vitro* cultures with the blood from human volunteer donors.

Specific Aims

- Produce a protocol that allows the culture of P. falciparum in a condition that replicates the conditions in the blood.
- Compare the growth of the parasite in blood from different donors, before and after the ingestion of a previously defined meal.

Strategy (setting up an *in-vitro* culture with high serum content)

Since the routine continuous culture is performed with the minimum blood constituents possible, using Albumax as a serum substitute, a solution to reverse this had to be found. First, the literature had to be thoroughly examined. This investigation was performed in order to understand what had already been done in the past, before the achievement of Trager and Jensen's method, and what could be learned from these older methods. The investigation

included a search for methods from the past when there were no substitutes for blood to be used. With the resources available in the beginning of the 20th century, trying to mimic the conditions which the parasite faced in the host was the most apparent choice. Both Russel *et al* (128), in Practical Malariology and Boyd *et al* in Malariology(129), reviewed some of the more important attempts to keep a continuous culture of *Plasmodium* parasites. In both these textbooks one can see that different nutritive culture media were used by several researchers with the purpose of achieving a continuous method for the culture of *Plasmodium* parasites. Ball *et al*, cited by Russel *et al(128)*, were able to accomplish a continuous culture of *P. knowlesi* using a nutrient fluid and parasitized blood in an atmosphere of 5% CO_2. The candle jar method by Trager and Jensen(126), which was mentioned earlier, was also performed using serum at 10% in a nutritive medium. Both these methods are interesting for the establishment of a culture method that mimics at the most what happens in the human host, the former because of the use of whole blood and the latter for the use of serum instead of a substitute.

The first step has to be concerning whether is possible or not to keep a continuous culture of *P. falciparum* with serum. For these experiments the concentration of serum to be used must be the currently used (10%). Since both the RBC and the serum are needed for this culture, it is reasonable to think of plasma as an alternative to serum, as it would be possible to take advantage of the collected blood in its entirety, with no waste (RBC have to be collected with anticoagulant). However, aspects such as the effects of anticoagulants must be considered. Heparin, for instance, appears to have some nefarious effects on parasite growth, namely on reinvasion(130, 131).

The underlying workplan was to revert to the use of serum (instead of Albumax) and try to increase the concentration of serum, while reducing the concentration of glucose with the aim to develop a novel culture protocol.

After the optimization of the protocol to the growth of *P. falciparum*, volunteers were recruited. The blood from the volunteers was collected before and after the intake of a meal given specifically for the study, with the concentrations of plasma glucose measured on both occasions. The blood was then used in the cultures both as culture medium and RBC as the previously mentioned protocol and incubated for 96 hours and comparing the growth of the parasite in both cultures. Using this approach, the culture with blood collected before the ingestion of the meal was the control. Therefore, other factors inherent to the blood from the donor would have an impact in both cultures. Nevertheless, confounding factors associated with the blood or the volunteers had to be investigated regarding their effect on parasite growth.

II. Material and Methods

Ethics

Any culture, including the *ex-vivo* study of the effect of nutrition on the growth of *P. falciparum.* Required not only the collection of blood samples from volunteers, but also offering different food items. All blood was drawn by the PI, a registered physician. This study was submitted and approved by the Ethic Committee from the *Centro Académico de Medicina de Lisboa*.

Reagents and measurement of parasitaemia

Reagents

The reagents used for the culture of the parasite are indicated in table six.

Table 6 Reagents used for complete culture medium

Reagent	**Amount**	**Reference/Supplier**
Liquid RPMI 1640 medium (without L-glutamine, with $NaHCO_3$)	500 ml	(31870-025) Life Technologies Corporation (Paisley, PA49RF, UK)
Hepes 1M	12 ml	(15630-080) Life Technologies Corporation
Gentamycin (50mg/ml)	500 µl	(15750037) Life Technologies Corporation
L-glutamine (200mM)	5 ml	(G6182) Sigma-Aldrich Chemie GmbH (Riedstr., 2D-89555 Steinheim)
Albumax II solution*:	50 ml	
RPMI (with L-glutamine, without $NaHCO_3$) in powder	5,2 g	(11021-067) Life Technologies Corporation
Albumax II	25 g	(11021-029) Life Technologies Corporation
Glucose	1 g	(G6152) Sigma-Aldrich Chemie GmbH
Gentamycin (50mg/ml)	10 µl	(15750037) Life Technologies Corporation
Hepes	2,98 g	(G6172) Sigma-Aldrich Chemie GmbH
Hypoxanthine	0,1 g	(G6135) Sigma-Aldrich Chemie GmbH
$NaHCO_3$	1,67 g	(G6148) Sigma-Aldrich Chemie GmbH

*The amounts for the Albumax II solution are relative to 500ml and is filtered to sterilise. Stored at -20ºC.

Flow cytometry and Microscopy

The SYBR green used in the flow cytometry protocol was obtained from Life Technologies Corporations. The Giemsa stain used in the microscopy protocol was obtained from Merk.

Blood Collection

For the collection of blood, the S-Monovette® system from Sarstedt (Nürmbrecht, Germany) was used. Tubes with sodium citrate were used for the collection of anticoagulated blood destined to be transformed into plasma and RBC, while tubes with a clot inducer were used for the collection of serum. To complement this system for blood collection, a Safety-Multifly® needle, from the same company, was used.

Meals offered to the volunteers

To test the effect of nutrition in the growth of *Plasmodium*, a high calorie and high sugary meal was offered to all volunteers. To increase the acceptance from the volunteers, commercially available 50 grams "chocolate bars" were chosen as the "meal" for the volunteers. This meal will further be referred as High Calorie-High Sugar (HCS) meal. The nutrition composition of each "chocolate bar" are described in table seven.

Table 7 Nutritional description of the "chocolate bars" ingested by the volunteers.

Type of chocolate	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Serving (g)	50	45	43	39	51	50	50
Energy (kcal)	136	234	244	177	229	242	248
Lipids (g)	8,3	12,5	16	8,8	4,3	11,4	12
Saturated (g)	5	7	7,4	5,1		4,2	7
Carbohydrates(g)	14,1	27	21,2	22,9	31,5	30,1	32,2
Sugars(g)	13,8	22,1	17,8	16	31,5	25,8	24,4
Protein (g)	1,6	3,1	3,6	2,1	2	4,3	2,2

On the other hand, to test the effect of different types of carbohydrates, 35g of commercially available glucose and fructose were mixed with water and given to drink to the volunteers.

Measurement of serum glucose

The device used to measure the volunteer's glycaemia was a home measuring device, *OneTouch Select® Plus*, which is commercially available in any pharmacy. The test strips were the recommended to the glucometer, from the same company.

Volunteers

This study involved the participation of healthy volunteers, both male and female with ages between 20 and 55 years old from a non-endemic country for malaria. To test the effect that the intake of at least meal might have on parasites growth, the participants had to be submitted to a blood collection and serum glucose measurement before the intake of a High Calorie-High Sugar

(HCS) meal. Then the volunteers ingested the HCS meal and were submitted to another venepuncture 30 minutes later, followed by the measurement of the glycaemia.

Blood Samples

Blood collection

The blood was obtained from healthy volunteers and collected under sterile conditions. The system for the blood collection was S-Monovette®. Tubes with citrate, lithium heparin and clot activator were used, depending if the blood was to extract RBC (the former two) or serum (the latter).

Preparation of blood samples

Serum was separated from RBC after the blood had completely clotted by centrifugation at 2000g at 8ºC. The serum was then stored in a refrigerated environment (4ºC). The blood to be separated into plasma and red blood cells (RBC) was centrifuged at 400g, followed by the separation of the plasma from the RBC. Furthermore, the isolation of the RBC was accomplished by centrifugation of the samples, removing of the supernatant. The cells were then washed, at least twice, with RPMI 1640, followed by centrifugation for five minutes at 8ºC, at 400g.

For some preliminary experiments RBC obtained from the National Blood Bank (NBB) in the form of buffy coats or low volume units were used. Both these types of units contain citrate phosphate dextrose (CPD) as anticoagulant, in the amount of 63 ml per blood bag of 450 mL. The composition of the CPD is described in the table below. It is important to point out that the low volume units do not have the total volume of the bag (450 mL) and thus have a higher concentration of CPD than that of the buffy coat.

Table 8 Composition of CPD.

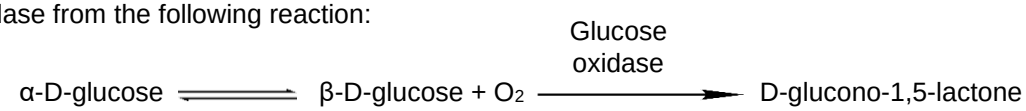
Component	**Concentration (g/L)**
Citric Acid Monohydrate	3,27
Sodium Citrate Dihydrate	26,3
Glucose Monohydrate	25,5
Sodium dihydrogen phosphate dihydrate	2,51
Water for injections to 1000 mL	

Defibrination of blood

The defibrination of the blood for the isolation of the RBCs was performed by smooth and continuous agitation of a small volume of blood (about 5 mL) in an Erlenmeyer flask containing small glass beads at the bottom. The fibrinogen attached to the beads and the remaining non-coagulated blood was extracted to a tube to isolate the RBC as described before.

Measurement of serum glucose

To measure the serum glucose of each participant, both before and after the ingestion of the HCS meal, a home device specific for the purpose was used. The device used measures the concentration of glucose in the blood by measuring the amount of glucose that is used by glucose oxidase from the following reaction:



Cultures

Strains

The strain of Plasmodium falciparum 3D7 was obtained from BEI Resources Repository, NIAID, NIH, contributed by Daniel J. Carucci.

Routine in-vitro culture of P. falciparum

The cultures of *Plasmodium falciparum* were performed according to Trager and Jensen's methodology(126) (figure eight) . A haematocrit of 5% was used in all cultures and the medium changed every day. Parasitaemia was assessed daily. When it was superior to 2% the culture was diluted with new RBCs. The cultures were always incubated in a 37°C in an atmosphere of 5% CO_2. The parasitaemia was checked by microscopy and flow cytometry as described before.

Standard Medium for P. falciparum culture

The standard medium for the continuous culture of *P. falciparum* was composed by 88%(v/v) of RPMI 1640, 9% of Albumax solution, 2% of Hepes buffer, 0.9% of L-glutamine and 0,09 of gentamycin. The 50ml of Albumax solution already have RPMI (0,52g), gentamycin (50µl), Hepes (0,298g), sodium bicarbonate (0,167g), glucose (0,1g), hypoxanthine (0,01g) and Albumax II (2,5g). The components of the medium are described in table nine.

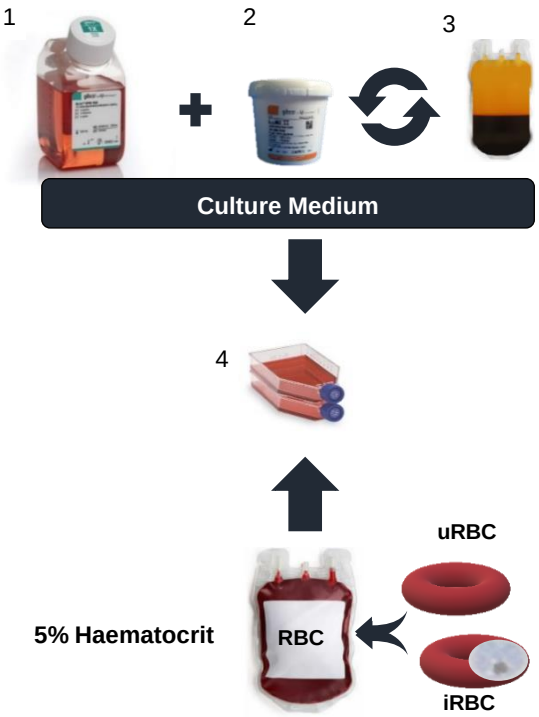


Figure 8 Method for the culture of *Plasmodium falciparum*.
The culture medium is prepared with RPMI 1640 and 10% of either serum or serum substitute, such as Albumax. This is added to a culture flask. Then RBC (infected and uninfected) are added to the culture medium for a total haematocrit of 5% and a parasitaemia below 5%. 1- RPMI 1640; 2- Albumax; 3- Serum; 4- Culture flask; uRBC- uninfected red blood cells; iRBC- infected red blood cells

Table 9 Description of the constituents of the medium.

RPMI 1640 (Roswell Park Memorial Institute 1640)	Medium used for cell culture and is formulated for use in an atmosphere of 5% CO_2. RPMI 1640 contains glucose, phenol red as a pH indicator, salts, amino acids and vitamins. It has to be supplemented with serum or a substitute for cell culture(132).
Hepes	Buffering agent commonly used in cell culture due to its ability to maintain the physiological pH despite CO_2 changes caused by cellular respiration(133).
L-glutamine	Most abundant free amino acid in the human body and it is necessary for the production of NAD^+, very important in oxidative stress(134).
Gentamycin	Broad-spectrum antibiotic used in cell cultures because of its non-toxicity to the cell cultures, stability independent on both pH and temperature, and effectiveness against several strains of *Mycoplasmas*(135).
Hypoxathine	Purine, necessary as a building block for nucleic acids as a nitrogen source as well as an energy source(136).
Albumax	Supplement used as a substitute of serum in cell culture. It is a highly purified lipid-rich bovine serum albumin (BSA)(127, 137).

Serum media

The medium with serum was made of RPMI, Hepes buffer, L-glutamine, gentamycin and Sodium hydroxide (NaOH). The quantities of the constituents had to be adjusted along with

increasing the concentration of serum, namely the volumes of RPMI 1640 and Hepes. The remaining constituents were used always in the same concentration, gentamycin to avoid contaminations, L-glutamine as an important nutrient and NaOH to adjust the pH. The quantities of each constituent are described in table ten.

Table 10 Composition of the medium with different concentrations of serum

	10%	**20%**	**30%**	**40%**	**50%**	**70%**	**80%**	**90%**
Liquid RPMI 1640 medium (without L-glutamine, with $NaHCO_3$) **(ml)**	90	80	70	60	50	30	20	10
Hepes 1M (ml)	2,16	1,92	1,68	1,2	0,96	0,72	0,48	0,24
Serum (ml)	10	20	30	40	50	70	80	90
Gentamycin (50mg/ml) (ml)	100							
L-glutamine (200mM) (ml)	1							
NaOH (ml)	0,2							

*The values are marked for 100ml of total medium.

It is necessary to add hepes buffer to the RPMI. In the end, the serum is added to the mixture.

In-vitro culture with different conditions

The first step was to thaw a frozen culture of *P. falciparum* and keep a continuous culture viable. This culture had to infect a sufficient volume of RBC, so the culture had enough volume to be divided into the different conditions. All experiments started with a parasitaemia of 0,5% and were incubated for two cycles (96h).

To infect the RBC from the volunteer, 12,5 µl of infected RBC from a pre-existing culture were added to 250 µl of RBC from the volunteer and incubated in culture medium until the parasitaemia was equal or higher than 0,5%. This method was the same used to compare the parasite's growth before and after the ingestion of the HCS meal, which is represented in figure eleven. The use of parasite cycle synchronization leads to a decrease in parasitaemia, prolonged preparation (with decrease in blood quality) and consequently, non-synchronized cultures were used.

The first aspect which was investigated was whether *P. falciparum* was able to grow in a medium with serum or plasma instead of the currently used Albumax. In this experiment, 10% of the medium was replaced with the same amount of serum or plasma, instead of the Albumax.

For the experiment with different concentrations of serum, the medium was prepared as described before. The assay was performed with 10%, 20%, 30%, 40%,50% 70%,80% and 90% of serum.

To test different types of RBC, blood was collected from the same donor to tubes containing citrate, CPD and to an Erlenmeyer with glass beads for defibrination. Then 12,5 µl of infected RBC from the same culture were added to 250 µl in culture medium and incubated for 120 h.

Assessment of Parasitaemia

Flow cytometry

Flow cytometry was used to measure the parasitaemia of the cultures. The analysis of SYBR Green stained samples of *P. falciparum* was performed using a CyFlow Blue (Partec, Munster, Germany). The analysis of the result was performed using the program FlowJo (TreeStar). The method used to assess parasite growth is described in (138).

Microscopy

Microscopy was used to assess the state of the cultures as well as the development of the parasites and respective parasitaemia. Blood smears of the cultures were performed, followed by a methanol fixation and a Giemsa staining for 20 minutes according to the protocol described in (139). This consisted in 10% Giemsa stain diluted in 1x PBS.

Analysis of Parasitaemia

Photos of smears were taken with the software Leica FireCam 3.4.1, from Leica microsystems (Switzerland) Ltd. Afterwards, the parasitaemia was determined using ImageJ 1.49v (Wayne Rasband, National Institutes of Health, USA). A minimum of 1000 cells were counted for each smear.

Test the effect of nutrition in *P. falciparum*'s growth

When the protocol was established and checked for functionality, it was possible to start the assays with volunteer donors' blood. These included the collection of blood before and after the intake of an HCS meal. To ease the intake of the said meal for the volunteers, a commercially available chocolate bar was chosen as the reference meal. This way it was possible to produce an effect on the volunteers' blood composition, characteristic of nutrient intake. The blood collection after the intake of the meal had to be in the time period when the effects are most explicit, in the case of the chosen meal (high sugar). Considering the biphasic response, it was 30 minutes(140, 141) as represented in figure nine. The 30 minutes time point was chosen considering the "biphasic-shape" curve, despite the higher prevalence observed of the "monophasic-shaped" curve, in the sample studied by the authors. By using the "biphasic-shaped" curve, it is easier to have a difference in glycaemia between before and after the ingestion of a high-carbohydrate meal. Whereas this difference is not so prominent in the "monophasic", and a bigger effect would be lost if this was the chosen shape. Moreover, in the results described by Tschritter *et al*, the individuals that presented a "biphasic-shaped" curve revealed some

characteristics that were closer to the volunteers that participated in this study, such as age, body mass index and gender.

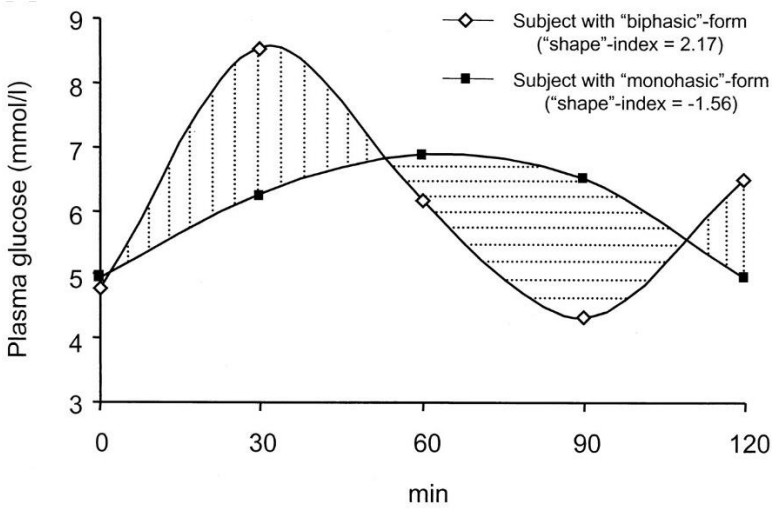


Figure 9 Glucose curves during an oral glucose tolerance test.
The glucose curves from 551 healthy individuals are represented. There are two shapes occurring, a biphasic with a peak at 30 minutes, and a monophasic which has a steadier increase, with a maximum at 60 minutes. These curves were obtained from reference (141).

The blood collected was then processed to produce serum, followed by the preparation of the culture medium, according to the established protocol, and incubation of the parasites with the same medium for at least two cycles. This process is summarized in figure ten. The results for the differences in the parasites' growth was accessed through the parasitaemia in each culture followed by comparison between the two conditions.

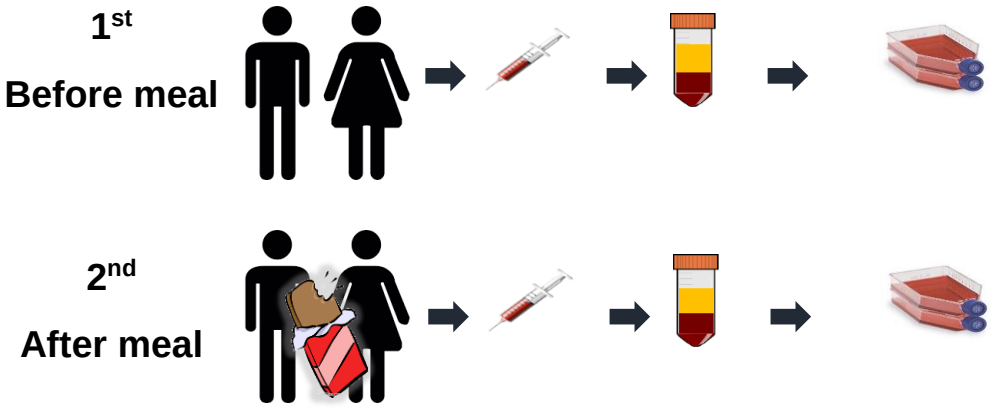


Figure 10 Schematic representation of the collection protocol.
The blood will be collected before and after the consumption of a meal and let to clot. Then it must be centrifuged to separate the serum from the clot. The serum is then mixed with the medium solution, which will be used to the *Plasmodium* culture. At last, the parasite is incubated with medium, with 5% haematocrit.

General procedure

For the cultures, both RBC and serum from the volunteer were used. The RBC were infected as described before and with non-synchronized parasites. For these experiments, medium with 90% serum was used to incubate the cultures. Cultures were performed in 24 well plates, with 1 ml of medium and 50 µl of cells for 96h, with the medium being changed every day. To avoid deterioration of the serum in the medium, small aliquots were made and frozen, thawed only on the day they were used for the culture. The parasitaemia was measured by flow cytometry and microscopy at 0, 48 and 96 hours. The whole protocol is represented in figure eleven.

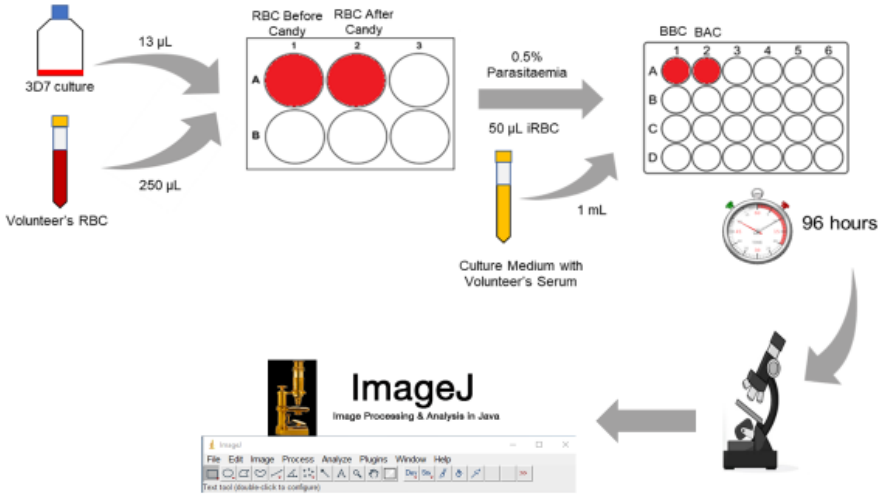


Figure 11 Schematic representation of the protocol.
The volunteer's RBC, after isolated, are infected with 13µL of infected RBC (iRBC). This culture is incubated until a parasitaemia of 0.5% is achieved. Then these RBC are incubated with the medium with the volunteer's serum in a 5% haematocrit. The final parasitaemias were obtained by microscopy. BBC - Blood Before Candy; BAC - Blood After Candy.

III. Results

1. Establishment of the experimental protocol

In order to detect differences in growth caused by the effect of nutrition on the parasite it was necessary to adapt the culture protocol to one that was closer to the conditions in the human host. With this objective in mind, the culture medium was modified as described in table eleven.

Table 11 Necessary changes to the standard protocol for the culture of *P. falciparum*

Standard medium to continuous culture		Substitution to mimic conditions *in vivo*
RPMI 1640	→	Minimum % of RPMI 1640
10% Albumax II	→	Maximum % of Serum or Plasma
11 mM of glucose added	→	0 mM of glucose
RBC from the NBB, with CPD	→	RBC from the donor, with citrate or defibrinated

NBB- National Blood Bank; CPD- Citrate Phosphate dextrose.

Before starting the experiments with different culture conditions, a culture of *P. falciparum* was successfully kept in the standard culture conditions mentioned before, after thawing. This culture grew through the whole cycle and was also able to reinvade.

a) Growth with serum and plasma

The first thing to be tested was if the parasite was able to grow in medium supplemented with blood constituents such as serum or plasma, instead of Albumax. Parasites grew in the media, supplemented with 10% of serum or Albumax (figure twelve). However, the culture incubated with plasma (Lithium-Heparin) started to die at the end of the first cycle.

It was noted in further assays, that even with RBCs collected with the same Lithium-Heparin tubes, no parasites grew after the first cycle. The blood group of the donor was not determined since previous studies observed that its influence was not relevant(142).

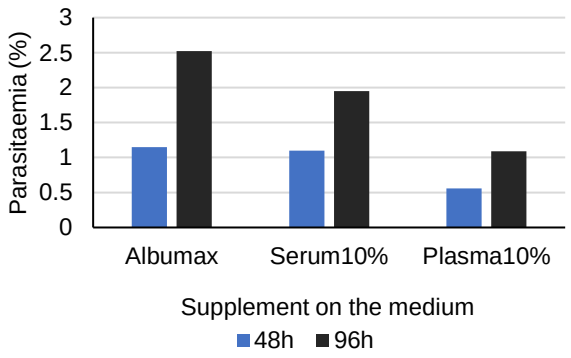


Figure 12 Growth of *P. falciparum* depending on supplement, by time point.
The graph shows the parasitaemia of the cultures incubated in medium supplemented with 10% Albumax, 10% plasma and 10% serum, measured at both 48 and 96 hours. Error bars are not present, because experiments could not be performed in triplicate, because the maximum amount of blood did not permit it (maximum amount as authorized by ethical approval).

b) Different concentrations of serum

Growth was observed in all the serum concentrations tested (from 10% to 90%). In a first round, tests were performed with 10%, 30% and 50% of serum, while the concentration of added glucose was the same from that used in continuous cultures (11mM). The higher growth was registered in the culture incubated in medium with 10% serum, followed by 30% and 50% serum medium. The culture where the parasite grew best was the one incubated with the standard medium, represented as control in figure thirteen.

Subsequently, cultures were incubated in media supplemented with 70%, 80% and 90% of serum. No additional glucose was added to the media for these cultures (glucose provided only by the added serum). The results are shown in figure thirteen. Growth was registerd up to 90% serum. Unexpectedly, a large growth was noted at 80%, which was interpreted as an error because of the extend of the standard deviation.

The result indicated that the experiments could be performed with a concentration of 90% serum whith a similar growth as cultures performed with 10% serum or with Albumax, which is represented as the control.

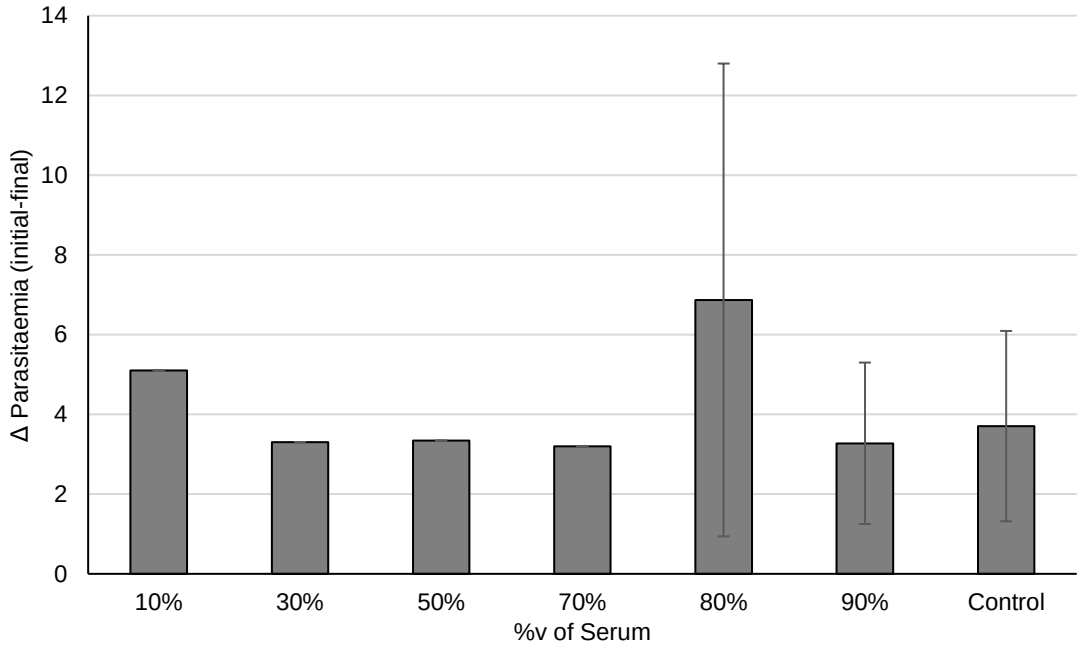


Figure 13 Growth of *P. falciparum* depending on percentage of serum.
The values for growth were obtained through the difference between the parasitaemia at 96 hours the initial parasitaemia, of the cultures incubated with concentrations of serum between 10% and 90% serum. Note that there was growth in all conditions, though the best growth was observed in the cultures with 80% but with a large error associated. The control represents a culture in the standard culture conditions explained in the methods.

c) Different concentrations of glucose

The objective of this work is related to the glucose in the blood and consequently, this nutrient should be provided only by the blood constituents (serum) in the medium. To test the lower concentration of glucose in which the parasite can grow, five cultures were incubated with different

concentrations of glucose, 2, 3 and 5 mM as well as the standard concentration (11mM) and a control with Albumax supplemented medium (11mM). Growth was observed in all cultures, independently of the glucose concentration. The culture with the highest parasitaemia was the one with the standard amount of glucose in the medium (11mM), although the total difference in growth did not exceed 0,6% (figure fourteen).

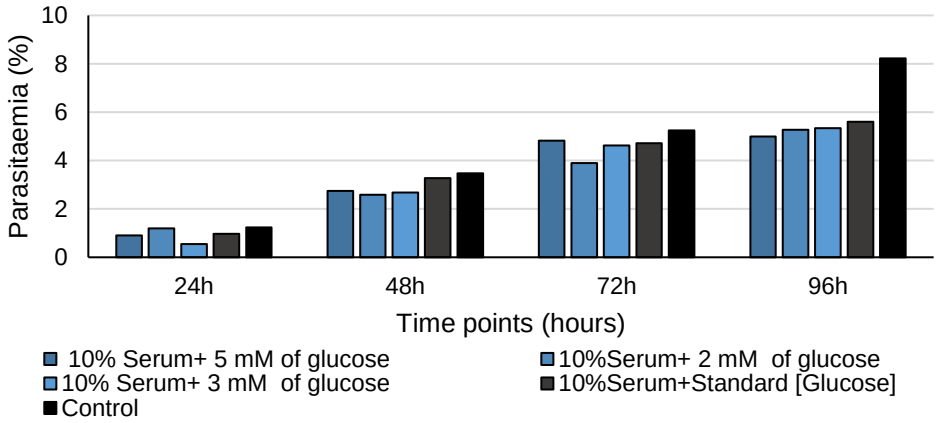


Figure 14 Parasitaemia of *P. falciparum* in 10% serum, different concentrations of glucose for 96h
Parasitaemia of the cultures incubated in medium supplemented with 10% Albumax and 10% serum with different concentrations of glucose. The three concentrations used were 5mM, 3mM, 2mM and the standard concentration (11mM) used in the continuous cultures.

d) Combining serum with glucose to determine the lowest threshold for parasite growth

With the results from the previous experiments, it was necessary to test the same concentrations of glucose but in a using a higher percentage of serum in the media. The usual concentration of glucose in the blood of fasting healthy humans is around 5 mM. Therefore, in a medium with 50% serum, the concentration of glucose would be expected to round 2,5 mM (table twelve). Based on this reasoning, no glucose was added to the medium at serum percentages exceeding 50%. The different concentrations of glucose from the serum are represented in table twelve. As it is possible to understand from the interpretation of the table, the concentration of glucose in the different media (with different concentrations of serum) was not the same, hence the results are not comparable as such. However, the objective of this part of the work concerned the establishment of a protocol that allows the growth of *P. falciparum*. The remaining concentrations were the same as the ones tested with 10% serum (3mM and 5 mM). The results obtained from this experiment were also positive, showing growth at all concentrations (figure fifteen). The culture incubated with 2,5 mM of glucose grew 1% more than the 5 mM and the 3 mM cultures at 96 hours.

Table 12 Glucose concentration from serum.

% Serum	[Glucose] from Serum (mM)	[Glucose] added (mM)
10	0,5	11
20	1	11
30	1,5	11
50	2,5	-
70	3,5	-
80	4	-
90	4,5	-
Control	0	11

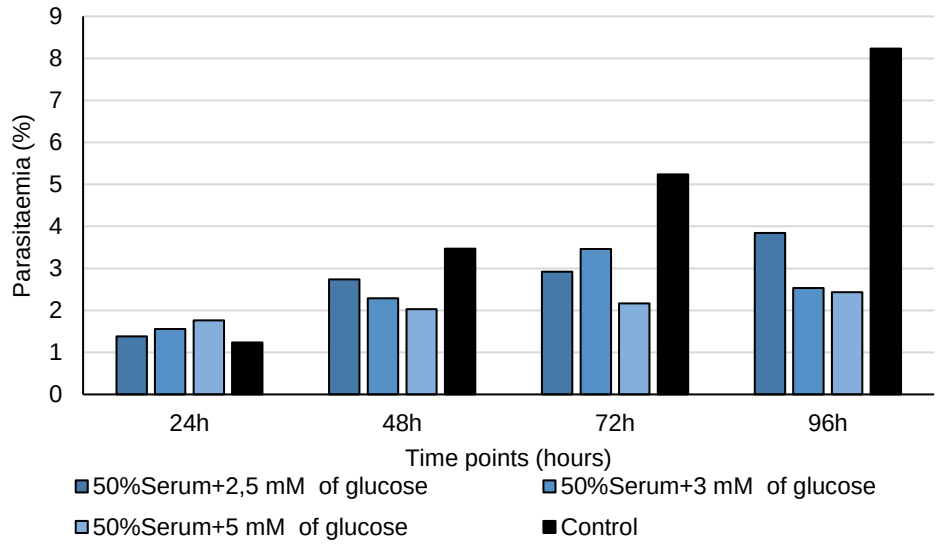


Figure 15 Determination of a lower threshold of glucose to maintain parasite growth.
Parasitaemia of the cultures incubated in medium supplemented with 10% Albumax, 50% serum with different concentrations of glucose, followed over 96h. The concentrations used were similar to the ones used in figure fourteen, with the exception of the lowest concentration (2,5mM instead of 2mM).

e) Growth in RBC from different sources

In the previous experiments, one blood component was not varied, the RBCs which were from the NBB, anticoagulated in CPD. However, in the context of this study, RBCs and serum should be from the same donor. Because the RBCs could not be coagulated, it was necessary to use either an anticoagulant or to defibrinate the blood. CPD and Sodium Citrate were studied (results for Lithium-Heparin were negative as described above).

To test the effect of the CPD on the cells, also considering that RBCs from the NBB are in this anticoagulant for days, a part of the RBCs were isolated only 24hours after incubation in CPD. To assess the possible effect that the glucose present in the CPD might have, to a part of the defibrinated cells glucose at the concentration of 3,6g/L (the same as the CPD) was added and incubated for 24 hours. Thus 5 conditions were tested: RBC in CPD, sodium citrate and defibrinated (processed immediately) and RBCs incubated for 24h in CPD or glucose.

It was observed that the parasite grew better when incubated in RBC collected with CPD as anticoagulant. When the RCB were exposed to CPD for 24 hours, the growth was close to the growth registered with CPD (figure sixteen). The growth of the parasite in the remaining RBCs is shown in figure sixteen. The CPD was excluded as its composition apparently seemed to enhance growths and thus could falsify the results. The preferred methods were thus defibrination or sodium citrate.

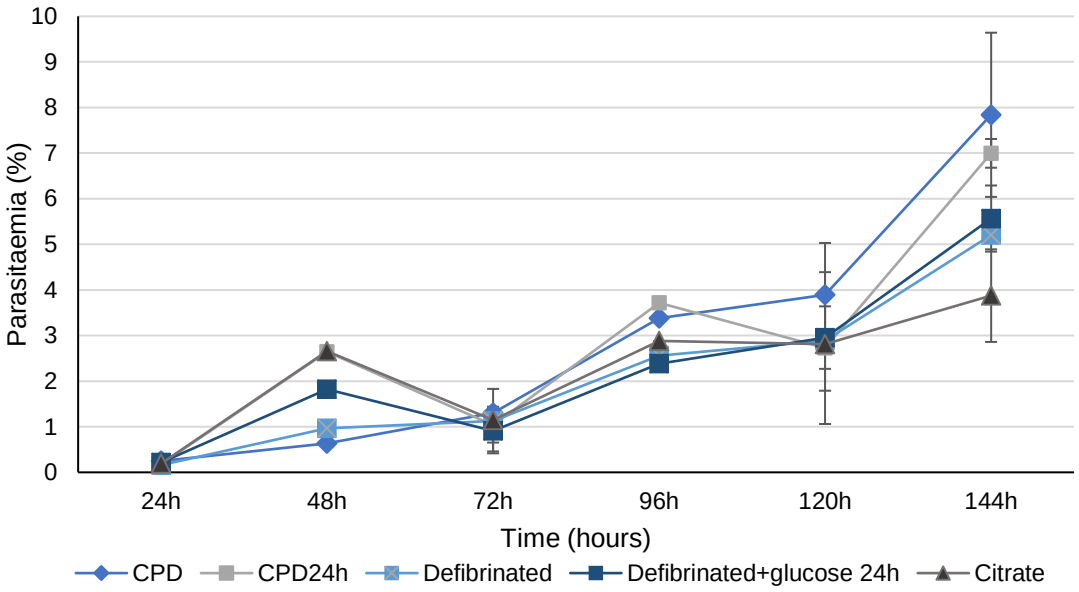


Figure 16 Parasitaemia of *P. falciparum* according to different sources of RBC through time. Cells obtained from the same donor but with different types of anticoagulation methods were infected with *P. falciparum* and the parasitaemias were measured by flow cytometry. The methods used for anticoagulation were CPD, citrate and defibrination, both the first and the latter had a second culture in which the cells rested for 24 hours in the presence of glucose (either from the CPD or added, in the case of the defibrinated).

f) Validation of the protocol using blood from different donors

To be able to start to test the influence of the diet on the parasite's growth, the established protocol was first validated with eight volunteers and compared with the growth in a standard culture medium. It was observed that the parasites were able to grow in all the cultures incubated with 90% serum and defibrinated RBC. In some cultures, the growth was better than in the one incubated with the standard medium, supplemented with Albumax (figure seventeen).

However, the parasite grow less well in the cultures from three donors. This was interpreted as a natural variations occuring in humans, where in some individuals additional factors might be present in the blood that prevent the parasite from growing well.

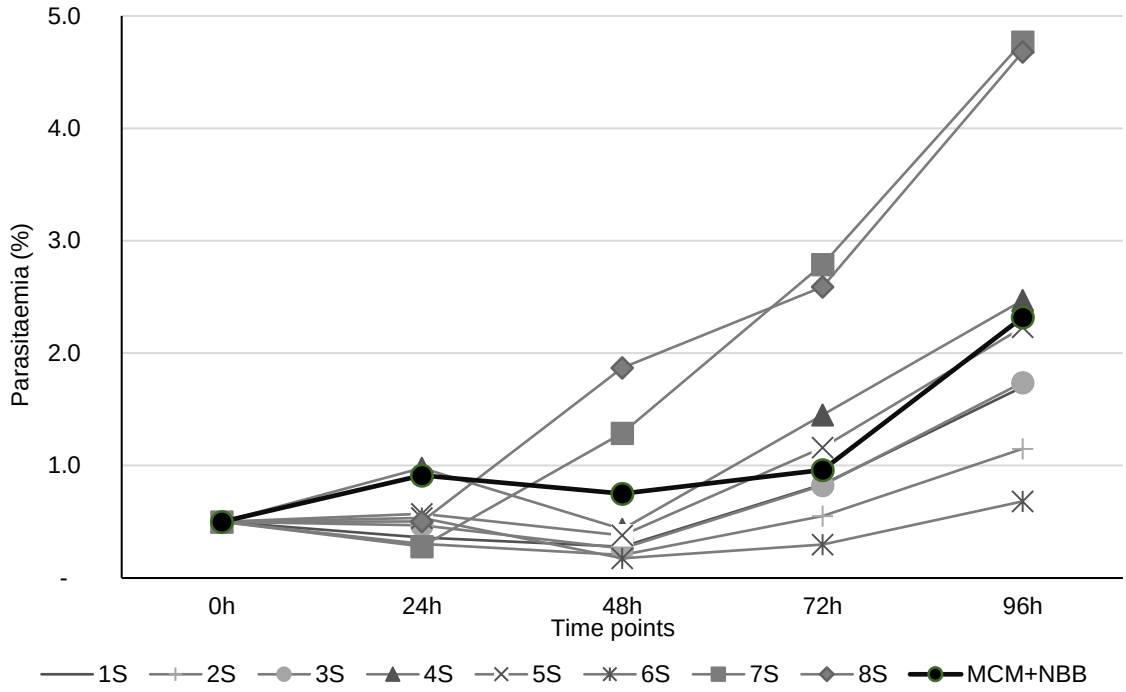


Figure 17 Parasitaemia of *P. falciparum* using the established protocol with eight different donors. Growth curves of cultures incubated with medium supplemented with 90% serum and defibrinated RBC from each eight donors and compared with one culture supplemented with Albumax. 1S – 8S – code number attributed to the volunteer donor; MCM + NBB – Complete Medium and cells from NBB.

Because of the potentially large volume of blood needed to obtain serum for the experiments, the same protocol was tested with plasma as a substitute for serum. The plasma used was the remaining plasma after isolation of the RBC, hence collected in sodium citrate. The idea was that this method would allow, with the collection of the same volume of blood, to have more medium and thus might have been possible to perform duplicates or even triplicates of each culture (if serum is used, the coagulated RBC have to be discarded). The obtained results were, however, not favourable for this idea, because none of the cultures was able to grow in medium with 90% sodium-citrate plasma, while the culture performed with the standard culture medium presented a twelve-fold increase in parasitaemia (figure eighteen).

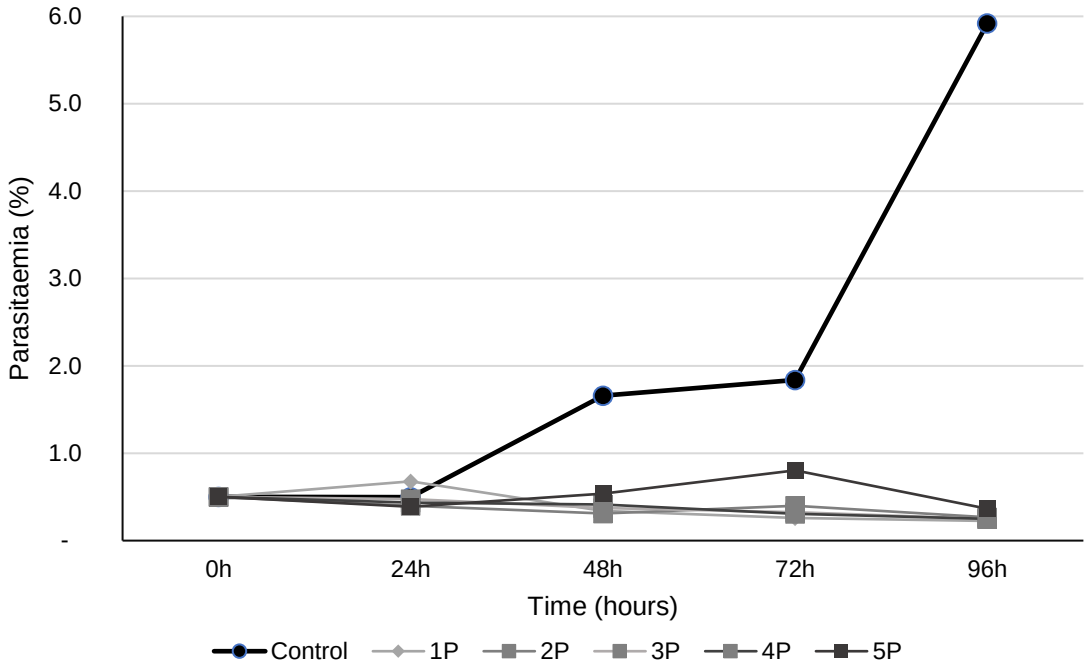


Figure 18 Parasitaemia of *P. falciparum* using the established protocol with five different donors
Growth curves of cultures incubated with medium supplemented with 90% plasma and RBC, collected to sodium citrate tubes, from each five donors and compared with one culture in the standard culture medium. 1P – 8P – code number attributed to the volunteer donor; Control – culture in the standard culture medium and cells from the NBB.

g) *Do higher concentrations of glucose produce better parasite growth?*

The parasite was cultured with a medium supplemented with 10% serum and to each medium a different concentration of glucose was added, from 70 mg/dl (3,5 mM) to 198 mg/dl (11 mM), simulating concentrations usually observed in humans.

It was observed that the percentage of growth was smaller when the concentration of glucose was 3,5 mM, slightly increasing and stabilizing between 5 mM and 7,5 mM (100-150 mg/dl) while, unexpectedly falling at 11 mM (198 mg/dl) (figure nineteen), the concentration in the standard culture (using Albumax). Interestingly, parasites in the negative control, which had no added glucose apart from the already existent in the serum, were able to grow.

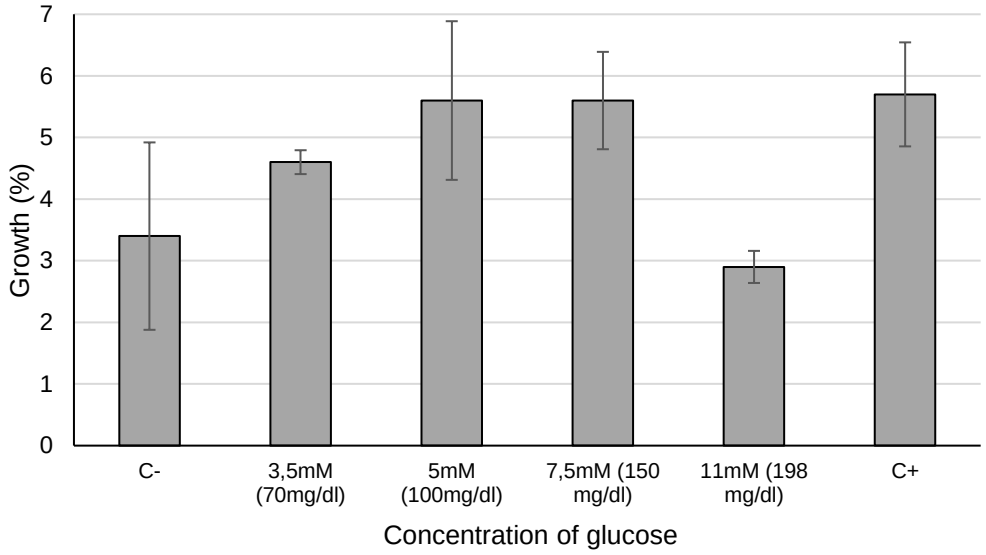


Figure 19 Growth of *P. falciparum in-vitro,* in function of the concentration of glucose in the medium. Comparison between *P. falciparum*'s growth *in-vitro*, in different concentrations of glucose. The growth is the difference between the parasitaemia at 96 hours and the initial parasitaemia. Triplicates for every condition were performed, the average is represented by this figure. Positive and negative controls were used, the former with the standard medium and the latter with no glucose added. C^+ - positive control; C^-- negative control

2. Impact of nutrition on *Plasmodium* growth

After the establishment of the protocol, it was possible to begin with the study of the growth of *P. falciparum*, both before and after the intake of nutrients, and compare the results. Fifty-three volunteeres participated in the study. The results from sixteen volunteers had to be removed from the study, because of absence of growth or a high percentage of unhealthy parasites (higher than 35%), such as the represented in figure twenty. The sample of participants in the study is described in table thirteen.

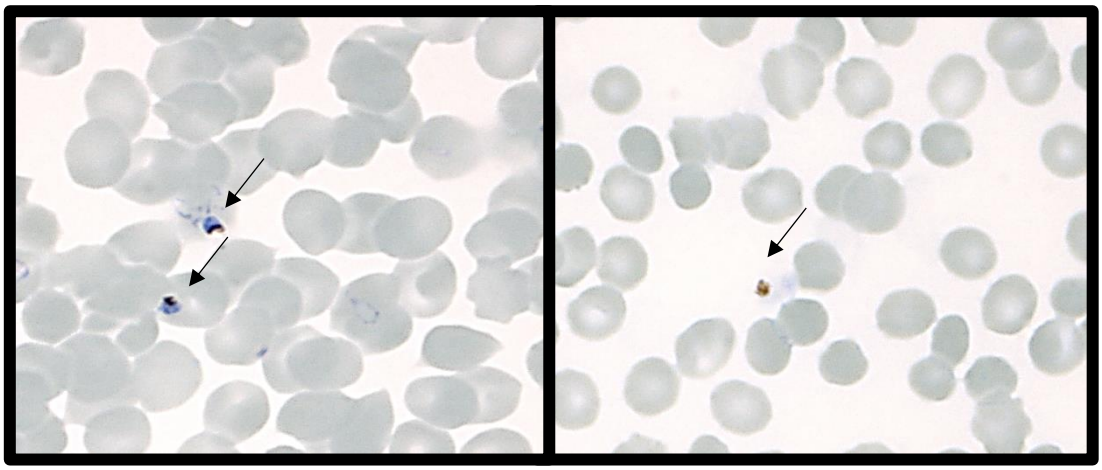


Figure 20 Representative images from a smear of RBC infected with *P. falciparum*.
Cells infected with "unhealthy" parasites are highlighted by the arrows. The left figure represents a small schizont which is not able to multiply. Other signs of the parasite being unhealthy is if they, not releasing the merozoites, were in the extracellular space, as is represented in the figure on the right. (Giemsa stain, 1000x magnification)

Table 13 Description of the sample according to gender, age and blood type.

		Total results	Included
		N	N
Gender	**Female**	37	27
	Male	14	8
Age	**20-30**	21	14
	31-40	11	8
	>40	6	4
	ND	13	9
Blood type	**A**	14	11
	B	4	2
	O	9	7
	ND	24	15

N – absolute number of the population; ND – Not determined.

During analysing of the results, comparing the growth before and after the intake of the HCS meal, possible confounding factors were analysed such as blood group or gender.

a) Influence of blood type on parasite growth

In the beginning of the study, the blood group of the volunteers was not accounted for, since the aim was to check for differences in growth when the donors ingested an HCS meal comparing

with before. The control, in this case would be the culture with blood before the ingestion of the meal, so if the blood group was to influence growth, it would do it in both cultures at the same level. However, with the observation of different outcomes, from the cultures, it was thought that the blood group might have something to do with the referred differences, hence the volunteers' blood group started to be registered. This, adding to the fact that some of the volunteers were not aware of their blood group, is the reason why the number of not determined results is larger than the remaining groups (A, B and O). There was no volunteer with blood group AB, as mentioned in table twelve.

It was observed that there was no great impact of the blood type in parasites growth. In figure twenty-one it is possible to notice that there is no clear increase in growth in any of the groups. The test of Kruskal-Wallis was used to assess the significance of these result, with which no significant difference was found (P=0,7).

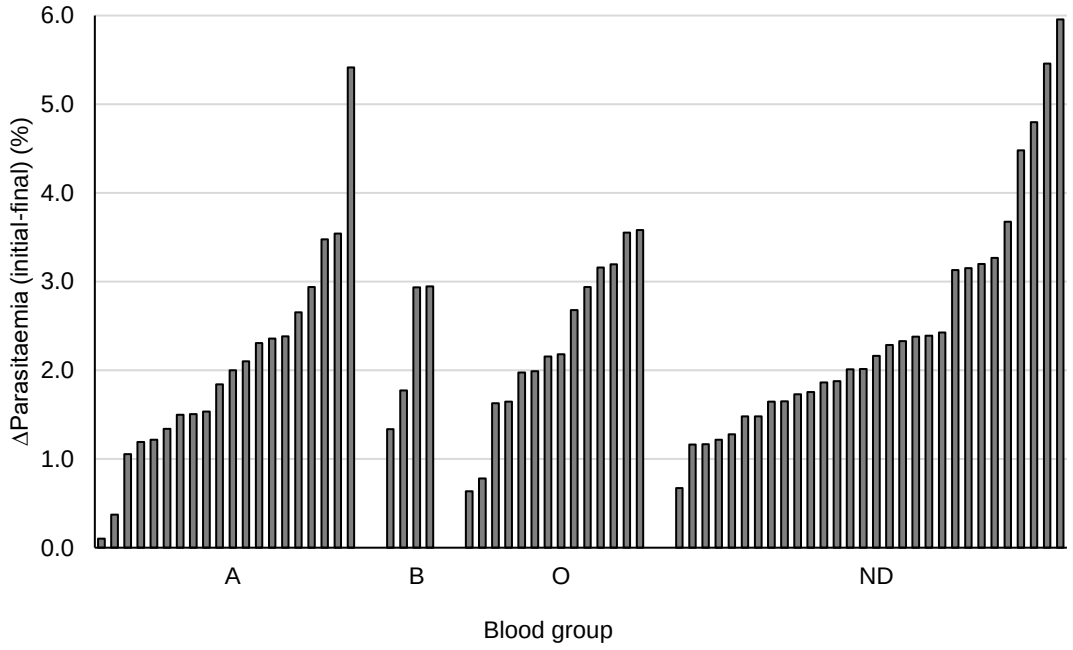


Figure 21 Growth of *P. falciparum* depending on blood type in different volunteer donors.
Difference between initial and final parasitaemia per volunteer, according with the blood types. ND – Not determined.

b) Influence of gender in parasite growth

Regarding gender, although its possible influence not being thought in the beginning, it was easy to look back and know. As it is clear in figure twenty-two, in agreement with the data presented in table thirteen, the sample was mainly composed by female volunteers. There is not a clear difference concerning the parasite's growth in function of gender. The same test of Kruskal-Wallis was used to assess the significance of the results, with which none was found (P=0,2). There was no association observed between the donors' gender and the cultures' growth.

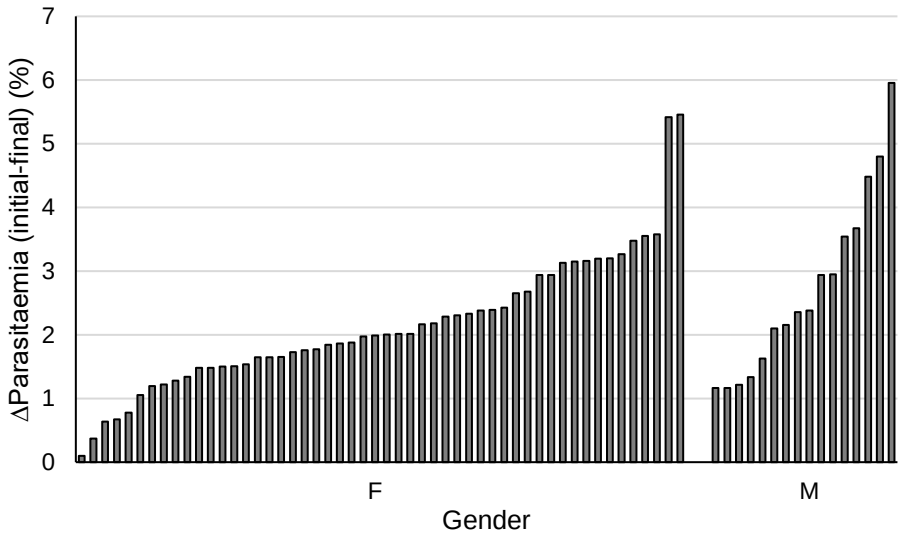


Figure 22 Growth of *P. falciparum* depending on gender.
Difference between initial and final parasitaemia per volunteer, according with the gender. F – Female; M – Male.

c) Influence of fasting in parasite growth

As the volunteers were not asked to be fasting by the time of the first venepuncture, most of the volunteers had had a meal, according to their personal habits. Although having a meal may influence the reaction to the meal provided for the study, it would create a strain when recruiting volunteers. Having the culture with blood collected before the intake of the meal as a control could already give some information regarding the reaction to the meal. The obtained results for the influence of fasting in the growth of the parasite showed, as the results for the influence of blood group and gender, no association with parasite's growth (P=0,8). There is, however, an increased growth in the not-fasting group which, not being very significant, it cannot be disregarded as a possible indicator (figure twenty-three).

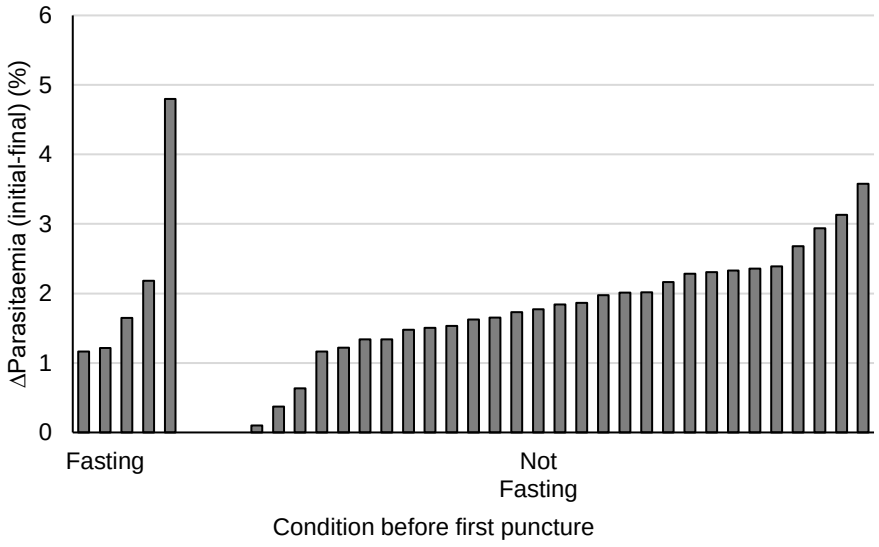


Figure 23 Growth of *P. falciparum* depending on fasting by the time of the first venepuncture.
Difference between initial and final parasitaemia per volunteer, in function of being or not fasting by the time of the first collection of blood.

a) Interindividual variability

Looking to the results its easily spotted the variability among the participants in the study. It is rather striking that both the reaction from the volunteer to the meal and the reaction from the parasite to the volunteer's blood presented differences. While some volunteers registered an increased glycaemia after the ingestion of the HCS meal, others had the opposite reaction and there was even another group that where almost no changes were registered in glycaemia values after consuming the HSC meal (figure twenty-four).

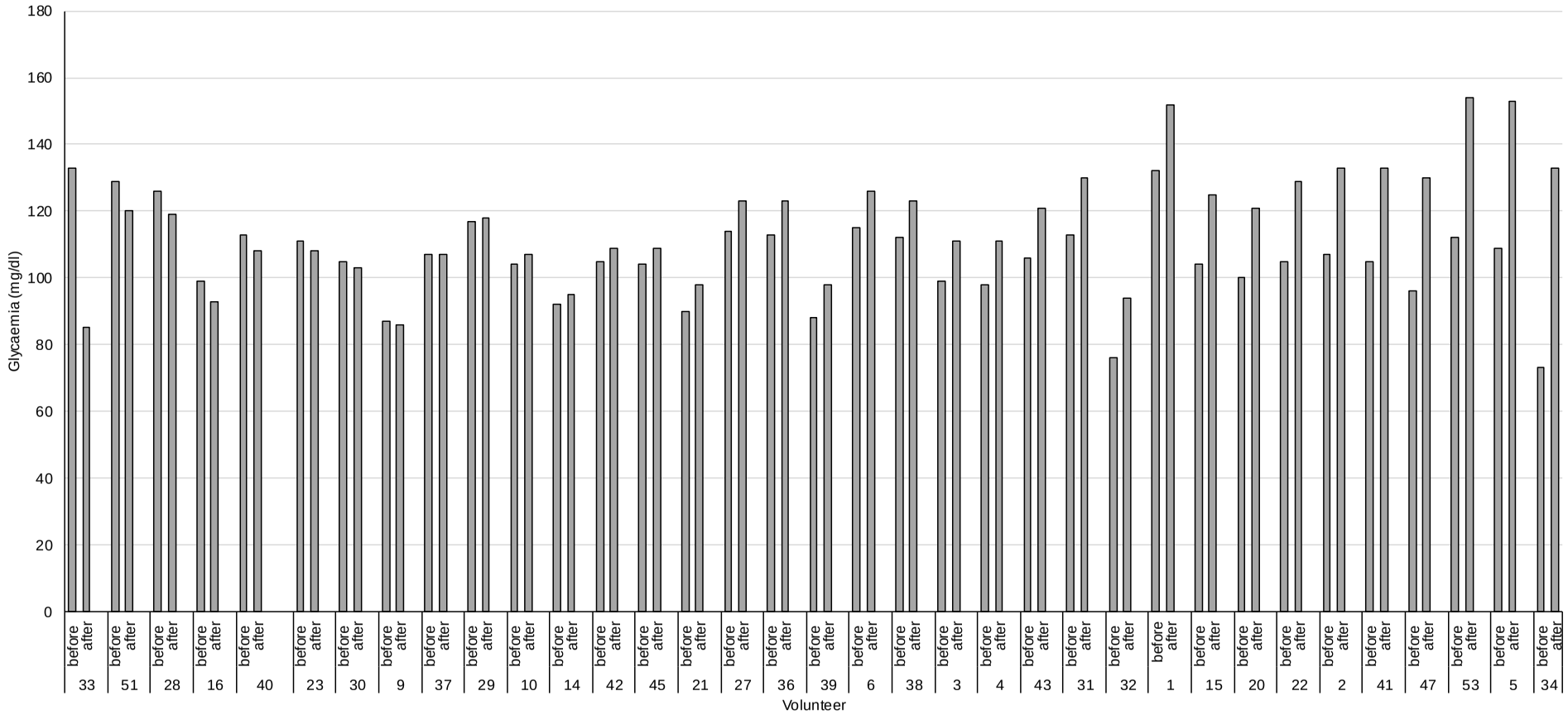


Figure 24 Glycaemia per Volunteer ordered by difference in glycaemia after – before.
The glycaemia was measured in all volunteers before and after the ingestion of a HSC meal and the difference between the glycaemia after-before was calculated. The results are organized according to the difference in glycaemia per volunteer as in figures 20 and 21. It is clear that the reaction to the HCS meal was different among the volunteers.

b) Comparison of growth before and after the intake of the HCS meal

After observing that none of the studied factors had a significant impact on the result, it was compared if the parasites growth incubated in the donors' blood before and after the intake of a HCS meal was different. Since at first sight the results would not allow an easy analyses (figure twenty-five), the results were sorted in different ways in order to find patterns. The criteria that provided a clear view of the results was the difference between the glycaemia after and before the intake of the HCS meal. It was calculated both the difference and fold difference for all volunteers.

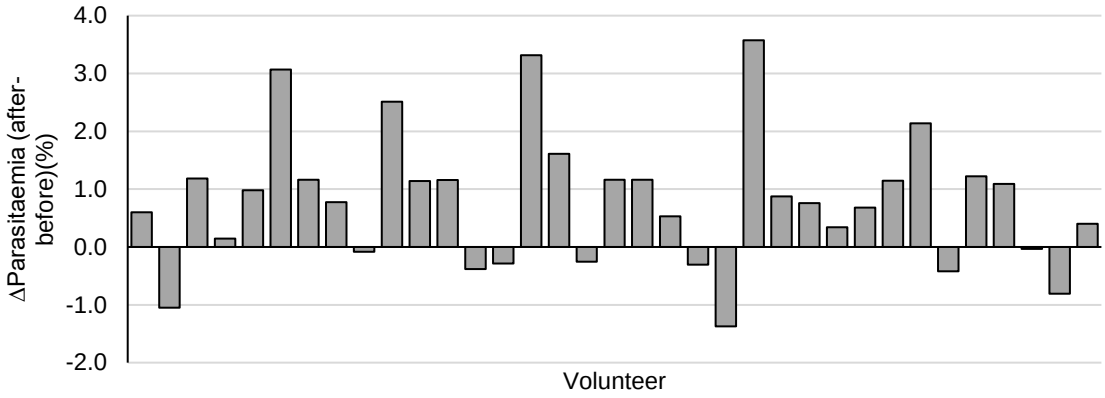


Figure 25 Comparison of the difference between growth after and before consumption of HCS meal. The growth is the difference between the parasitaemia at 96 hours and the initial parasitaemia. Then, the difference between the growth registered after and before the consume of the HCS meal was calculated. The results are organized by chronologic order only.

As it can be observed in the figure twenty-six, most of the cultures registered a better growth when incubated in blood collected after the ingestions of the meal. There were some cases in which the glicaemia after the consumption of the HCS meal was lower then the one measured before. Those are the ones in which there was a fall in the growth values after the intake of the HCS meal. Is is important to highlight that 71% of the cultures registerd an increased growth after the ingestion of the HCS meal, whereas only 11% had the opposite result. There were also some cases (17%) where the growth was very similar between the two conditions. The distribution of the results did not obeyed to the normal distribution, therefore, non-parametric Wilcoxon signed-rank test was used. There is a significant difference between the growth before the ingestion of the HCS meal and after ($P<0,001$).

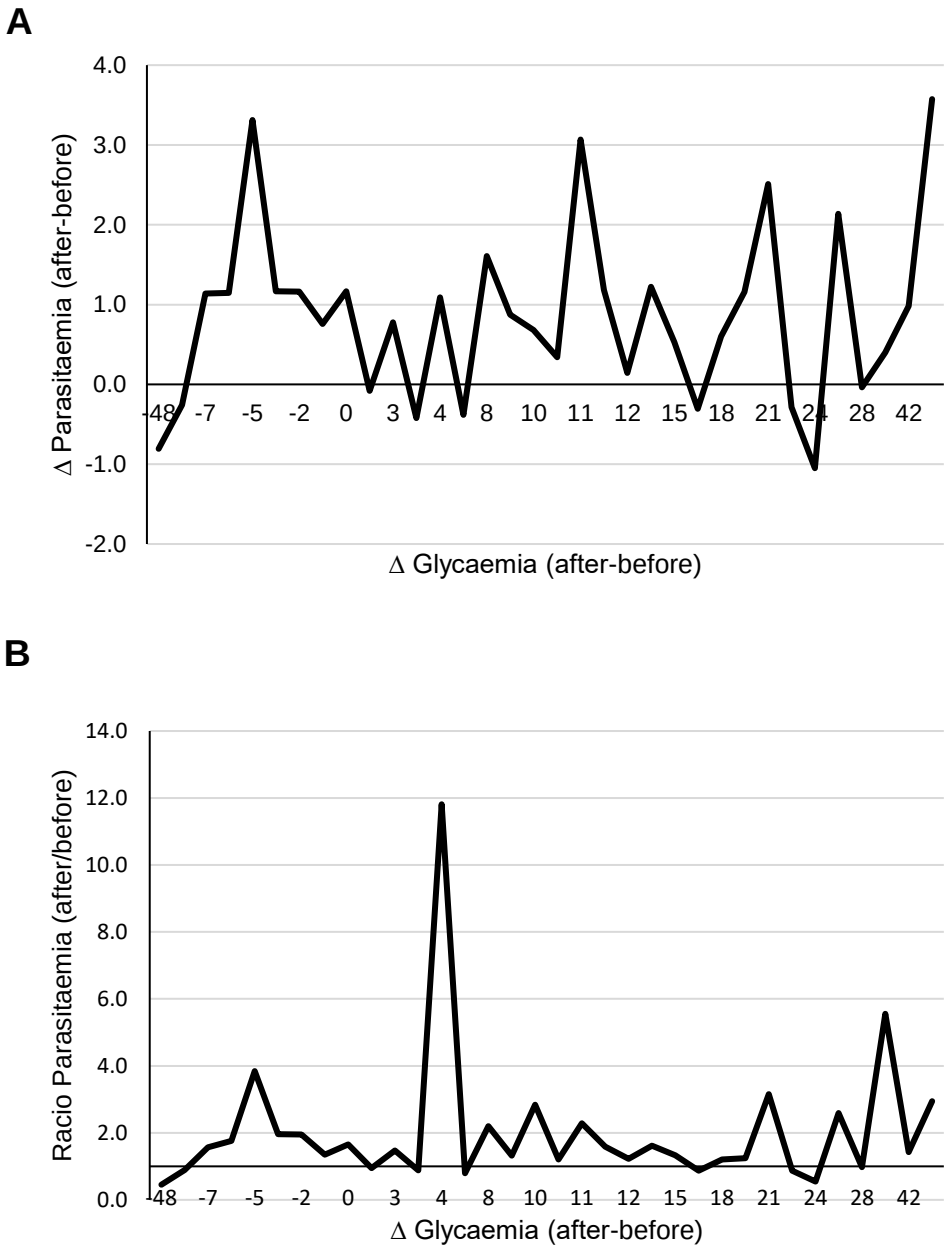


Figure 26 Comparison between growth after and before consumption of HCS meal.
In B, the difference between the growth registered after and before the consume of the HCS meal was calculated. In A, the ratio between the growth registered after and before the consume of the HCS meal was calculated. The results are organized in function of the difference between the glycaemia after and before.

3. Effect of different types of carbohydrates on the growth of *P. falciparum*

a) In-vitro testing of the effects of different carbohydrates

After the observation that the glycaemia appears to be the main factor influencing the growth of the parasite, the question on whether the type of carbohydrate also had an impact was raised. For this, cultures with the standard culture medium supplemented with 10% serum were incubated with the same concentration of carbohydrates used for continuous cultures (11mM). The carbon sources used were glucose, fructose and a mixture of both in equal parts. As a pilot experiment, both glucose and the fructose used were the commercially available for cooking purposes.

For these experiments, a culture of *P. falciparum* was incubated with standard culture medium supplemented with 10% serum from only one volunteer and 11mM of each carbohydrate, as it is used for the continuous culture of *P. falciparum*. The positive control was incubated with standard medium, whereas the negative control was incubated with the same medium as the remaining cultures but with no glucose added.

The parasite registered the highest growth when incubated with a mixture of glucose and fructose in two of the three cultures. This best growth was followed by the growth in the cultures incubated with fructose and at last by the ones incubated with glucose, with a difference of about 1% between the former and the latter (figure twenty-seven). As already observed in figure nineteen, there was growth registered for the negative control which had no glucose added.

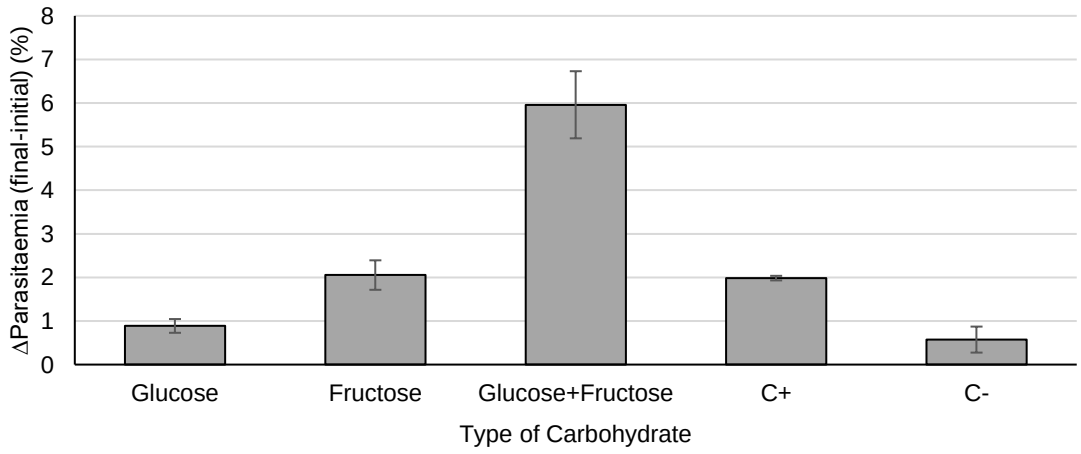


Figure 27 Growth of *P. falciparum in-vitro,* according with carbon source and controls.
Cultures of *P. falciparum* were incubated with media with different carbon sources (glucose, fructose and a mixture of both in the same total concentration). The results of growth comparing with the controls are represented. Triplicates for every condition were performed. Positive and negative controls were used, the former with the standard medium e and the latter with no glucose added. C^+ - positive control; C^--negative control.

b) Testing of the effects of different carbohydrates in blood from donors

Then it became interesting to study the same effect but *ex-vivo*, using the same protocol that was used to investigate the impact of the ingestion of the HCS meal. For this, blood was collected

from six volunteers before and after the ingestion of 35 g of glucose or fructose (diluted in water) in two consecutive days. The results are represented in figure twenty-eight, where it is possible to see that the reaction from the parasite to the ingestion of both glucose and fructose was not constant among the volunteers, existing even cultures where de parasite was not able to grow. In most cases, the growth after the ingestion of the solution was higher than before, being the cultures from volunteers three and four with glucose and volunteer one with fructose, the exceptions. It does not seem to exist a difference between the growth in the blood from when the volunteer ingested the glucose (BG) and the fructose (BF). Although the values for the glycaemia raised less after the ingestion of fructose, compared with glucose. Indicating that only a portion of the fructose was converted into glucose and that the parasite can use the remaining fructose as a carbon source. These results are somehow weakened by the standard deviation represented in figure twenty-eight, where the percentage of growth for most volunteers had a variance around 1,5%. The error associated to this experiment is, in some cases, very large, which can put the quality of these results somehow in jeopardy.

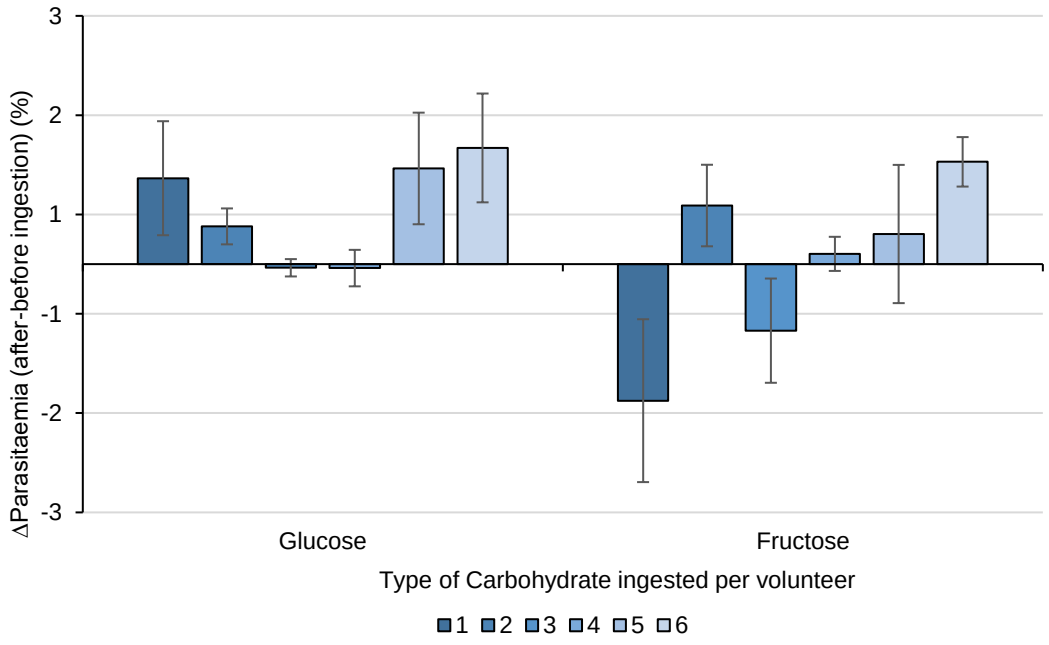


Figure 28 Difference in growth of *P. falciparum* *ex-vivo*, by carbon source in different donors.
Cultures of *P. falciparum* were incubated with media with blood from different donors whom had drank a solution with one carbon source each time (glucose and fructose). The difference between the average growth, after and before drinking the solution, in six different volunteer donors according with carbon source is represented. In the legend, the number is associated to each volunteer.

IV. Discussion

This work has two distinct parts: the establishment of a novel culture protocol with a maximum amount of human blood components and the study of the effect of a determined meal in healthy volunteers on the growth of *P. falciparum ex-vivo*. The first part involved an adaptation of the procedures for the *in-vitro* culture of *P. falciparum*, to get it closer to the condition that the parasite encounters in the host. This protocol was then applied to the blood from 51 volunteers, before and after they had ingested a previously determined meal.

1. Establishment of the protocol

It would be foreseeable that the culture incubated in medium supplemented with 10% serum would present a higher growth than the one incubated with Albumax, since the latter is a substitute for the former. Basco, in his experiments with several animal substitutes of human serum for the culture of *P. falciparum* reported precisely that neither of the substitutes, including Albumax, could achieve the same level of growth as the human serum(127). However, when testing cultures incubated in medium supplemented with plasma, it was expected that the results were similar to the ones in serum, since the biological material has very similar features. There is, however, an important aspect which distinguish plasma from serum, the anticoagulant. In this experiment, the plasma was collected to heparin tubes, because of its easy availability. The fact that the cultures did not grew the same way in plasma and serum may be associated with the inhibitory effect heparin is thought to have regarding the growth of *P. falciparum*(143, 144).

The observed results were not completely in concordance with the ones described in the literature. The growth observed in the cultures incubated with 10% serum was slightly lower than the growth observed in the cultures with Albumax. Although the reason why this occurred was not determined, it is possible that the parasite, being cultured for a long time in Albumax, had adapted to this condition in detriment to serum. However, it was possible to confirm that the parasite was able to grow in medium supplemented with serum, so the next step was to increase this concentration to one closer to what the parasite encounters in the human.

The descriptions for the use of large amounts of human serum date from the beginning of the 20th century, a time when the first attempts to culture *P. falciparum* occurred. By 1912, Bass *et al* accomplishing the culture of *P. falciparum* using whole blood, though they did not manage to grow more than one to two cycles(125). It is noteworthy to refer that, in that time, it was not possible to have a controlled atmosphere and other features that were possible later. Another important aspect of the method from Bass et al is the fact that whole blood was used, thus the haematocrit was very high (45%). While *in vivo* the nutrients and ions of the blood are always being replenished, this does not happen in culture. Thus, a higher haematocrit might mean a higher rate of metabolism in the blood (RBCs) which could lead to a change or even exhaustion of the medium. This can be an explanation for the difficulties in growing more than two cycles with this method.

Only in the 1970s, Trager and Jensen created a new method, which allowed the continuous culture of *P. falciparum*. In this method, the recommended percentage of serum is 10%(126). However, it is important to mention that, while they were trying to create the best protocol, serum was a culture component that was difficult to get hold of (such blood products are not usually commercially available) and hence, the objective would have been to use the minimum amount possible.

While the results from Bass *et al* and the recommendations from Trager and Jensen may be seen as an indicator that low concentrations of serum are good, if not the best conditions for the parasite to grow, this does not mean that the parasite might not grow (well) in higher concentrations of serum.

Certainly, culturing the parasite in a medium with a maximum concentration of serum possible (as in our study) is likely to be the opposite of the objective Trager and Jensen had. Importantly, it means that one cannot exclude that the use of higher concentrations of serum might work well. The ambition to reduce or eliminate human blood components means that the literature is conspicuously non-informative with regards to such conditions. In fact, the observations from our study indicate that, although the maximum growth appears to be in medium with 10% serum, it is possible to culture the parasite in medium with concentrations of serum as high as 90% with similar levels of parasitaemia. To better understand the protocol used in this study and its implications, figure twenty-nine represents the differences between the conditions *in-vivo* and in the *in-vitro* cultures (standard continuous culture and the protocol used for this study).

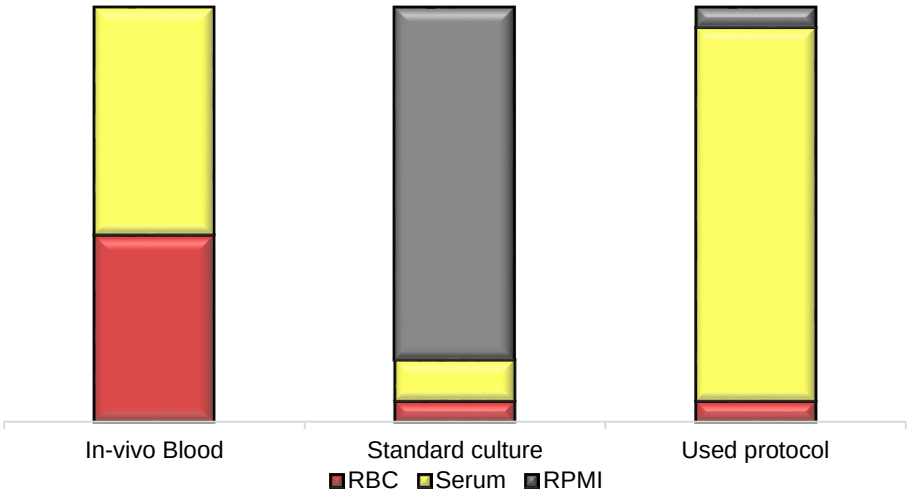


Figure 29 Representation of the proportion of components in the different media
Whole blood has much higher haematocrit than it is used in culture (45%) completed with a 55% of serum, whereas the remaining two culture protocols use a haematocrit of 5%. In the standard culture the medium is composed by 10% serum and 85% RPMI, while the protocol used in this study used 90% serum and 5% RPMI.

With the possibility of maintaining the cultures of *P. falciparum* in medium with 90% serum, it became necessary to look into the concentration of glucose in the medium. As the objective of the study was to investigate nutrients in the blood, crucially one component of the culture had to be addressed: the added glucose. The standard concentration used for the cultures of *P. falciparum* is 11mM. Interestingly, taking into account that the "normal" concentration of glucose in Humans (around 5mM), this concentration is more than double that. In fact, the concentration of 11mM (or 200mg/dl) glucose is well above the threshold for the diagnosis of diabetes in humans. This invites speculation that the parasite prefers a growth environment, certainly *in-vitro*, with higher concentrations of glucose. Or even, is this high concentration better for *in-vitro* cultures to avoid the total consume of the glucose in the medium during the usual 24hours that it is in culture? This hypothesis considers that, while in the body the nutrients are always being replenished, *in-vitro* this only happens each 24hours. During this period, if the concentration of glucose is low, it is possible that it is exhausted, thus the parasite is not able to grow.

A more recent *in-vitro* study by Humeida *et al* tested different concentrations of glucose (74). According to this study, the parasite appeared to grow only with concentrations of glucose above 11mM, which is consistent with the theory mentioned earlier. On the other hand, Mancio-Silva et al were able to use concentrations as low as 5mM(78) to maintain growth, results replicated in our study. Because 5mM is the "normal" concentration found in human blood, values close to it could be achieved in culture by increasing the serum concentration to 90%, thus making the addition of glucose to the medium obsolete.

Interestingly, in our study, the parasite appeared to grow as well in 2mM as in 5mM of glucose (figure fifteen), a rather unexpected result considering what has been described before. It raises the intriguing question if the parasite may find nutrients elsewhere.

Of course, the two more obvious sources of nutrients for the parasite are the medium and the RBC. The latter depends crucially on glucose metabolism (pentose-phosphate-pathway) as an energy supply, mainly to maintain membrane integrity. Due to the intra-erythrocytic location of the parasite, the glucose that enters the RBC may serve as a nutrient for the parasite as well. This idea is even more important, as RBC from the blood bank are kept in an anticoagulant solution (CPD, which is very rich in glucose (the D of CPD stands for dextrose). However, the preliminary results performed in our study did not seem to confirm this idea, as cultures with RBC which had been incubated with glucose or CPD for 24 showed a similar growth as the cells isolated one hour after collection (figure sixteen). However, RBC from the blood bank seemed to favour an increased growth than RBC from healthy volunteers, and it could be that other components of the CPD anticoagulant might be responsible for the increased growth. Phosphate figures as a candidate, as it is an important part of the production of energy in the form of adenosine triphosphate and phosphorylation is important in a number of metabolic reactions(145).

With the novel working protocol for *in-vitro* cultures established, it had to be validated using blood from different donors. It was striking that the parasite grew better in the blood from some

donors as compared with others. The rather large variability of adapting parasites in culture was already noted in field studies(146). Rebelo et al noticed four different growth patterns, where the cultures from seven of 46 patients registered no maturation of the parasites, while in 17 cases the parasites were not able to reinvade(146).Thus the difference in the growth curves in our study are likely indicators of some inter-individual variability (figures seventeen and twenty-four). Human volunteers mean a sample from a population of genetically different individuals, with different life styles, background and perhaps even environments.

Certainly, this strongly contrasts with animal studies, especially when using inbred rodents, which is a very homogeneous population. However, studies trying to investigate the effect of a particular factor in a population *in vivo*, are recurring to a genetically heterogeneous population of rodents(147). For example, studies testing the effect of cigarette smoke already follow this strategy. Regarding nutrition, though, it is mostly studied in inbred rodents with the inherent limitations when generalizing results to population level. Hughey *et al*, in a review about studies of glucose homeostasis in rodents, referred studies using different strains of rodents and with different outcomes regarding some aspects, in the respective strains(148). Moreover, how rodents react to a diet may not be very similar to the reaction seen in humans. For example, when rodents are submitted to a high fructose diet, they do not seem to gain weight as was observed in several studies and reviewed by Tran *et al*(149). This is the opposite to what seems to happen in humans(150). While research on nutrition in animal models on malaria may provide important mechanistic insights, it is crucial to study this subject in humans.

2. Impact of nutrition in *Plasmodium* growth

Before analysing the results regarding the possible impact of nutrition on the growth of *P. falciparum* in *in-vitro/ex-vivo* cultures, it was necessary to see if any other factors might have influenced/biased the results, especially considering the possible large inter-individual variation, already discussed above. The factors that could be investigated more thoroughly were: blood type, gender and whether the volunteer donor was, or was not fasting at the time of blood collection.

Some studies observed a higher incidence of malaria in individuals with blood types A, B or AB as opposed to blood type O, as described in a metanalysis by Loscetales *et al* (151, 152). This apparent influence of blood type seems to be related to different receptors in the surface of the RBC that allows a better or worse immune reaction(152). However, this subject is controversial. Degarege *et al* (151), observed the contrary, that the blood type was not likely to be an important factor for the parasite to grow. The results of this study are rather in agreement with the results obtained by Degarege (figure twenty-one). All these were epidemiological studies thus may not correspond to the effect that blood group antigens could have in *in-vitro* cultures. Basco, however, when describing the culture conditions for *in-vitro* drug tests, suggests that the influence of the blood group can be discarded (142). As the present study was conducted *ex-vivo*, this effect may no longer have existed and possibly, did not interfere with the parasite's growth.

Gender could be another possible confounding factor. Studies with animal models refer that males seem to be more susceptible to malaria than females (153, 154). Dkhil realized that this observations could be an immunosuppressive effect of testosterone(154). Lopes *et al* reached the same conclusion when testing the effect of sex hormones on rodent infected with *P. berghei(155)*. Furthermore, the authors also observed a decreased survival rate in castrated female rodents, indicating that female hormones might play some protective role against *Plasmodium.* The described associations in rodents between gender and *Plasmodium* growth were not observed in our sample of humans (figure twenty-two). Certainly, the possible effect that hormones could produce might be different *in vivo* as compared with *ex-vivo*.

The last factor which was investigated was the fasting status (overnight) of the volunteers at the time of blood collection. No significant difference was observed between both groups (figure twenty-three).

Interestingly, none of these three factors showed any significant influence on the results. When comparing the percentage of growth between the cultures in blood before ingestion of the meal (BBC) and blood after ingestion of the meal (BAC) some results were unexpected. While it would be expected that the cultures with BAC registered a higher growth than the ones with BBC, in some cases the opposite was observed. However, it appears that this happened in those cases where the values for the glycaemia at the BAC time point were either very close to the iinitial values or even lower than those seen at the BBC time point (figure twenty-six).

The analysis of the overall results showed a statistically significant difference ($P<0,001$), indicating that that the ingestion of the HCS meal might be causaly related to the increased parasite growth. Although the observed reaction might be explained by other factors of the meal, one key component is likely to be the glucose.

The obtained results point toward the hypothesis that the parasite grows better in an environment with a higher concentration of glucose, with the difference in glycaemia being the greatest factor influencing the results. These observations are consistent with results from previous studies which observed thay glucose is a limiting factor for *P. falciparum (74)* to grow, as well as with the observation, that rodents with higher glycaemia also present higher parasitaemias(78).

Since the sample of donors was mostly composed by females it is relevant to mention the menstrual cycle, which influences the blood composition(156). One of the features changed during the cycle is the tolerance to glucose, which is higher during the sercretory phase than in both proliferative and menstrual phase. Therefore, the small increases and decreases in blood glucose seen after the ingestion of the chocolate might be explained by the fact that these volunteers might have been in either the proliferative or menstrual phases of the cycle and thus had lower glucose tolerance.

Another observation is, that the observed relationship is not linear, and even some volunteers showed decreases in the growth. The exact reasons remain unknwown, but one could specualte that the parasites might grow better in the blood from some donors and worse in the blood of others, casued by additional, yet to be indentified factors.

Considering that the results indicate that the parasites might grow better in the blood of those with higher glycaemia, these results are a great cause for concern. Sugar consumption is increasing in most developing countries, such as the countries in the African continent and Brazil(81). Diabetes is also increasingly more prevalent in these countries(83), which is likely linked to the high intake of sugar. Our study results may indicate that these new dietary habits could have an influence on parasite growth *in vivo* and thus, eventually, indicate a worse prognosis of malaria (1, 157, 158).

Interestingly, the changes in metabolism and blood composition in the volunteers after a meal might go beyond glucose and glycaemia. The intake of the HCS meal triggers a series of reactions in the volunteer's organism, such as hormonal reactions(140). There are also other nutrients in the HCS meal, apart from the glucose, that might be metabolised by the parasite and contribute to its better growth. One such nutrient is fructose, a sugar commonly used in food products as a sweetener (often in the form of high-fructose-corn-syrup) such as the chocolates given to the volunteers. In fact, most chocolates contain sucrose a disaccharide of glucose-fructose.

The metabolism of fructose is different from the metabolism of glucose; therefore the consequences of its ingestion may not be the same. Since the glucometer used could only measure the glucose concentration in the blood, the remaining sugar and carbohydrates are not measured unless they are converted into glucose. Woodrow *et al* observed that *P. falciparum* parasites were able to use fructose as a replacement of glucose as an energy source (159). Thus, the values for the glycaemia might not indicate the true energy supply to the parasite in the blood. Following this line of thought, experiments using each of these sugars were performed in order to investigate growth of *P. falciparum* with these two carbohydrates as energy source.

The most common sugar used by food industry is sucrose, a disaccharide of glucose and fructose(160). The latter two have different metabolic pathways and, therefore, might have different influences in the human body as was thoroughly reviewed by Sun and Empie (161) and is summarized by figure thirty. With this knowledge in mind, the question arises: Could *P. falciparum* use fructose as a relevant energy source?

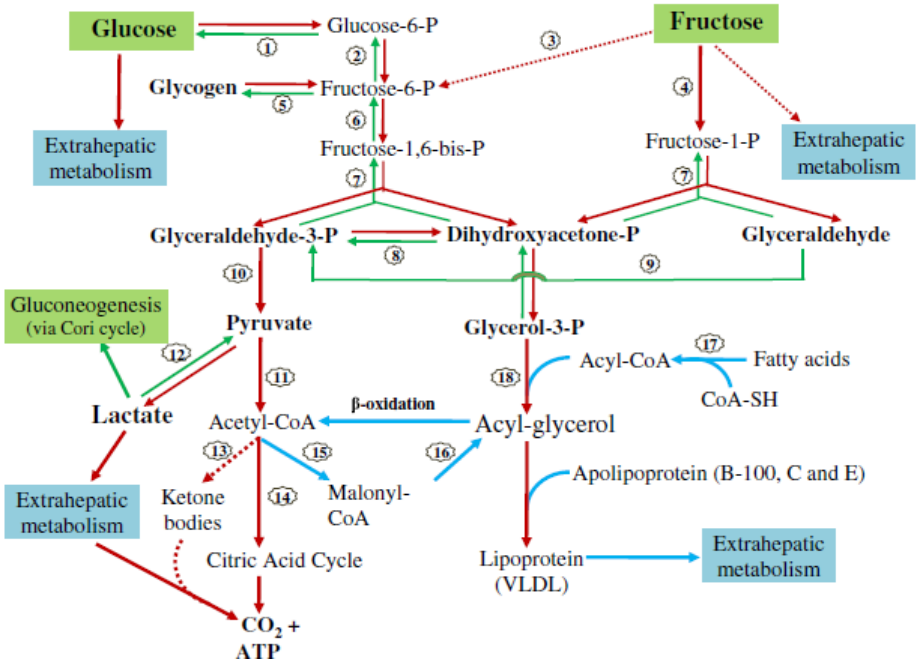


Figure 30 Major metabolic pathways of dietary glucose and fructose.
Metabolic pathways of both glucose and fructose in the human. It is clear that, although the fructose can be converted into glucose, it is mostly metabolised into other pathways.(161)

Woodrow *et al* tried to answer this question and described that *P. falciparum* was able to use fructose as a replacement for glucose(159). When looking into the effect that glucose or fructose *ex-vivo* on *P. falciparum* growth, the results were more similar to the ones described by Geary *et al* and by Woodrow *et al*. In both studies observed growth in cultures incubated with fructose however in slightly lower than the ones incubated with glucose. This observation was more consistent with the results we obtained *ex-vivo* than *in-vivo* (figures twenty-seven and twenty-eight). The apparent similar growth in before glucose (BG) and in before fructose (BF) cultures points towards the parasite's ability to use both glucose and fructose as a carbon source. The difference in the values of glycaemia suggests that only a portion of the fructose was converted into glucose and that the parasite can use the remaining fructose as a carbon source.

Woodrow reported that the parasite stopped growing with a negligible concentration of glucose (0,5 mM) but partially restored its growth with 20 mM of fructose. The growth in fructose was lower than the growth observed with glucose but at a concentration of 40 mM of fructose the growth was completely restored. Interestingly, these results present two major differences from the results obtained in our study, which were:

1. The parasite was able to grow in the culture with no glucose added with a negligible concentration of glucose (0.5 mM).
2. The growth registered for the cultures incubated with fructose was higher than the growth with glucose.

Regarding the first point, the growth registered in the cultures with negligible concentrations of glucose was low and not consistent as observed, for example, in the positive controls. This

might be in line with the previous thought that the parasite might be able to use glucose from other sources, like the one inside the RBC. This concentration of glucose might have been enough to survive for two cycles. This would need further investigation to understand if the parasite uses the glucose inside the RBC and if it is of greater importance than the glucose in the medium or serum.

On the other hand, the best growth was registered in the cultures incubated with a mixture of glucose and fructose. An outcome which might indicate that, while the parasite is able to grow in both glucose and fructose, using both together increases its growth significantly. Since the parasite has both sugars available it can use different metabolic pathways to create energy and thus grows better then with only one of the two carbon sources. This result should be explored further in future studies.

V. Conclusions and Future work

During this study it was possible to create a protocol that allows the *ex-vivo/in-vitro* study of the effects of different conditions, especially effects of nutrition, on the growth of *P. falciparum.* This *ex-vivo*/*in-vitro* approach is likely to mimic in some ways the conditions that the parasite encounters in the blood of humans. One application of this protocol, and, in fact the one that was used in this study, is nutrition. By manipulating the diet of the host (human) and consequently the composition of the nutrients in the blood, it is possible to study the effect that these nutrients might have for the parasite during its growth in *in-vitro* cultures. In this study, the protocol was used to study the effect of a high-carbohydrate meal on the growth of *P. falciparum.*

It was possible to exclude the interfering effects of some confounding factors, such as blood group, gender and the fact some volunteers were fasting. When analysing the effects of the meal provided to the volunteers on the growth of the parasite, the ingestion of high-carbohydrate meals seemed to increase the growth of the parasite. The association between the ingestion of carbohydrates and the growth of the parasite appeared to be related with the glycaemia of the donor, with a tendency that those who had larger increases in glycaemia tended to have a better parasite growth in culture. However, further results show that fructose might also play an important role in parasite growth. Interestingly, preliminary results suggest that fructose can be used as a substitute of glucose in the culture, while the combination of both, glucose and fructose, seem to produce the best parasite growth.

Another important observation was the variability of parasite growth among the volunteers with regard to the glycaemic reaction after the meal. However, the variability was such, that it seems likely, that other host factors may influence (especially impair) parasite growth in the blood from some donors.

There are aspects that need to be investigated further when trying to disentangle the effect which nutrition has on *P. falciparum* growth. The new protocol still has some limitations, considering the way human metabolism works *in-vivo*. In a human, constant metabolism and fed-back mechanisms mean that nutrients in the blood change over a 24hour period. In the culture protocol, however, each culture condition only represented a single time-point, for example the one before and the one after the ingestion of the meal. Of course, one possible solution for this problem would be to increase the blood sampling along the day (for example 4hourly sampling) and substitute the serum in the culture accordingly. However, this is technically demanding apart from the discomfort of the volunteers (let alone their availability to participate in such a study). The presented set-up was thought best to test the hypothesis of a nutritional effect on parasite growth.

Another, rather interesting issue of this study is that we do not really know all nutrients (or constituents) which are increased (or changed) in the volunteer's blood after he/she takes a meal and which of these may be influencing the parasite growth. The food intake can lead to the

production of some metabolites or sub-products that would, in turn, be beneficial (or not) for parasite growth. Certainly, this could be solved with a metabolomic study of the blood before and after the ingestion of the meal. With this approach, it might be possible to compare the blood composition in both conditions.

An interesting, and possibly the most relevant implication of this study is the relationship between glycaemia and the growth of *P. falciparum*: Does the parasite grow better in the blood from diabetic patients? This can be addressed in future studies using blood from diabetic donors. This finding might have some implications for malaria control in Africa, where the number of diabetes case are increasing.

Overall, though the information gathered with this study already provides some understanding of the influence of nutrition on malaria. Further investigations are still needed in order to fully understand what role nutrition plays in malaria. With a better understanding, hopefully, dietary recommendations/guidelines could be produced as a useful part in the fight against malaria.

VI. References

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