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

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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 or to iron. The effect of macronutrients, especially carbohydrates, is hardly explored. Furthermore, existing studies have inherent limitations, such as rodent models.

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.

Background

Malaria is still a serious disease worldwide, that affected, in 2016 alone, 216 million people (1). In the last report from the World Health Organization (WHO), malaria figured as the responsible 16 000 more deaths than the previous year(1), an alarming increase when the objective is towards elimination of the disease. This increase shows that, instead of moving forward on the way of elimination of the disease, it may herald a step backwards. Belying these large numbers of morbidity and mortality, there is a lot of knowledge about malaria and the parasite that causes it, *Plasmodium*.

This parasite has a complex life cycle, composed by a sexual and an asexual stage and in two different hosts, the mosquito and the human(2). When the parasite infects the human, it infects primarily the liver and then migrates to the blood stream where it invades the Red Blood Cells (RBC). Here starts the blood stage where the parasite undergo an asexual cycle. While in the liver there is only a small number of parasites, when the schizont reaches the blood stream the multiplication much faster, as each time that a schizont bursts, at least 16 new RBC are infected. With this high increase in the numer of parasites in the host's body, the symptoms and thus the disease start to manifest only at this stage. In the blood the parasite goes from its earlier ring to the trophozoite form and ultimately the schizont form.

Based on the incidence numbers mentioned previously it would seem that something is happening regarding the control of malaria, lowering the efficacy of measures that have been of the utmost importance in the fight against malaria.

When searching the more recent literature in PubMed, it appears that nutrition is the subject with the lowest number of publications when compared to vaccine, vector control, diagnosis and drugs. Of note, most results obtained with the search terms "Nutrition AND Malaria", were related to iron and malnutrition; while other subjects were very scarce. Understandably, malnutrition was the main focus in the past, more than likely because of wars and prevalent poverty. 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 malnutrition status. 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.

In order to better understand how nutrition can influence the course of malaria it may be helpful to look into the broader picture, such as the role of nutrition on infection in general. That way it may be possible to understand which the most relevant nutrients are, regarding infection, 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.

Without a doubt, a relationship between nutrition and infection exists, as described in a recent textbook(3). Basically, one simple relationship is that nutrition is essential for a good immune response and thus the development of the disease, while many infections, especially more severe ones, may affect the nutritional status of the patients(4).

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 an uncomplicated urinary-tract-infection, but may have a large impact in other infections, such as tuberculosis or malaria.

The underlying hypothesis is that nutrition may influence rather directly the development of pathogens. 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

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

Undernutrition, often associated with PEM, is the most studied nutrition-related subject affecting patients with malaria, mostly because it was almost a common denominator for most malaria patients, apart from being the easiest nutrition deficiency to spot(7). Interestingly, in the past, undernutrition was thought to protect against malaria, a finding that was reported in several studies, although mainly in the older ones(8, 9). However, the analysis of such result must be made carefully, especially in older studies, because in the past the definition of malnutrition was based merely on anthropometrics(10), while nowadays it also takes into account growth, development, chronicity, cause of malnutrition, and the impact of malnutrition (undernutrition) on functional status(10, 11). Nevertheless, recent studies observed that undernutrition was in fact a risk factor for malaria(12, 13). This disparities in the classification of the nutritional status might explain why the results on the effect of malnutrition in malaria are so different.

Some authors suggested that this detrimental effect, which malnutrition appears to have during malaria. 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 caused by severe wasting(14).

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. Robert *et al* observed that obese mice were able to resist better to increased parasitaemia and did not develop cerebral malaria than normal mice(15). 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(16).

In fact, Wyss *et al* published one of the few reports investigating the relationship between obesity and malaria in a Swedish national wide study(17), in which obesity seemed to be related to more severe cases of malaria disease.

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.

Objective

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

Material and Methods

This study was approved by the Ethic Committee from the *Centro Académico de Medicina de Lisboa*.

Blood Collection

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

Meals offered to the volunteers

To test the effect of nutrition in *Plasmodium*'s growth, a high calorie and high sugary meal was offered to all volunteers. These features were chosen due to the fact that the glucose is one, if not the most important nutrient for the parasite to grow, as well for its prevalence in most people's diet. To increase the acceptance from the volunteers, commercially available 50 grams "chocolate bars" were chosen as the meal for the volunteers' ingestion. This meal will further be referred as High Calorie-High Sugar (HCS) meal.

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 in two consecutive days. Both the glucose and the fructose used, were the ones used for domestic purposes.

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 50 healthy volunteers, from a non-endemic country for malaria. To test the effect that the intake the meal might have on parasites growth, the participants had to be submitted to two blood collections and serum glucose measurements. The punctures were performed before and one after the ingestion of a High Calorie-High Sugar (HCS) meal, with 30 minutes between collections.

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

To extract the serum from the blood, it was first let to clot and then centrifuged at 2000g at 8ºC. 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, followed by 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.

Defibrination of blood

The defibrination of the blood was performed by smooth and continuous agitation of 5 ml of blood in an Erlenmeyer flask with the bottom full of glass beads.

Measurement of serum glucose

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

Cultures

Culture maintenance, Giemsa and SYBR green staining were performed as described by Rebelo *et al* (18).

Serum media

The medium with serum was made of RPMI, Hepes buffer, L-glutamine, gentamycin and Sodium hydroxide. 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. The quantities of each constituent are described in table 10.

To the RPMI it is necessary to add hepes buffer In the end, the serum is to be 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 a parasitaemia higher than 0.5% was met. 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 9. As the synchronization leads to a decrease in parasitaemia, non-synchronized cultures were used. The use of synchronized cultures would lead to more time of incubation after the infection of the RBC and before the synchronization. This would, in turn extend the time of culture and may lead to the deterioration of the blood's quality.

The first condition that 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% serum.

To test different types of RBC, blood was collected from the same donor to be anticoagulated by citrate, CPD and 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

Photos of cultures' smear were taken with the software Leica FireCam 3.4.1, from Leica microsystems (Switzerland) Ltd. Afterwards, the parasitaemias were counted using ImageJ 1.49v (Wayne Rasband, National Institutes of Health, USA).

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

When the protocol was established and checked for reproducibility, 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. 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) it was 30 minutes(19, 20).

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. The results on the differences in parasite growth was accessed through the parasitaemia in each culture followed by comparison between conditions.

For the cultures, both RBC and serum from the volunteer were used. The RBC were infected as described before and were not synchronized. For these experiments, medium with 90% serum was used to incubate the cultures. These 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 frosted, warmed only in the day they were put into the culture. The parasitaemia was measured mainly by both flow cytometry and microscopy at 0, 48 and 96 hours.

Results

1. Establishment of the 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. The necessary changes to the protocol are described in table 1.

Table 1 Changes to be made to the standard protocol for continuous 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

a) Confirmation of protocol in different donors

To be able to start to test the influence of the diet on the parasite's growth, the established protocol, with 90% serum and no glucose added, was tested with eight volunteers and compared with the growth in a standard culture medium. It was observed that the parasite was able to grow in all the cultures incubated with 90% serum and defibrinated RBC as established previously. In some cultures, the growth was better than in the one incubated with the standard medium, supplemented with Albumax (figure 1).

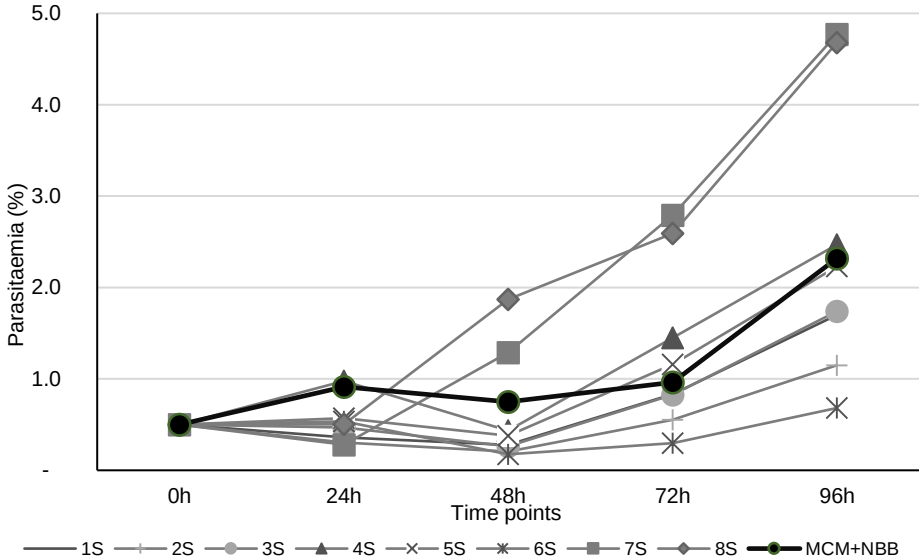

Figure 1 Parasitaemia of *P. falciparum* using the established protocol with eight different donors. Growth curves of cultures incubated with medium supplemented with 90% serum and defibrinated RBC from each eight donors and compared with one culture supplemented with Albumax. 1S – 8S – code number attributed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

Impact of nutrition in *Plasmodium*

growth

Fifty-one volunteeres participated in the study. The results from 16 volunteers had to be removed from the study, because of either absence of growth or a high percentage of unhealthy parasites. It was considered absence of growth when the difference between the final and initial parasitaemias was lower than 0.5% . The sample of participants in the study is described in table 3.

Table 2 Description of the sample according to gender, age and blood type.
N – absolute number of the population; ND – Not determined.

		Total results	Included
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

a) 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 blood from the donors before and after the intake of a HCS meal was different

As it can be observed in the figure two, the bigger the difference between the glicaemia after and before, the bigger is the difference in growth between the respective cultures. The donors whose glycaemia was lower after the ingestion of the meal than if was before, were mainly the ones in which there was a fall in the growth values after the intake of the HCS meal. The higher values follow this trend, occuring when the difference in glicaemia is bigger. 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. There is a significant difference between the growth before the ingestion of the HCS meal and after ($P<0.001$).

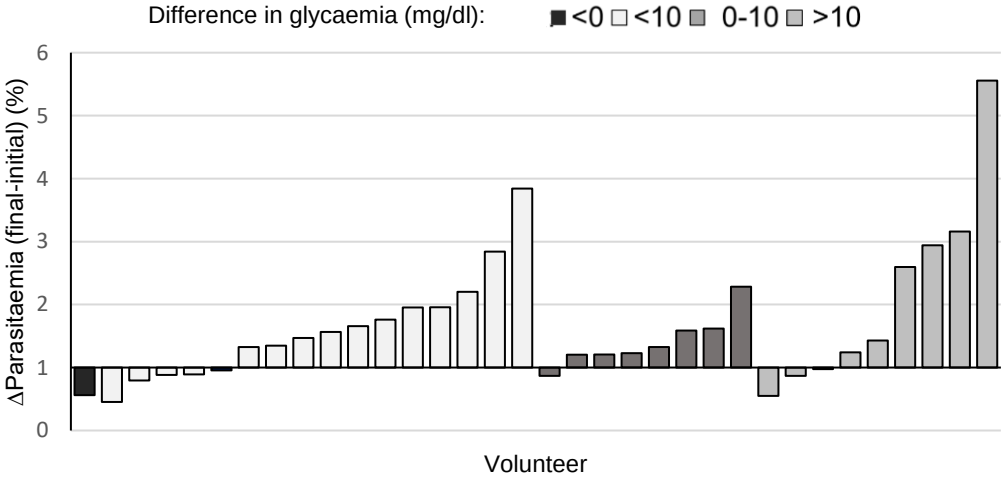

Figure 2 Comparison between growth after and before consumption of HCS meal.
The growth is the difference between the parasitaemia at 96 hours and the initial parasitaemia. The ratio between the growth registered after and before the consume of the HCS meal was calculated. The results are organized in function of the difference between the glycaemia after and before according to classes (less than 0, less than 10, between 0 and 10, and more than 10) as represented.

2.* 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 parasites growth, the question on whether the type of carbohydrate had also 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.

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 three).

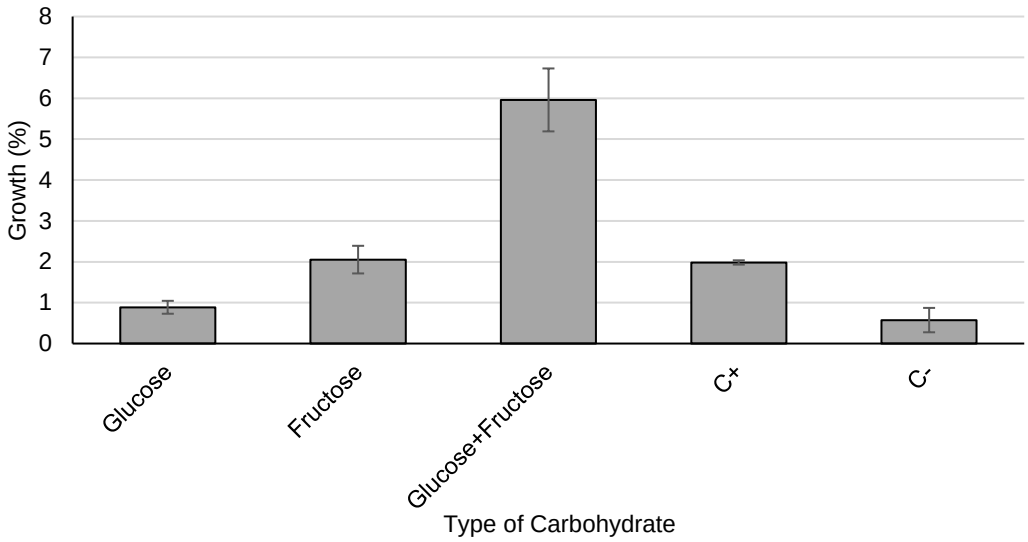

Figure 3 Growth of *P. falciparum in vitro,* according with carbon source and controls.
Cultures of *P. falciparum* were incubated with media with different carbon sources (glucose, fructose and a mixture of both in the same total concentration Positive and negative controls were used, the former with 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 in two consecutive days. The results are represented in figure four, 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.

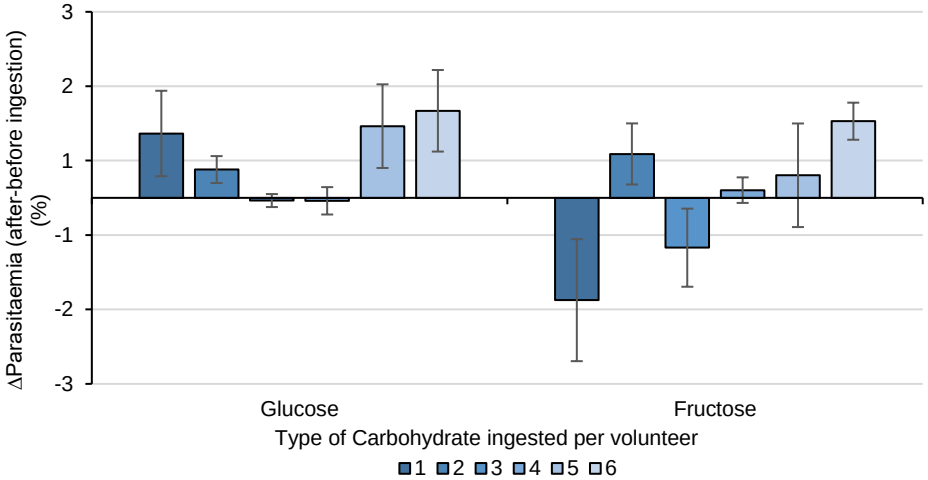

Figure 4 Difference in growth of *P. falciparum ex* vivo, by carbon source in different donors.
Cultures of *P. falciparum* were incubated with media with blood from different donors whom had drank a solution with one carbon source each time (glucose and fructose).

Discussion

This 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* in *ex-vivo*.

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(21).

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. 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%(22). 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 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.

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 on 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 (23). 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(24) 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, 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. RBC 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 incubated with glucose or CPD for 24 showed a similar growth as the cells isolated one hour after collection. 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(25).

With the novel working protocol for in-vitro cultures establihsed, it had to be validated using blood from different donors. The rather large variability of adapting parasites in culture was already noted in field stduies(26). Rebelo et al noticed four different growth patterns in cultures from the blood of 47 patients suffering with malaria(26).Thus the difference in the growth curves in our study are likely indicators of some inter-individual variability. 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(27). 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(32). Basco, however, when describing the culture conditions for in vitro drug tests, suggests that the influence of the blood group can be discarded (33). 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. The described associations in rodents between gender and *Plasmodium* growth(34) were not observed in our sample of humans. 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.

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 ABC time point were either very close to the iinitial values or even lower than those seen at the BBC time point. The analysis of the overall results showed a statistically signifficant 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 (23)* to grow, as well as with the observation, that rodents with higher glycaemia also present higher parasitaemias(24). 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(36). Diabetes is also increasingly more prevalent in these countries(37), 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, 38, 39).

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(19). 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, 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 (40). 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.

Woodrow *et al* described that *P. falciparum* was able to use fructose as a replacement for glucose(40). 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. The apparent similar growth in before glucose (BG) and in (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.

References

1.World Malaria Report. 2017.

2.Farrar J, Hotez P, Junghanss T, Kang G, Lalloo D, White NJ. Manson's Tropical Diseases Elsevier Health Sciences; 2013.

3.Pammi M, Vallejo JG, Abrams SA. Nutrition-infection interactions and impacts on human health: CRC Press; 2014.

4.Hughes DA, Darlington LG, Bendich A. Diet and human immune function: Springer Science & Business Media; 2003.

5.Collins J, Robinson C, Danhof H, Knetsch CW, van Leeuwen HC, Lawley TD, et al. Dietary trehalose enhances virulence of epidemic Clostridium difficile. Nature. 2018;553:291.

6.Higashiyama T. Novel functions and applications of trehalose. Pure and Applied Chemistry2002. p. 1263.

7.Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. The Lancet. 2016;387(10026):1377-96.

8.Genton B, Al-Yaman F, Ginny M, Taraika J, Alpers MP. Relation of anthropometry to malaria morbidity and immunity in Papua New Guinean children. The American Journal of Clinical Nutrition. 1998;68(3):734-41.

9.Charchuk R, Houston S, Hawkes MT. Elevated prevalence of malnutrition and malaria among school-aged children and adolescents in war-ravaged South Sudan. Pathogens and Global Health. 2015;109(8):395-400.

10.Mehta NM, Corkins MR, Lyman B, Malone A, Goday PS, Carney L, et al. Defining pediatric malnutrition: a paradigm shift toward etiology-related definitions. Journal of Parenteral and Enteral Nutrition. 2013;37(4):460-81.

11.White JV, Guenter P, Jensen G, Malone A, Schofield M. Consensus Statement of the Academy of Nutrition and Dietetics/American Society for Parenteral and Enteral Nutrition: Characteristics Recommended for the Identification and Documentation of Adult Malnutrition (Undernutrition). Journal of the Academy of Nutrition and Dietetics. 2012;112(5):730-8.

12.Rice AL, Sacco L, Hyder A, Black RE. Malnutrition as an underlying cause of childhood deaths associated with infectious diseases in developing countries. Bulletin of the World Health Organization. 2000;78(10):1207-21.

13.Ehrhardt S, Burchard GD, Mantel C, Cramer JP, Kaiser S, Kubo M, et al. Malaria, Anemia, and Malnutrition in African Children—Defining Intervention Priorities. The Journal of Infectious Diseases. 2006;194(1):108-14.

14.Shikur B, Deressa W, Lindtjørn B. Association between malaria and malnutrition among children aged under-five years in Adami Tulu District, south-central Ethiopia: a case–control study. BMC Public Health. 2016;16:174.

15.Robert V, Bourgouin C, Depoix D, Thouvenot C, Lombard M-N, Grellier P. Malaria and obesity: obese mice are resistant to cerebral malaria. Malaria J. 2008;7:81-.

16.Visser BJ, de Vries SG, Vingerling R, Gritter M, Kroon D, Aguilar LC, et al. Serum Lipids and Lipoproteins During Uncomplicated Malaria: A Cohort Study in Lambaréné, Gabon. The American Journal of Tropical Medicine and Hygiene. 2017;96(5):1205-14.

17.Wyss K, Wångdahl A, Vesterlund M, Hammar U, Dashti S, Naucler P, et al. Obesity and Diabetes as Risk Factors for Severe Plasmodium falciparum Malaria: Results from a Swedish Nationwide Study. Clinical Infectious Diseases. 2017:cix437.

18.Rebelo M, Sousa C, Shapiro H, Mota M, Grobusch M, Hanscheid T. A Novel Flow Cytometric Hemozoin Detection Assay for Real-Time Sensitivity Testing of Plasmodium falciparum2013. e61606 p.

19.Giugliano D, Ceriello A, Esposito K. Glucose metabolism and hyperglycemia. The American Journal of Clinical Nutrition. 2008;87(1):217S-22S.

20.Tschritter O, Fritsche A, Shirkavand F, Machicao F, Häring H, Stumvoll M. Assessing the Shape of the Glucose Curve During an Oral Glucose Tolerance Test. Diabetes Care. 2003;26(4):1026.

21.Basco LK. Molecular epidemiology of malaria in Cameroon. XV. Experimental studies on serum substitutes and supplements and alternative culture

media for in vitro drug sensitivity assays using fresh isolates of Plasmodium falciparum. Am J Trop Med Hyg. 2003;69(2):168-73.
22.Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976;193(4254):673-5.
23.Humeida H, Pradel G, Stich A, Krawinkel M. The effect of glucose and insulin on in vitro proliferation of Plasmodium falciparum. Journal of Diabetology. 2011;3:6.
24.Mancio-Silva L, Slavic K, Ruivo MTG, Grosso AR, Modrzynska KK, Vera IM, et al. Nutrient sensing modulates malaria parasite virulence. Nature. 2017.
25.Kanaani J, Ginsburg H. Metabolic interconnection between the human malarial parasite Plasmodium falciparum and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase. Journal of Biological Chemistry. 1989;264(6):3194-9.
26.Rebelo M, Tempera C, Fernandes JF, Grobusch MP, Hänscheid T. Assessing anti-malarial drug effects ex vivo using the haemozoin detection assay. Malaria J. 2015;14(1):140.
27.Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzo H, et al. The Development of Emphysema in Cigarette Smoke-exposed Mice Is Strain Dependent. American Journal of Respiratory and Critical Care Medicine. 2004;170(9):974-80.
28.Hughey CC, Wasserman DH, Lee-Young RS, Lantier L. Approach to Assessing Determinants of Glucose Homeostasis in the Conscious Mouse. Mammalian genome : official journal of the International Mammalian Genome Society. 2014;25(0):522-38.
29.Tran LT, Yuen VG, McNeill JH. The fructose-fed rat: a review on the mechanisms of fructose-induced insulin resistance and hypertension. Molecular and Cellular Biochemistry. 2009;332(1):145-59.
30.Basciano H, Federico L, Adeli K. Fructose, insulin resistance, and metabolic dyslipidemia. Nutrition & Metabolism. 2005;2(1):5.
31.Degarege A, Medhin G, Animut A, Legess M, Erko B. Association of ABO blood group and P. falciparum malaria related outcomes: A cross-sectional study in Ethiopia. Acta Tropica. 2012;123(3):164-9.
32.Loscertales MP, Owens S, O'Donnell J, Bunn J, Bosch-Capblanch X, Brabin BJ. ABO Blood Group Phenotypes and Plasmodium falciparum Malaria: Unlocking a Pivotal Mechanism. Advances in Parasitology. 65: Academic Press; 2007. p. 1-50.
33.Basco LK, Heseltine E, Organization WH. Field application of in vitro assays for the sensitivity of human malaria parasites to antimalarial drugs. 2007.
34.Calero-Riestra M, García JT. Sex-dependent differences in avian malaria prevalence and consequences of infections on nestling growth and adult condition in the Tawny pipit, Anthus campestris. Malaria J. 2016;15(1):178.
35.Dkhil MA. estosterone and malaria infection. Austin J Endocrinol Diabetes 2014;1(2):1009.
36.OECD/FAO. OECD-FAO Agricultural Outlook 2016-2025: OECD Publishing.
37.Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. The Lancet. 2016;387(10027):1513-30.
38.Dondorp AM, Desakorn V, Pongtavornpinyo W, Sahassananda D, Silamut K, Chotivanich K, et al. Estimation of the Total Parasite Biomass in Acute Falciparum Malaria from Plasma PfHRP2. PLOS Medicine. 2005;2(8):e204.
39.Kingston HW, Ghose A, Plewes K, Ishioka H, Leopold SJ, Maude RJ, et al. Disease Severity and Effective Parasite Multiplication Rate in Falciparum Malaria. Open Forum Infectious Diseases. 2017;4(4):ofx169-ofx.
40.Woodrow CJ, Burchmore RJ, Krishna S. Hexose permeation pathways in Plasmodium falciparum-infected erythrocytes. P Natl Acad Sci USA. 2000;97(18):9931-6.