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Plasmodium exploitation of host methionine metabolism

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Preface and Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

This Master's dissertation was performed in the Maria Mota's laboratory, from the Instituto de Medicina Molecular (iMM), during the academic year of 2018/2019.

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Abstract

Plasmodium parasites are obligate intracellular eukaryotic organisms, that cause, in humans, one of the most devastating diseases of all times, malaria. Before being released in the blood and cause malaria symptoms, *Plasmodium* parasites infect the liver of their mammalian hosts, a pre-erythrocytic stage that even though clinically silent, allows the parasite to overcome a population bottleneck, by multiplying its population size up to tens of thousands of merozoites. *In vitro P. berghei* infection of hepatoma cells, shows that, when depleting the essential amino acid methionine, the percentage of infected cells is not altered, while parasite development and growth inside hepatocytes is extremely affected. When compensating methionine absence with S-adenosylmethionine (SAM), the major methyl group donor in all living organisms, that results from the enzymatic activity of Methionine Adenosyltransferase 1 (MAT1), parasite development is restored. Furthermore, time course analysis demonstrated that SAM is essential for the parasite during the second half of liver stage infection, when the peak of replication by schizogony occurs. Finally, quantifications of SAM inside the parasite, show that not only SAM levels depend on the exogenous supply but also that, in *wild type* and *Pb*Mat deficient parasites, the measures of this molecule are similar, meaning that *Plasmodium* is relying on the host SAM metabolic pool. Homology search revealed three possible candidates for *Plasmodium* SAM transporter, two of them showing a critical role during liver stage, that might explain the exploitation of host produced SAM. Overall, the results of this work may contribute to elucidate the fundamental role of hepatic methionine metabolism for *Plasmodium* development.

Keywords

Methionine; S-adenosylmethionine; hepatocyte; *Plasmodium*; replication; malaria

Resumo

Parasitas do género *Plasmodium* são organismos eucariotas intracelulares, que causam malária. Antes de serem libertados no sangue e provocar os sintomas da malária, *Plasmodium* infecta o fígado dos seus hospedeiros mamíferos, uma fase pré-eritrocítica, que apesar de sintomaticamente silenciosa, permite aos parasitas ultrapassar um evento de diminuição da população, ao dar origem a dezenas de milhares de merozoítos. Na infeção de células de hepatocarcinoma por *P.berghei*, ao depletar o aminoácido essencial metionina, a percentagem de células infetadas não é alterada, mas o desenvolvimento do parasita é extremamente afetado. Ao compensar a ausência de metionina, com S-adenosilmetionina (SAM), o principal dador de grupos metilo e produto da atividade enzimática da Metionina Adenosiltransferase 1 (MAT1), o desenvolvimento do parasita é reposto. Adicionalmente, análise de intervalos de tempo mostram que SAM é essencial para o parasita, durante a segunda metade da infeção no fígado, quando o pico de replicação por esquizogonia ocorre. Finalmente, quantificações de SAM dentro do parasita, revelam não só que os níveis de SAM são dependentes do fornecimento exterior, mas também que em parasitas *wild type* e *knocked down* para o gene *Pb*Mat, os níveis medidos de SAM são iguais, o que significa que *Plasmodium* está dependente da produção de SAM pelo hospedeiro. Pesquisas por homologia, identificaram três possíveis candidatos para o transportador de SAM em *Plasmodium*, dois deles com funções essenciais durante a infeção no fígado, que podem explicar a exploração de SAM produzido neste órgão e a elevada taxa de replicação durante a infeção por *Plasmodium* em hepatócitos.

Palavras-chave

Metionina; S-adenosilmetionina; hepatócito; *Plasmodium*; replicação; malária

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Abbreviations

ACT	Artemisin-based Combination Therapies	MFR	Major Facilitator Related Superfamily
ATP	Adenosine Triphosphate	MFS	Major Facilitator Superfamily
BSA	Bovine Serum Albumine	MS	Methionine Synthase
CBS	Cystathionine-β-Synthase	MTA	Methylthioadenosine
CGL	Cystathionine-γ-Lyase	MTHF	5-methyltetrahydrofolate
CHO	Chinese Hamster Ovary	NAAT	Neutral Amino Acid Transporters
CSP	Circumsporozoite protein	NEA	Non Essential Amino acids
DMEM	Dulbecco's Modified Eagle's Medium	NPP	New Permeability Pathways
DMEMc	Complete DMEM	NT	Non-Transfected
DNA	Desoxyribonucleic Acid	PBS	Phosphate-Buffered Saline
EEF	Exoerythrocytic Forms	PFA	Paraformaldehyde
ER	Endoplasmatic Reticulum	PV	Parasitophorous Vacuole
EV	Empty Vector	PVM	Parasitophorous Vacuole Membrane
FBS	Fetal Bovine Serum	RBC	Reb Blood Cell
FSC	Forward Scatter	RNA	Ribonucleic Acid
GFP	Green Fluorescent Protein	RPMI	Roswell Park Memorial Institute
GSH	Glutathione	SAH	S-adenosylhomocysteine
HA	Hemagglutinin	SAM	S-adenosylmethionine
Hcy	Homocysteine	SAMS	S-adenosylmethionine synthase
HSPG	Heparan Sulfate Proteoglycan	SNF	Sinefungin
IMC	Inner Membrane Complex	SOB	Super Optimal Broth
kDa	kilo Dalton	SSC	Side Scatter
MAT	Methionine Adenosyltransferase	TMP	Trimethoprim
Mc	Micronemes	WHO	World Health Organization

A. Introduction

1. An ancient killer

Humankind has been known to fight malaria since the Neolitic dwellers and Mesopotamian era (4500 - 3200 B.C), as suggested by the findings of ancient writings in clay tablets and malaria antigens in Egyptian remains. Similarly, in the Vedic period (1500 - 800 B.C) Indian texts mention malaria as “the king of diseases” and later, in 270 B.C, Chinese medical records, refer to symptoms such fevers, enlargement of spleen, headaches and chills, common conditions in malaria, blamed on what was thought to be the action of three demons, supporting the long-lasting battle of men with this fatal disease. The following centuries, would enabled the flourishing of malaria, traveling from Africa, through the Nile, to the Mediterranean, reaching Europe and then the American continent, with the movement of explorers, conquerors and colonists, the New World immigrants and travellers, and mostly slave trades from African countries [1].

More than 2000 years after, malaria is still a predominant high-risk infectious disease, that occurs primarily in tropical and subtropical regions of Africa, Central and South America and Asia (Figure 1), and that accounted for 435 000 deaths in the world in 2017 [2]. However, due to climate, ecology and poverty, sub-Saharan African countries have been pinpointed for 90% of both cases and deaths worldwide [2].

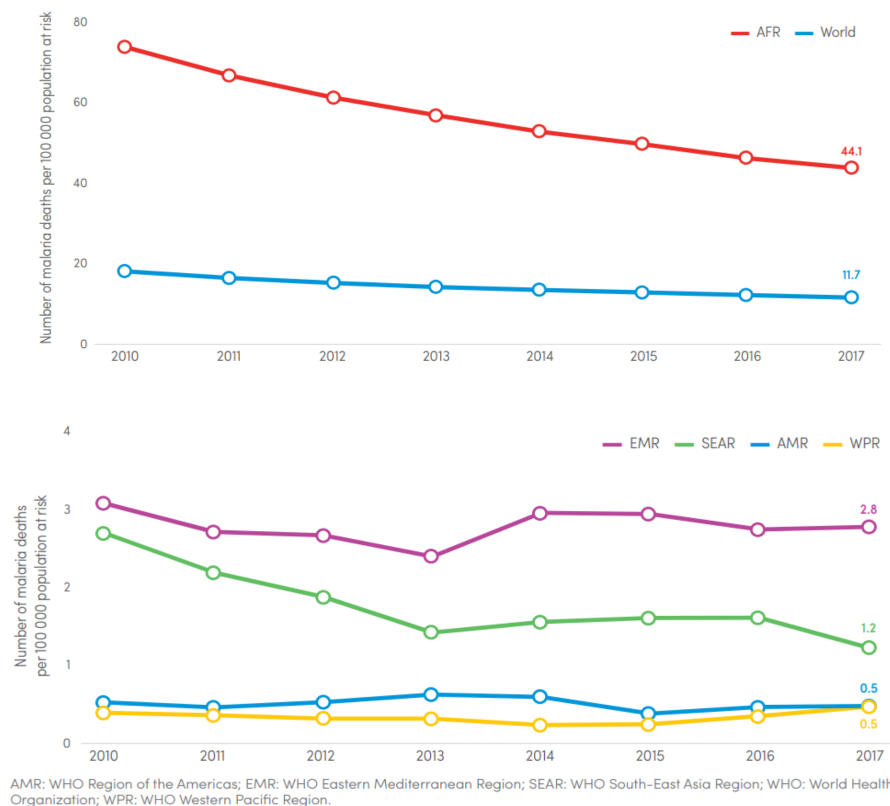

Figure 1 - **Trends in malaria mortality rate:** deaths per 100 000 population at risk, in the WHO regions, in 2010-2017. AFR – WHO African Region; AMR – WHO Region of the Americas; EMR – WHO Eastern Mediterranean Region; SEAR – WHO South-East Asia Region; WPR – WHO Western Pacific Region. (From WHO, World malaria report 2018) .

Malaria's clinical symptoms can be compared, and frequently confused, with a flu-like condition, gastroenteritis, typhoid fever or even dengue, in the sense that the outcome of the disease can be summed up to fever, malaise, headache, myalgias, jaundice and gastrointestinal symptoms of nausea, vomiting and diarrhoea, that can vary according to the stage of disease, ranging from uncomplicated to severe malaria[3]. Alongside with these symptoms, anaemia (characterized as decreased in the number of red blood cells in the blood) is a direct consequence of malaria infection, which aggravates the incidence of the already existing anaemia, as a public health problem in sub Saharan Africa, with significant morbidity and mortality, making malarial severe anaemia the responsible for more than half childhood deaths in Africa [2,4].

While uncomplicated malaria is characterized by a combination of fever, headaches, nausea and general malaise, severe malaria occurs when the infection evolves to organ failures and abnormalities in the blood or metabolism, which can result in cerebral malaria, severe anaemia and consequent haemolysis, acute respiratory distress syndrome (ARDS), acute kidney failure and other medical conditions, that could eventually result in death, if not treated urgently [5].

Even though a decrease of 84 000 deaths has been registered between 2010 and 2016, in 2017 malaria was still responsible for 219 million cases, being 266 000 deaths accountable in children under 5 years old, corresponding to the death of one child every two minutes [2].

2. The parasite

For many years, malaria was thought to be caused by miasmas from swaps. However, with the discovery of bacteria by Antoni van Leeuwenhoek in 1676 and the finding of microorganisms as the cause of infectious diseases, the search for the causative agent of malaria deepened, and it was Charles Louis Alphonse Laveran in 1880 that identified the parasites. This finding was followed by the discovery of the disease carrying vector, the mosquito, first for avian malaria by Ronald Ross in 1897 and later for human malaria, by Italian scientists between 1898 and 1900 [6].

The genus *Plasmodium* comprises five clinically relevant species responsible for infections in human beings: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium malariae*, and *Plasmodium knowlesi*. *P. falciparum* predominates in the African WHO Region and is the deadliest form of human malaria, being accountable for 193.9 million of cases and 618 700 deaths, in 2017. Meanwhile, *P.vivax* is the second most prevalent species, able to undergo dormant stages, termed hypnozoites, which makes it the most widespread malaria parasite and also very difficult to eradicate. *P.vivax* is the predominant parasite in WHO Region of Americas and is also very frequent in the WHO South-East Asia Region. This species was responsible for 14.3 million of cases worldwide, in 2017. *P.ovale* is common in West Africa and some islands in the western Pacific. Like *P.vivax*, *P.ovale* is also able to form hypnozoites, however, unlike *P.vivax*, *P.ovale* can infect individuals who are negative for the Duffy antigen blood group, explaining the greater prevalence of *P.ovale* rather than *P.vivax* in Africa. On the other hand, while *P.malariae* is found worldwide and is the only human infective parasite that has a three-day cycle, named quartan cycle (*P.falciparum*, *P.vivax* and *P.ovale* have a tertian two-day

cycle), *P.knowlesi*, which is the only zoonotic malaria parasite and a natural pathogen of macaques, is particularly frequent in Malaysia [7–9].

2.1. *Plasmodium* lifecycle

Plasmodium parasites are transmitted to humans through the bite of infected female *Anopheles* mosquitoes, which inject sporozoites in the skin that migrate to establish in the liver prior to infecting red blood cells (RBCs) and cause malaria (Figure 2).

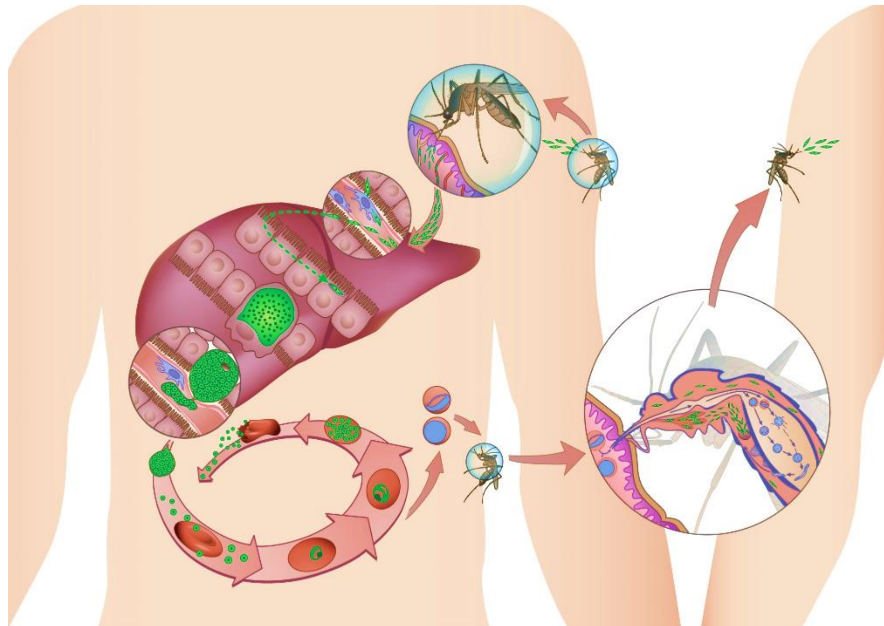

Figure 2 - ***Plasmodium* life cycle** (Portugal *et al.,* 2011).

Since the bite site in the skin, until reaching the liver, sporozoites need to traverse different types of host cells, both in the dermis, such as fibroblasts and phagocytes, and in the liver, such as Kupffer cells and sinusoidal endothelial cells [10]. When in the liver, each sporozoite continues to migrate until it successfully infects one hepatocyte through the formation of a parasitophorous vacuole (PV) inside which the exoerythrocytic form (EEF) develops [11]. The EEFs divide inside the PV into thousands of merozoites, through schizogony (Figure 3 A). The erythrocyte-infective merozoites are then released into the blood stream initiating the erythrocytic stage of the infection [12]. When released in the blood stream, merozoites invade RBCs. In this stage, *Plasmodium* parasites undergo ring, trophozoite and schizont forms, inside the RBC, and replicate to produce between 16 to 32 daughter merozoites, that, when released, can infect other erythrocytes and continue the asexual lifecycle (Figure 3 B). Still in the blood stage, while some merozoites leave the RBC to infect new ones, some intraerythrocytic stages evolve to male or female gametocytes, becoming the sexual forms of the parasite (Figure 3 C). Later, when the mosquito feeds again on the host, it will ingest the gametocytes, that will develop in the insect gut into gametes, to form zygotes. At this point, the parasite's lifecycle is complete, and zygotes will mature to ookinetes and after to oocysts, from which sporozoites will be released and migrate to the mosquito's salivary glands (Figure 3 D), ready to be injected in a new vertebrate host during the next blood meal [13].

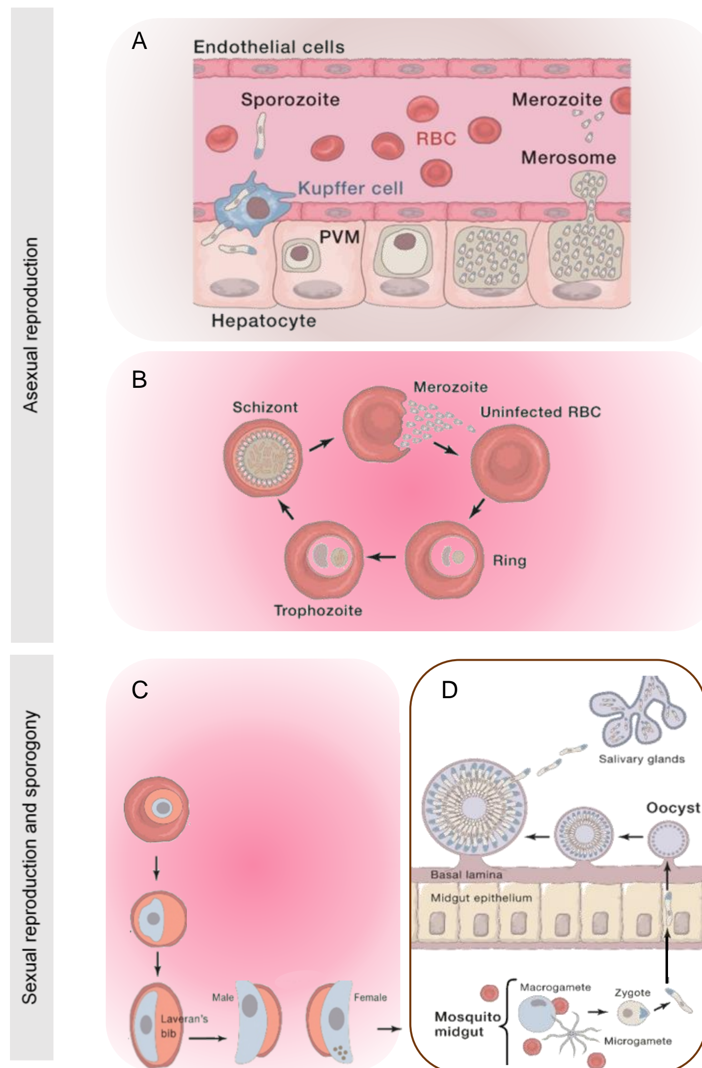

Figure 3 - **Different stages of *Plasmodium* life cycle. A.** Liver stage: *Plasmodium* sporozoites enter in the vasculature and migrate to the liver, where they traverse sinusoidal endothelial cells and Kupffer cells, until invasion of the hepatocyte. When stablished in the hepatocyte, sporozoites form a PV and initiate the division and replication process trough schizogony, to originate tens of thousands of merozoites that will be released in the blood stream trough the "budding" of merosomes; **B.** Blood stage: merozoites released from the liver encounter erythrocytes and start multiples rounds of red blood cells infection and asexual schizogony; **C.** Sexual reproduction: a fraction of merozoites are reprogrammed to differentiate into gametocytes, going through several stages of development, including Laveran's bib stage, which is characterized by visible remains of the infected and distorted RBC, before disappearing completely, to form male and female gametocytes. After gametocytes sequestration and development in the host bone marrow, they will enter the peripheral circulation to be ingested by a mosquito, during its blood meal. **D.**Sporogony: Inside the mosquito midgut, male and female gametes, micro and macrogametes, respectively, will mate by fusion, to forma zygote that will mature to ookinete and oocyst, from which asexual sporogenic replication will occur, forming motile sporozoites. This sporozoites will be released upon oocyst rupture and will migrate to the mosquito salivary glands, where they can be injected again (Adapted from Cowman *et al.*, 2016).

2.2. Apicomplexan heritage

Plasmodium parasites are obligate intracellular organisms, that belong to the Phylum Apicomplexa and order Haemosporida, alongside with *Toxoplasma gondii*, *Cryptosporidium parvum*, *Babesia* and other disease causing endoparasites. The eukaryotic Phylum Apicomplexa is characterized by more than 5000 species of mostly obligate intracellular parasitic protists, that together with two others, Ciliophora and Dinoflagellata form the superphylum Alveolata under the protistan kingdom Chromalveolata (Figure 4) [14–16].

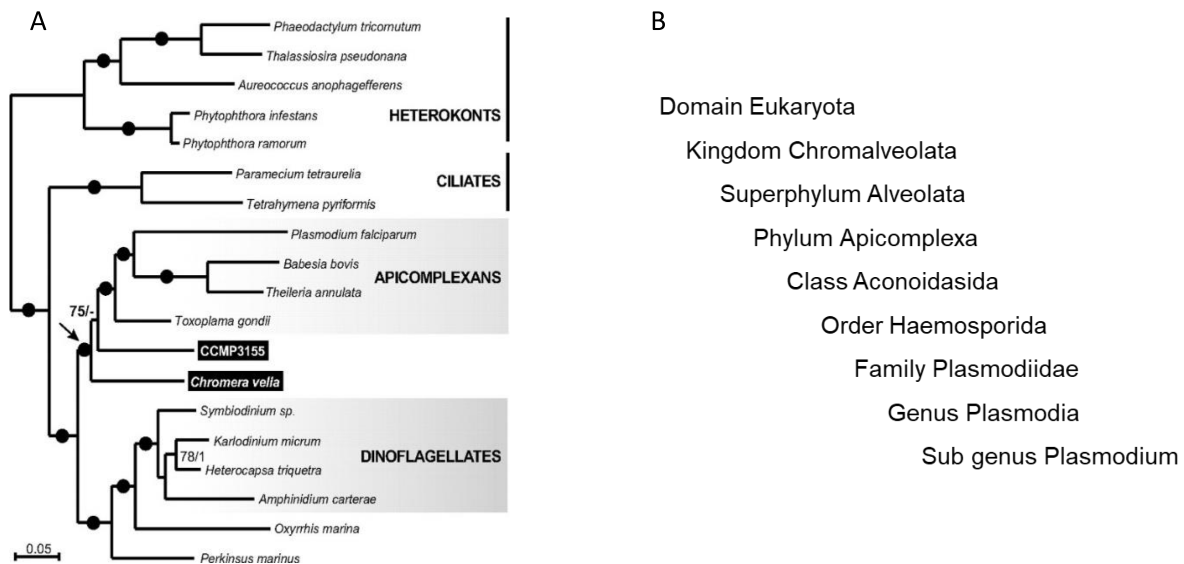

Figure 4 - ***Plasmodium* phylogenetic position and relations** (From Janouškovec *et al.*, 2010).

Apicomplexan (from the two Latin words, *apex* (top) and *complexus* (infolds)) are named after their apical complex of secretory organelles, such as microtubules, polar ring(s), and secretory bodies, that allow these parasites to invade and establish within the host cells [17]. Surprisingly, in 1991, the discovery of a third genome in malaria parasites from a non-photosynthetic plastidic organelle, the apicoplast, besides the mitochondrial and chromosomal genomes, came to revolutionize biology, that at the time firmly believed the only eukaryotic cells with three different genomes were plants and algae, unravelling the “green past” of Apicomplexan parasites [18,19].

As most of its closest Apicomplexa relatives, the sub cellular constitution of *Plasmodium* parasites is a balanced combination of typical eukaryotic organelles combined with the cytoskeletal architecture of an apicomplexan cell. A common organelle to all Apicomplexan is the apicoplast, an unpigmented chloroplast, like plants chloroplasts, that lost its photosynthetic abilities. Although its functions are not conserved among the various apicomplexan parasites, in *Plasmodium* the apicoplast houses pathways for: fatty acid synthesis, necessary for membrane lipid production, during sporozoite development in the mosquito midgut and during the late stage of liver development; isoprenoid synthesis which are precursors of ubiquinones, important for mitochondrion electron transport and formation of glycoproteins; iron-sulphur clusters and haem synthesis, essential during blood stage of the infection [20,21].

Besides harbouring these important metabolic pathways, the apicoplast is also an important "gate" for import of metabolites, such as carbon sources necessary to fuel different metabolic pathways and export the by-products to the cytoplasm, all this crucial for the parasite survival [22].

Apicomplexan organisms are also characterized by a set of apical secretory organelles, localized in the apical end of the parasite and involved in invasion mechanisms: micronemes, rhoptries and dense granules.

In the case of *Plasmodium*, after traversing several hepatocytes, sporozoites switch to the "invasion mode" and establish contact with one particular hepatocyte, through the interaction between parasite and host proteins. These interactions somehow trigger the discharge of micronemes protein content, that will also bind to hepatocyte surface proteins, leading to the formation of a structure named the moving junction, which allow the parasite invasion of the host cell.

Besides the micronemes, the inner membrane complex (IMC), a membranous network of flattened vesicles, is also an important player, that allows the parasite movement into the hepatocyte. The invasion mechanism is preceded by the invagination of the hepatocyte plasma membrane and the release of proteins from the rhoptries, that will contribute for the PV formation. Surrounding the PV will be the parasitophorous vacuole membrane (PVM) of hepatocyte origin, that will be highly modified by the parasite and dense granule proteins, that will be important for the maturation of the PVM [23–25]. These set of secretory organelles is a constant during the life cycle of the parasite, being fundamental not only in the invasion of hepatocytes, but also during the cycle of invasion and re-infection of RBCs and mosquito salivary glands invasion, acting in a specific manner during each life cycle stage [26,27].

2.3. Malaria "bottlenecks"

While cycling between the vertebrate host and the mosquito vector, the size of *Plasmodium* parasite population varies immensely. This may be due to the exposure to the human and mosquito environments, but also to the characteristics of the *Plasmodium* lifecycle (Figure 5).

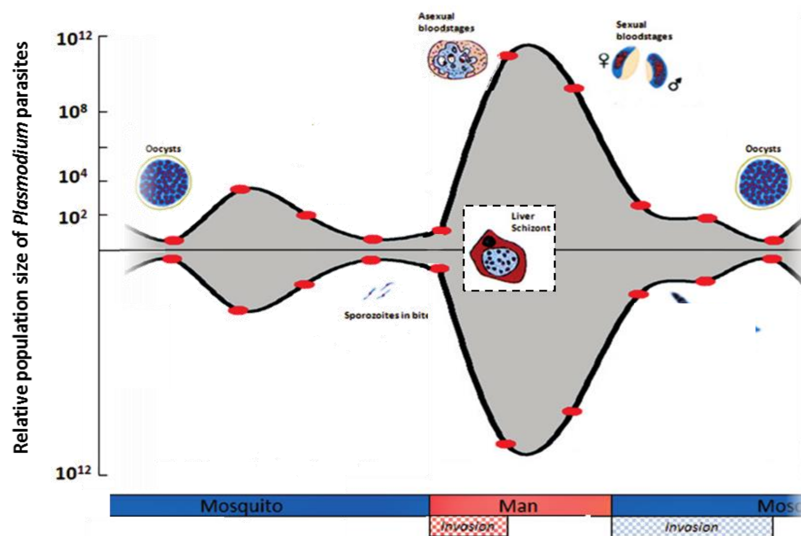

Figure 5 - **"Malaria bottlenecks":** *Plasmodium* population size fluctuations in the different stages of the life cycle, in the human host and the mosquito. (Adapted from Sinden, RE., 2010)

In particular, two population “bottlenecks” occur in the transmission phases:

(1) the transmission of gametocytes from the vertebrate blood to the mosquito midgut;
(2) the transmission of the sporozoites from the mosquito salivary glands into the skin of the vertebrate host.

In order to overcome these bottlenecks, the parasite has developed strategies to increase population numbers upon transmission. Upon ingestion of the gametocytes (haploid cells) by the mosquito and after several events of mitosis, the male gametes fertilize the female gametes, forming the zygotes (diploid cells), inside the mosquito midgut. Zygotes subsequently develop into ookinetes and oocysts, which after 10 or 11 rounds of DNA synthesis and mitosis, generate thousands of new sporozoites, resetting the population of parasites to a sustainable population number[28].

The second critical stage for the parasite’s population maintenance, occurs after the deposition of the sporozoites in the skin of the vertebrates. In mammals, the sporozoites migrate from the skin to the liver and develop inside hepatocytes. Interestingly, inside hepatocytes, not only *Plasmodium* is able to increase the number of parasites necessary to guarantee a successful infection, but also achieves one of the most extraordinary, and high multiplication rates observed in eukaryote organisms [29].

But not all *Plasmodium* species are able to achieve such massive numbers of parasites. Avian and reptile malaria parasites infect macrophages at the bite site of the mosquito bite, and differentiate into only a dozen of new parasites that will proceed to infect host erythrocytes [30]. The remarkable capacity of mammalian malaria parasites to originate thousands of new parasites and its specific tropism for hepatocytes might be highly interrelated, although this hypothesis has not been explored. However, it is interesting to hypothesize that there are mechanisms of host resources exploitation by the parasite, during the liver stage of infection, that help the (low) number of viable parasites that reaches the hepatocytes, to rise up to a sufficient number of merozoites capable of successfully infect RBC and ensuring transmission.

3. Hepatic methionine metabolism

Methionine is characterized as an essential amino acid, as it cannot be synthetized *de novo*, by parasites or animals, including humans. As such, the need of this amino acid must be fully met by the dietary supply. Methionine's crucial role in cell survival and proliferation derives from its input in numerous fundamental biological processes such as protein synthesis, being the first amino acid incorporated in proteins sequence and most methylation-dependent reactions, due to its conversion into S-adenosylmethionine (SAM or AdoMet), a major biological methylating agent.

Methionine adenosyltransferase (MAT) is the enzyme responsible for the conversion of methionine into SAM, using ATP as co-substract. While all tissues express *Mat2a* gene, which encodes for the MATII enzyme, the hepatocytes specifically express the *Mat1a* gene. *Mat1a* encodes for two different isoforms, MATI and MATIII. MAT enzymatic activity is specially enhanced in the liver, due to the activity of the liver MATIII isoform, which unlike the MATI isoform and the extrahepatic MATII enzyme, is able to produce unlimited amounts of SAM, without being inhibited by high levels of its product. Therefore, even though all mammalian cells can synthetize SAM, the major production of this

metabolite happens in hepatocytes, where approximately 50% of all diet supplied methionine is metabolized and most methylation reactions occur [31,32].

SAM is an important sulfonium compound with a sugar moiety and positively charged, which makes it a highly polar (and hydrophilic) molecule. In its native form is an extremely labile molecule, due to its ease of losing its positive charge, when giving away methyl groups to acceptor molecules, a reaction known as transmethylation and catalysed by methyltransferases. Being the major methyl group donor in cells, SAM participates in important methylation reactions such as DNA, RNA and protein methylation, which are crucial not only for the regulation of these molecules expression, but also for phospholipids methylation, that keep membranes and receptors fluid and mobile, and many others [33]. Besides participating in methylation reactions, SAM also sustains other two main pathways: the transsulfuration and aminopropylation pathways.

When losing its methyl groups, due to the enzymatic activity of methyltransferases, SAM is converted into S-adenosylhomocysteine (SAH) and subsequently into homocysteine (Hcy), initiating the transsulfuration pathway. By itself, homocysteine can either be remethylated into methionine, using the methyl group from 5-methyltetrahydrofolate (MTHF), catalysed by methionine synthase (MS), or converted to cystathionine, by a condensation reaction between homocysteine and serine, catalysed by cystathione β-synthase (CBS), that is then cleaved to cysteine, and can be used for protein synthesis or glutathione (GSH) production.

In the aminopropylation pathway, SAM acts as a donor of aminopropyl groups, in a reaction catalysed by propylamine transferase. After decarboxylation, SAM transfers its aminopropyl group to putrescine, allowing the formation of polyamines spermidine and spermine, that are essential for cell growth and proliferation (Figure 6) [33,34].

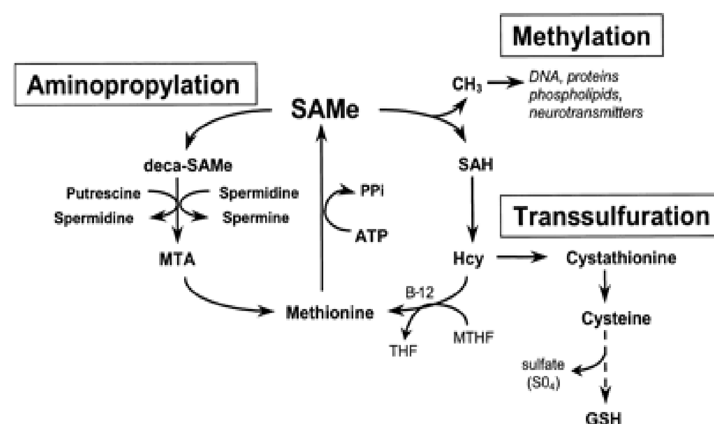

Figure 6 - **Liver methionine and s-adenosylmethionine metabolism**: SAM or SAMe – s-adenosylmethionine; SAH - s-adenosylhomocysteine; Hcy - homocysteine; MTHF - methyltetrahydrofolate; THF - tetrahydrofolate; GSH - glutathione; deca-SAMe - decarboxylated S-adenosyl-L-methionine; MTA - , methylthioadenosine; ATP - adenosine triphosphate; PPi - pyrophosphate. (Bottiglieri, T., 2002)

4. Parasite *vs.* Host

Being an obligatory intracellular organism, *Plasmodium* parasites, scavenge all the nutrients needed such as sugars, nucleosides, vitamins, amino acids, iron and others, from the host, through membrane transport proteins [35].

During replication in the liver stage, the rapid and extraordinary rate at what it occurs, must require the acquisition of many nutrients and metabolites by the parasite. However, little is known about which molecules is the parasite able to explore and the mechanisms employed behind this exploitation. Lipids are essential molecules for the PVM formation and growth, as well as for merozoites membrane neogenesis upon replication in the hepatocyte. *Plasmodium* is known to have some lipids synthesis pathways, however it does not encode for the enzymes needed for the synthesis of important lipids, such as cholesterol, phosphatidylcholine and lipoic acid. To surpass this obstacle, the parasite relies on the abundance of these molecules in hepatocytes and is able to scavenge it for its own benefit [35]. Moreover, small molecules such as glucose, arginine and iron, have also been demonstrated to be essential during exoerythrocytic development and can be obtained by the parasite through exploitation of host plasma membrane transporters, combined with the expression of transporter proteins from the parasite itself [35–38]. Even though methionine supply, is well understood in the blood stage of infection, whether and if the parasite exploits this amino acid metabolism during liver stage, remains poorly understood.

4.1. *Plasmodium* liver stage infection and methionine exploitation

Despite its importance, the bioavailability of SAM in blood and tissues is relatively low (in human plasma, methionine physiological concentration is between 10-40 µmol/l and SAM bioavailability is around 0.150 µmol/ L [39,40]). In the hepatocytes the uptake of SAM is about one third that of methionine. Both these observations are due to the positively charged sulfonium moiety, that not only gives the molecule an extremely polar (and therefore hydrophilic and labile) character but also limits SAM passage through cell membranes [41]. Instead, specifically in hepatocytes, it has been shown that transport of this molecule from the extracellular matrix is extremely low, which might be justified by the fact that, being the main producer of SAM, these cells have no need for extracellular uptake [42].

In the mammalian liver, *Plasmodium* is able to replicate its genome in 10^4-10^5 folds, to become a multinucleated schizont [43]. Such high rate of genome replication implies an efficient DNA methylation, which is not only essential for genome multiplication but also for its repair and regulation at gene expression level. Furthermore, the importance of DNA methylation is also shown in yeast or even bacteria, and is reflected in cell growth, cell cycle arrest and cell division defects with elongated chromatin, when SAM levels fall below an healthy and homeostatic threshold [44,45]. Being the mammalian liver the organ that ensures the major (and unlimited) production of SAM (approximately 6-8g/day [46]), the hepatocyte represents the perfect environment for any obligatory intracellular organism to develop and its extremely efficient methionine metabolism might be the key player that justifies such high rate of replication that is observed during *Plasmodium*'s liver stage of infection.

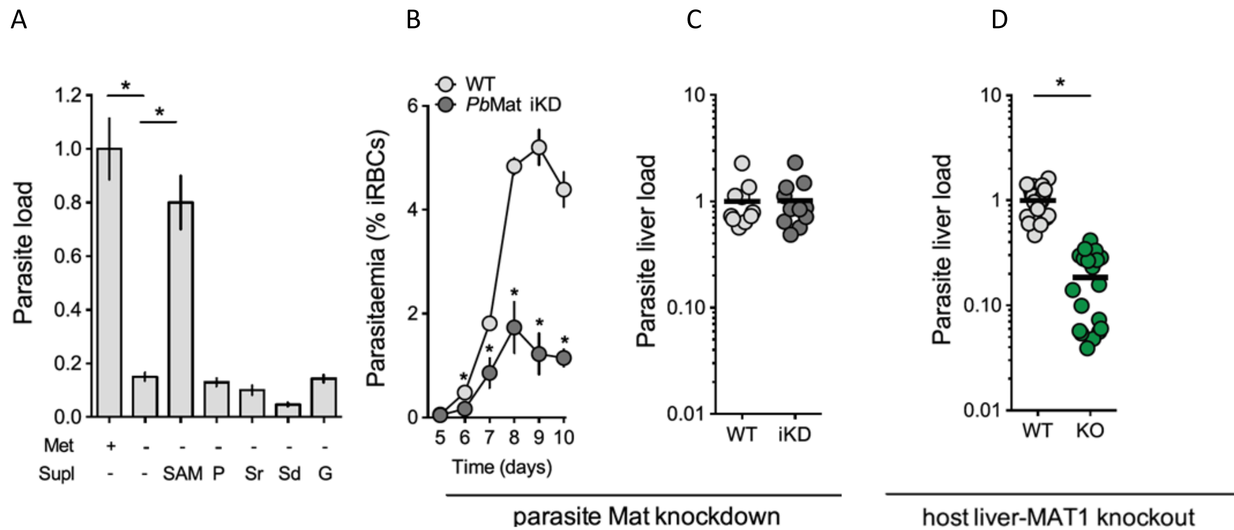

Figure 7 - ***P.berghei* infection is diminished in the absence of methionine produced by the host**. **A.** *P.berghei* infection in HepG2 cells cultured in the presence or absence of methionine (Met) and supplemented with SAM, the polyamines putresceine (P), spermine (Sr) or spermidine (Sd) and glutathione (G), analysed by relative luminescence units of luciferase expressing parasites, at 48hpi. **B.**Parasitaemia (% of infected RBCs) in mice infected with *wild type*(WT) or *Pb*Mat iKD parasites. **C.**Parasite liver load in mice infected with WT and *Pb*Mat iKD parasites, quantified by qRT-PCR of Pb18S rRNA, at 48 hpi. **D.**Parasite liver load in WT and MAT1KO mice infected with *P.berghei*, quantified as in **C**.*, p<0,05 (unpublished results from Maria Mota lab).

Corroborating this hypothesis, previous experiments from the host laboratory, showed that, HepG2 cells infected with firefly luciferase-expressing *P. berghei* sporozoites, present significantly lower parasite load, measured by luciferase activity, when growing in methionine depleted medium. Moreover, upon the administration of the methionine metabolism “downstream products”, SAM, polyamines (putresceine, spermine or spermidine) or glutathione, only SAM can fully revert the effect of methionine depletion (Figure 7 A).

Similarly, to its mammalian host, *Plasmodium* spp. also have a gene coding for MAT enzyme. However, previous experimental data show that, infection of mice with parasites deficient for the SAM synthetase enzyme, is only affected during blood stage (Figure 7 B) and not in liver stage leading to the conclusion that *Plasmodium* MAT enzyme is essential during the erythrocytic stage, but not in the replication inside hepatocytes, where infection load with *wild type* and knockout parasites is the same (Figure 7 C).

Considering that SAM is a crucial metabolite for the parasite’s replication during liver stage, these results suggest that *Plasmodium* does not rely on its own MAT enzyme to synthetize this compound. On the contrary, the host MAT enzyme seems to be essential, as *P.berghei* infection load is strongly reduced in MAT knockout mice (Figure 7 D).

5. S-adenosylmethionine: make it or take it?

Genome sequence analysis data show that almost all living organisms are able (and need) to synthesize S-adenosylmethionine, however, the number of organisms and/or cells that can uptake SAM from the environment remains uncertain. SAM plasma membrane transport has been demonstrated in some organisms, such as *Saccharomyces cerevisiae*, *Rickettsia prowazekki* and some protozoan parasites. In the case of *Rickettsia prowazekii*, the survival is totally dependent on uptake, since these bacteria are unable to produce SAM, due to a mutation in the metK gene, coding for MAT enzyme. Also, in *Chlamydia trachomatis* and *Amoebophilus asiaticus*, the methionine metabolism and methylation cycle are under reductive evolutionary pressure, which lead to the loss of some essential genes, including MAT enzyme [47,48]. For this bacterial intracellular species, having their genomes reduced throughout evolution as endosymbiont organisms, reflected on the incapacity to perform important metabolic reactions, such as conversion of methionine and ATP into SAM. So, not having a SAM synthesis pathway to rely on, results in the absolute need of expressing transport systems that guarantee that these organisms are able to scavenge this metabolite from the host. This data is in agreement with previous observations that most obligate intracellular organisms have been evolving in the way to exploit the host cells metabolic pools, using these transport systems [49].

On the other hand, the kinetoplastids *Leishmania infantum* and *Trypanosoma brucei*, phylogenetically close relatives to *Plasmodium* and the Apicomplexa Phylum, and evolutionary more complex than the bacterial species mentioned before, combine a SAM synthetase enzyme perfectly operable with the expression of plasma membrane transporter proteins used to exploit the host metabolism. While in *Trypanosome brucei*, the identity of this transporter remains unknown, in *Leishmania*, AdoMet T1 has been identified and characterized as SAM plasma membrane transporter [50,51]. In *Toxoplasma gondii*, a SAM plasma membrane transporter has never been characterized, although susceptibility to sinefungin, a structural analogue of SAM, was associated with a mutation in a putative amino acid transporter [52].

Given the phylogenetically close relation between *Plasmodium* and the previous mentioned parasites, and the importance of host provided SAM for the successful replication of *Plasmodium*, inside hepatocytes, there are enough evidences to hypothesize that malaria parasites also encode for a SAM transporter protein.

6. Aims

Considering all the literature and the preliminary data introduced above, and given the importance of SAM in *Plasmodium* replication inside the hepatocytes, the main aims of this thesis are to:

(1) characterize *Plasmodium* time dependency on host SAM;

(2) identify the *Plasmodium* SAM plasma membrane transporter;

The elucidation of these aims will contribute to the ultimate goal of clarifying the mechanism behind the exploitation of host produced SAM, that might represent a new anti-malarial target for liver stage, directing this knowledge to shape and provide new therapeutic options for this disease.

B. Materials and Methods

1. Ethics statement

All *in vivo* protocols were approved by the internal animal care committee of Instituto de Medicina Molecular and were performed according to the national and European regulations.

2. Parasites and mosquitoes

For this thesis three *Plasmodium berghei* lines were used: *Pb*_GFP_{CON}, that constitutively expresses GFP (259cl2) and *Pb*_GFP-Luc_{CON} that constitutively expresses a GFP-Luciferase fusion protein (676m1cl1) both under the control of ef1αa promotor, kindly provided by the laboratory of Chris J. Janse, were used for *in vitro* hepatoma cells infection. A parasite line deficient for *Pb*Mat enzyme (Pb SAMS-DD-HA) generated in the laboratory, was also used for the quantification of SAM levels, that expresses the MAT enzyme in fusion with a destabilizing domain (DD), that is stabilized in the presence of trimethoprim (TMP). *Anopheles stephensi* mosquitoes were obtained from the breeding facility at IMM. Mosquitoes were kept at 20ºC, 80% relative humidity and fed *ad libitum* with 10% glucose supplemented with 2% paraminobenzoic acid (PABA).

2.1. Mosquito salivary glands dissection

Sporozoites used in *in vitro* infections of hepatoma HepG2 cells were obtained through dissection of infected salivary glands, during the period of 21 to 28 days after *A.stephensi* female mosquito infection. Salivary glands were dissected in Dulbecco's Modified Eagle's Medium (DMEM). Sporozoite number was accessed using a Neubaeur cell counting chamber, by light microscopy.

3. Cell lines

HepG2 (human hepatoma) and Hek293T (human embryonic kidney) cells were cultured at 37°C with 5% of CO_2 in DMEM supplemented with 1% of glutamine and penicillin–streptomycin (PenStrep) mixture and 10% fecal bovine serum (FBS) (complete DMEM, DMEMc). CHO (chinese hamster ovary) cells were also maintained at 37°C with 5% of CO_2, in complete DMEMc supplemented with 1% of non-essential amino acids (NEA).

4. *In vitro P.berghei* infection and luminescence measurement

Hepatic infection of luciferase-expressing *P. berghei* parasites was assessed in HepG2 cells by measuring the luminescence. HepG2 cells were seeded in 96-well plate (5 x 10^4 per well) the day before infection, in DMEM. At the day of infection, freshly dissected firefly luciferase-expressing *P. berghei* sporozoites were added to the cells, followed by a centrifugation step at 3000xg for 5 min. Medium was replaced 2 hours post infection (hpi) to RPMI without methionine, containing 1 % PenStrep, 1 % Glutamine, 10 % FBS, 1:300 Fungizone, 1:1000 Gentamicin and 200 µM cysteine (Sigma-Aldrich®). Different concentrations of methionine and/or SAM were added according with the experimental design. Cells were maintained in a 5% CO_2 humidified incubator at 37 ^oC. At 48 hpi., parasite infection load was measured by bioluminescense assay using a multiplate reader Infinite M200 Tecan, and cell viability was measured by Cell Titer-Blue assay (Promega®).

5. Quantification of *P. berghei* infection and development by flow cytometry

For flow cytometry analysis of infection with GFP-expressing *P. berghei* parasites, HepG2 cells were seeded in 24-well plate the day before infection. Infection with sporozoites was performed as described above. Complete medium was replaced 2 hpi, for the appropriate medium containing different concentrations of methionine and SAM, throughout the next 46 hours. At 48 hpi, cells were trypsinized and collected for flow cytometry analysis on BD Accuri C6. Infection rate and intracellular parasite development was measured by determining the number of GFP positive cells and the geometric mean of intensity of the GFP signal. Data analysis was performed using FlowJo software, that enabled the selection of a population of cells with certain physical properties, measured by two optical detectors: the forward scatter (FSC), allowing for the discrimination of cells by size, and the side scatter (SSC) that provided information regarding the complexity of the cell, allowing for the differenciation of fit and metabolically active cells from dead cells and debries. After selecting the population of healthy cells, the population of cells expressing GFP, which correspond to cells infected with GFP-expressing *P.berghei*, was selected based on the pattern of GFP emission (FL1-A), derived from the incidence of an excitation laser (488nm).

6. Search for orthologues to AdoMet T1 and *Tg*Transp in *Plasmodium* genome

AdoMet T1 (accession number LINF_100008900-T1) and TGVAND_290860 (that for the sake of clarity will be referred to as *Tg*Transp) protein sequences were obtained from the *Leishmania* and *Toxoplasma* genome database (TriTryDB and ToxoDB, respectively) and aligned with *Plasmodium* genome in PlasmoDB using the bio-informatic tool BLAST, for homology search.

7. Cloning of *Plasmodium* putative transporter protein sequences in mammalian expression vector

Codon-optimized for mammalian expression, versions of the sequences of *Toxoplasma gondii* transporter TGVAND_290860 and *Plasmodium falciparum* candidate SAM transporter proteins, LH1, MFR5 and MFS6 synthesized by GenScript, were digested from the initial carrying vectors (pUC57) with Bgl-II and Not I - HF restriction endonucleases (New England Biolabs®). Digestion reactions were run in an agarose gel, and inserts were excised from the gel and purified using Qiagen's QIAquick Gel Extraction Kit. The mammalian expression vector pCDNA3 was linearized with BamHI-HF and Not I-HF restriction endonucleases (New England Biolabs®) and purified using Qiagen's QIAquick PCR Purification kit. Ligation of the inserts resulting from the digestion and the purified pCDNA3 mammalian expression vector was performed using T4 ligase enzyme (New England Biolabs®). Ligation mixtures containing insert and vector were used to transform *Escherichia coli* XL-10 gold strain ultracompetent cells at 5% v/v. Briefly, the cells and DNA mixture were maintained on ice for 30 minutes, followed by heat shock at 42ºC for 45 s and then 2 min again on ice. 500 µL of room temperature SOC medium (Super Optimal Broth - SOB - supplemented with 20 mM glucose) was added to transformation cocktail and incubated at 37ºC, 200 rpm for 1 hour. Transformed bacteria were then plated on LB Agar plates

supplemented with Ampicilin (1:1000), incubated overnight at 37ºC and colony DNA extracted using NZYMiniprep kits.

8. Mammalian cell transfection

The final vectors containing the cloned transporter sequences were transfected into Hek293T, HepG2 or CHO cells, using FuGENE® HD Transfection Reagent protocol, following specific manufacturer instructions. Cells were plated in 24 well-plate and transfected on the same day. DNA of the recombinant vectors was diluted in Opti-Mem I reduced serum medium, and the mixture was added to appropriate amount of FuGENE® HD Transfection Reagent to a 3:1 (FuGENE transfection reagent:DNA) ratio, and incubated for 15 min. The FuGENE® HD Transfection Reagent and DNA mixture was added to the cells, and incubated for 48 hours, in a 5% CO_2 humidified incubator at 37 ºC.

9. [^{3}H]-SAM uptake assay

Transport assay in transfected Hek293T, CHO and HepG2 cells, was performed 48 hours post transfection. The [^{3}H]-SAM (specific activity of 13.8 Ci/mmol, Perkin Elmer®) transport was initiated by the addition of 145 mM [^{3}H] SAM in RPMI (without methionine), for 30 minutes, at 37ºC. Transport was stopped by washing cells twice with ice cold Stop buffer (137mM NaCl and 10mM HEPES, pH 7,4). Cells were lysed with lysis buffer (100 mM NaOH and 0,1% SDS) at 37ºC for 15 minutes and radioactivity was assayed by liquid scintillation upon addition of scintillant solution to the cell lysate. Intracellular accumulation of [^{3}H]-SAM was measured using MicroBet Trilux, PerkinElmer®. Protein concentration was determined using Bio-Rad Bradford Reagent, and the amount of intracellular [^{3}H]-SAM was normalized to total protein content.

10. Immunofluorescent assay (IFA)

10.1. SAM quantification

HepG2 cells were seeded on coverslips or 96-well black imaging plates, infected with GFP-expressing *P.berghei* parasites, maintained in specific medium conditions and fixed at different time points. Cells were fixed with paraformaldehyde (PFA) 4% at room temperature for 10 min, followed by 3 times wash in 1x PBS, permeabilized with 0.1% v/v Triton X-100 in PBS for 10 min and blocked with 2% w/v bovine serum albumin (BSA) in 1x PBS for 30 minutes. Cells were incubated with Alexa Fluor 647 anti-SAM antibody (2 uL/mL), DAPI and Alexa Fluor 488 anti-GFP (1:400), for 2 hours at room temperature, followed by 3 washes in 1x PBS. Samples were mounted with Fluoromount-G™ (from Southern Biotech) and dried overnight before imaging. Images were obtained by fluorescence microscopy using Zeiss Cell Observer Microscope.

10.2. Protein cellular localization studies

Immunofluorescence assay for protein cellular localization of transfected (Hek293T/CHO/ HepG2) cells, was performed 48 hours post transfection. Cells were seeded in 96-well plate and transfected with the different HA-tagged transporter constructs. 48 hours post transfection, cells were fixed with 4% PFA for 10 minutes at room temperature, washed 3 times with 1x PBS, incubated with 0.1% v/v Triton X-100 permeabilization solution for 10 minutes room temperature and 2% w/v BSA in 1x PBS blocking solution

for 30 min at room temperature. After permeabilizing and blocking (as previously explained), cells were incubated with a rabbit anti-HA primary antibody (1:300, from Cell Signaling) in 2% BSA, for 1 hour at room temperature. After primary antibody incubation, cells were washed 3 times with 1x PBS, followed by incubation with donkey anti-rabbit conjugated to Alexa Fluor 488 (1:400, from Thermo Fisher Scientific), for HA-tagged protein staining and DAPI (1:1000, from Sigma) for nuclear staining, in 2% BSA for 1 hour at RT. Samples were mounted with Fluoromount-G™ (from Southern Biotech) and dried overnight before imaging. Images were obtained by fluorescence microscopy using LSM-710 Zeiss Microscope.

10.3. Analysis of *P. berghei* infection by microscopy

HepG2 cells were seeded on glass cover slips in 24-well plates and infected, the following day, with GFP-expressing *P. berghei* parasites. 48 hours post infection, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature, washed 3 times with 1x PBS, incubated with 0.1% v/v Triton X-100 permeabilization solution for 10 minutes and 2% w/v bovine serum albumin (BSA) in 1x PBS blocking solution, for 30 min at room temperature. Afterwards, infected cells were stained with an anti-UIS4 antibody (dilution 1:1000) for 1 hour at room temperature, followed by 3 washes with 1x PBS. Cells were then incubated in a 1:1000 dilution of Donkey anti-Goat Alexa Fluor 568, anti-GFP Alexa Fluor 488 and DAPI for nuclei staining, in blocking solution. Following 3 washes with PBS, coverslips were mounted on microscope slides with Fluoromount-G™ (from Southern Biotech) and dried overnight before imaging. Images were obtained by fluorescence microscopy using LSM-710 Zeiss Microscope.

11. Western blot analysis

Mammalian transfected cells were lysed in NP-40 buffer with benzonase (1:500) and proteinase inhibitor (1x) for 10 minutes, on ice. After lysis, protein samples were diluted in 5x SDS sample buffer (NZYTech), denatured at 56ºC for 10 min and resolved in an 10/12% polyacrylamide gel. Afterwards, proteins were blotted in a nitrocellulose membrane, trough wet transfer at 200 mA for 2 hours. Membrane blocking was made with 5% BSA in 0,05% PBST (PBS-Tween 20) for 1 hour in agitation. Primary antibody Rabbit anti-HA (1:1000) was incubated overnight at 4°C. Next day, membrane was rinsed in 0,05% PBST and after incubated with secondary antibody Goat anti Rabbit HRP (1:5000), at room temperature for 45 min. Protein bands were observed post addition of Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore®) to the membrane, on the ChemiDoc XRS+ Gel Imaging System (Bio-Rad®).

12. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 software. Statistically significant differences involving more than two conditions were analyzed using one-way ANOVA. Statistical comparison between two groups was performed using the Mann-Whitney test. For both tests, statistical meaningful differences were scored as * for p<0,05, ** for p<0,01, *** for p<0,001 and **** for p<0,0001. Non-significant differences were represented as *ns*.

C. Results and Discussion

1. *Plasmodium* dependency on host liver methionine metabolism

When comparing reptile and avian malaria parasites' lifecycles with that of mammalian-infectious *Plasmodium* parasites, the differential factor is the type of cells the different parasites infect in the pre-erythrocytic stage of the lifecycle. Since mammalian-infectious *Plasmodium* species, like *P.berghei* or *P.falciparum*, are the only ones able to generate thousands of new parasites, we hypothesize that the liver is supplying these malaria parasites with specific nutrients, to fuel the massive replication that generates the high number of merozoites that are released in the blood stream and cause malaria.

One of the unique features of the mammalian liver is the methionine metabolism. To access how this complex and dynamic pathway influences the outcome of *Plasmodium* liver infection, we used an *in vitro* system to mimic liver stage of infection: hepatoma cell line (HepG2) infected with either GFP- or firefly luciferase-expressing *P.berghei* parasites. The cultures were kept under different concentrations of methionine and/or SAM, the two major key players in the methionine metabolism that are object of our study to access not only the importance of these two metabolites for the parasite replications inside the hepatocyte, but also identify in which specific periods of the infection are these molecules essential.

1.1. Administration of SAM rescues the impairment in *Plasmodium* infection, caused by methionine deficiency

Previous experimental data from our laboratory have showed that, in the context of a deficient methionine supply, whether by administration of a methionine deficient diet to mice or reduction in the methionine concentration of hepatocytes medium, infection with *P.berghei* parasites is significantly reduced. To replicate the impaired infection phenotype upon methionine depletion, hepatoma HepG2 cells were infected with firefly luciferase-expressing *P.berghei* sporozoites.

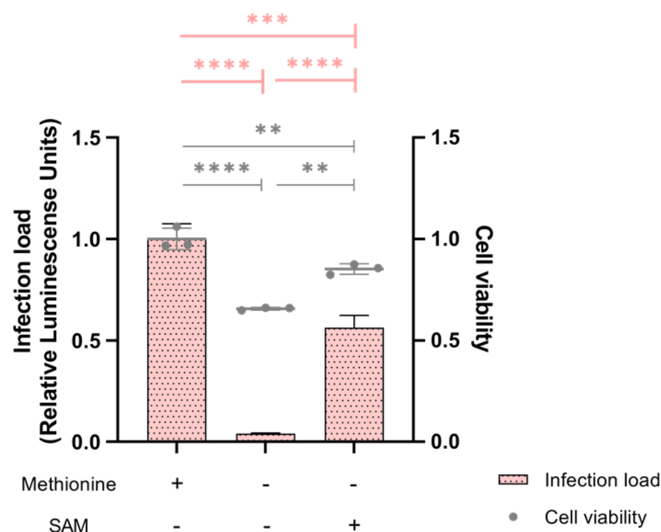

Figure 8 - **In the absence of methionine, SAM reverts the impairment in *Plasmodium* infection load.** Cell viability and *P.berghei* infection load in HepG2 cells cultured in the presence or absence of methionine and supplemented with SAM between 2 and 48 hpi, were assessed at 48 hpi. N=1 One way ANOVA test. ** p<0,01, *** p<0,001, **** p<0,0001

Two hours post infection, cell medium was exchanged to mimic the conditions of methionine deficiency (10 μM) and methionine sufficiency (100 μM) followed by incubation for 46 hours at 37ºC. A third experimental condition was also employed, where methionine deficient medium was supplemented with SAM (500 μM).

At 48 hpi, luciferase activity measurement showed that depletion of methionine from the culture medium led to a 96% decrease in parasite load, when compared to that of the methionine sufficient medium as previously demonstrated. Moreover, when compensating methionine deficiency with the supplementation of its derivative SAM, parasite load is restored to 60% of the total infection load, observed in methionine sufficient condition. Cell viability in this experiment was also slightly affected upon methionine deprivation (30%) (Figure 8).

Considering the decrease in infection resulting from methionine depletion, it was necessary to determine whether the deficiency in this amino acid was affecting parasite survival or its intra-hepatic growth and development.

While the luciferase reporter assay is useful to quickly assess infection load in the context of cells infected with luciferase-expressing *P.berghei* parasites, it does not allow for a quantitative (number of infected cells) nor qualitative (the size of the parasite, resulting from the amount of merozoites contained in a multinucleated schizont) analysis of infection. Flow cytometry analysis, on the other hand, is a technique applied for single-cell analysis, which allows not only the identification of different subpopulations of cells (infected cells from non-infected cells, in the same heterologous cell sample), but also to infer about cell size and relative expression of the reporter genes (parasite size in each one of the selected cells) (Figure 9).

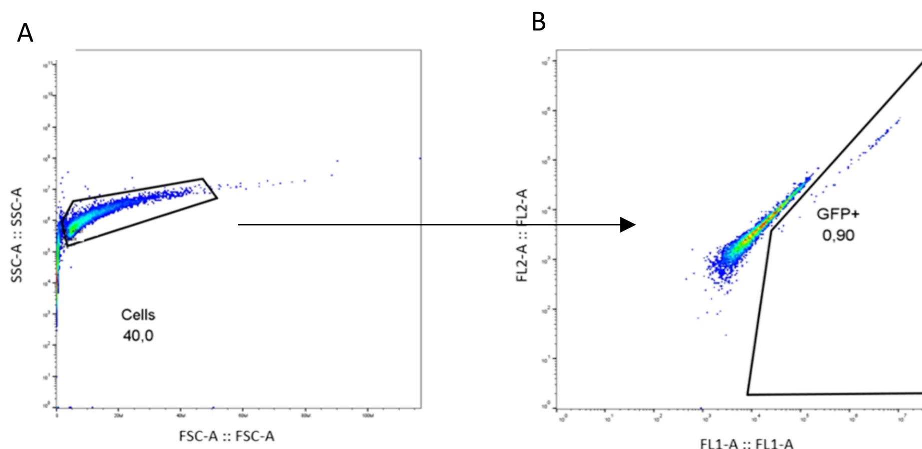

Figure 9 - **Gating strategy** used during the acquisition and analyses of flow cytometry data, to define **A.** live cells and **B**. infected (GFP positive) cells within the live cells gate.

As such, to understand exactly how methionine depletion is affecting infection, HepG2 cells were infected with GFP-expressing *P.berghei* sporozoites, exposed to the same experimental setup and

culture medium conditions as mentioned before, and at 48 hpi, the samples were analysed by flow cytometry .

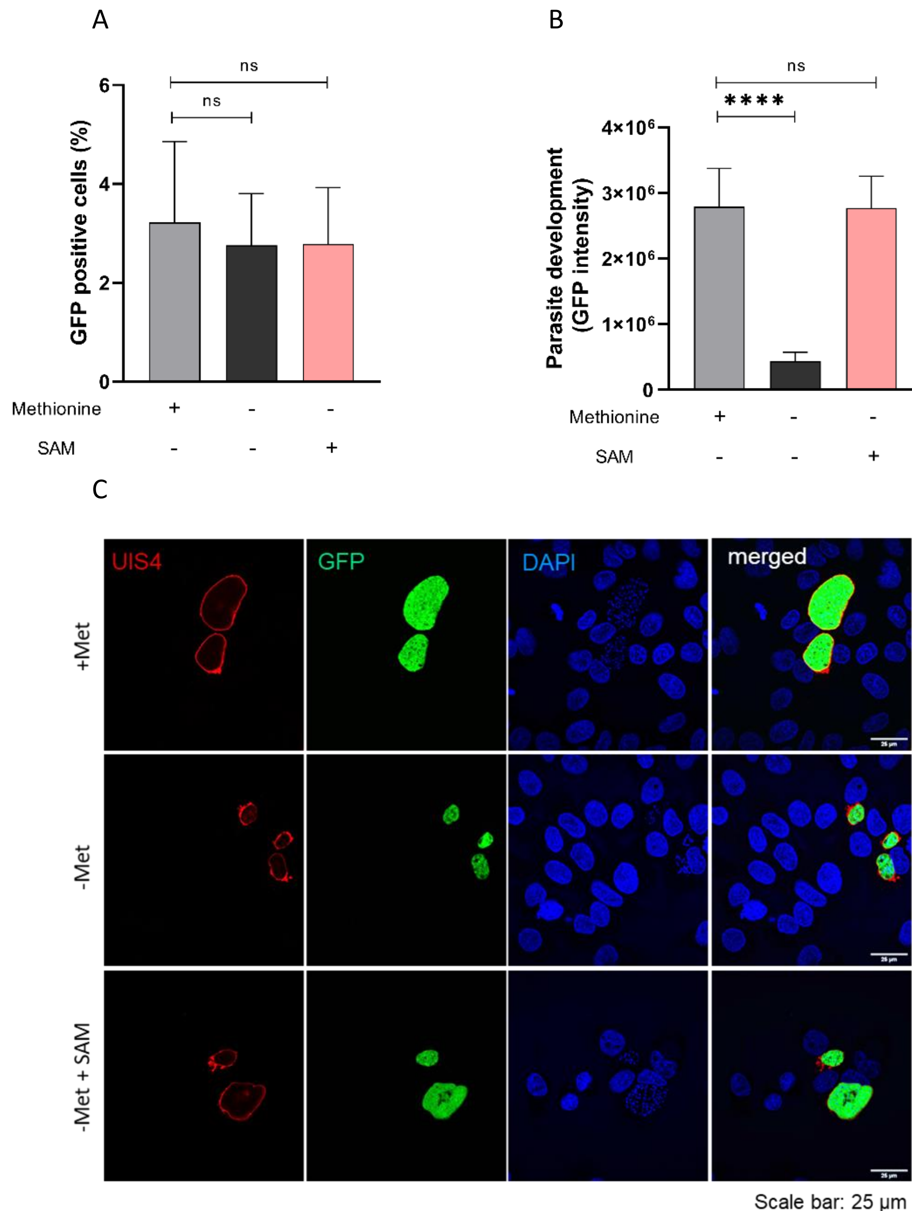

Figure 10 - ***Plasmodium* infection impairment in the absence of methionine, is reflected in the parasite replication and not in the number of infected cells.** HepG2 cells infected with GFP-expressing *P. berghei* sporozoites and maintained in the mentioned cell culture medium between 2 and 48 hpi. **A.** Number of infected cells and **B.** parasite development assessed by flow cytometry at 48 hpi. Both graphs: Mann-Whitney test. ns – not significant and **** p<0,0001. N=5 **C.** Immunofluorescence analysis of infected cells fixed with PFA at 48 hpi. Confocal images of liver stage parasites, stained with an anti-UIS4 antibody to detect the parasite's PVM (red), an anti-GFP to detect the parasites (green) and DAPI for nuclei staining (blue). Scale bar – 25 µm.

The analysis of the frequency of infected cells (GFP positive cells) showed that the number of infected cells was approximately the same between the three conditions (Figure 10 A). However, when accessing parasite development, inferred from the geometric mean of GFP intensity values which is directly proportional to intrahepatic growth and replication, it was evident that upon methionine depletion parasite development was reduced (Figure 10 B).

Importantly, the compensation of the methionine depletion with the addition of SAM, reestored parasite development to levels similar to the ones observed in methionine sufficient condition (Figure 10 B). The same results were corroborated by immunofluorescense microscopy analysis of these same conditions (Figure 10 C). These observations confirm that the defficiency in methionine affects, not the survival of the parasite inside cells, but EEF's development within the hepatocytes. Most importantly, this impairment in parasite growth, can be reverted by the administration of methionine's metabolite, SAM, which enables parasites development.

1.2. SAM is essential during the replication stage of *Plasmodium* liver infection

The completion of *Plasmodium berghei* development inside hepatocytes takes approximately 48 - 56 hours, and results in the release of erythrocyte-infectious merozoites into the blood stream. In the *in vitro* system, for the first two hours of infection, the parasite travels through layers of different cells, before invading the final host cell. Since hepatocyte invasion until approximately 16-20 hpi, *Plasmodium* maintains a single nucleus, that undergoes a transformation process in which it starts by rounding up and initiate DNA replication, a process that is accompanied by a growth and branching of both the mitochondrion and apicoplast [53]. It has been shown that by 18 hpi, the parasite has already started replicating its DNA to form the multinucleated schizont that is observed at the end of the liver stage infection[54]. However, it's not only until the last 20-24 hours of infection that the parasite starts to replicate, at a very fast and efficient multiplication rate (Figure 11) [53].

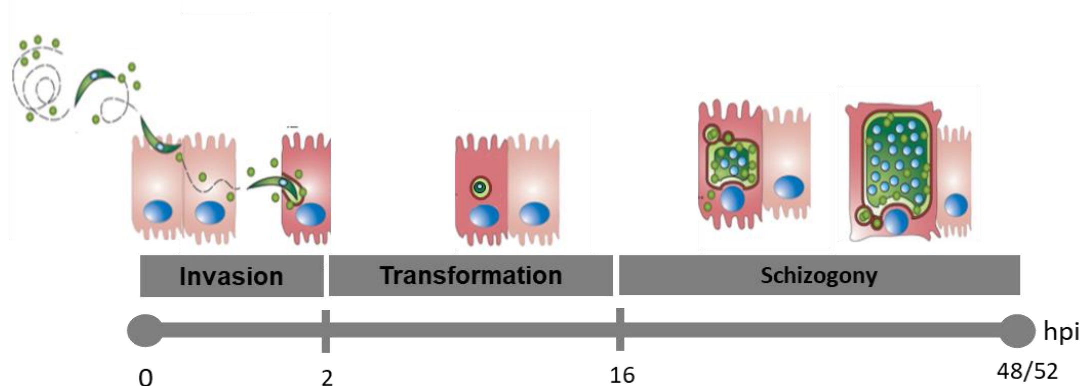

Figure 11 - **Different stages of *P.berghei* development inside the hepatocyte** (Adapted from Rennenberg, A. *et al.*, 2010).

Having in mind that SAM, appears to be critical for a successful intrahepatic development, it was necessary to establish at what specific time of infection this metabolite was being utilized by the parasite, seeking to determine for what specific parasite developmental processes was this compound required. To access this question, HepG2 cells were infected with GFP-expressing *P.berghei* sporozoites and maintained in methionine sufficient or methionine deficient medium, and supplemented or not with SAM for different periods and analysed at 48hpi by flow cytometry.

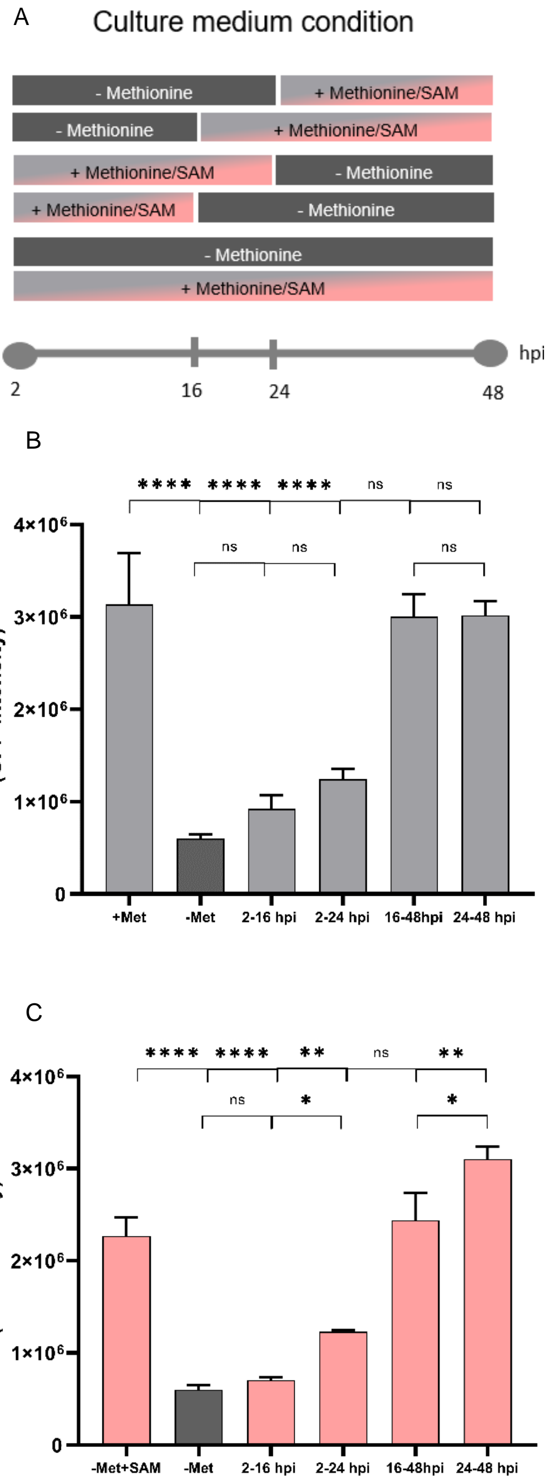

Figure 12 - **SAM is essential for *Plasmodium* replication inside the hepatocyte, in the late stage of liver infection**. **A.** Different medium conditions for the different timepoints. HepG2 cells infected with GFP-expressing *P. berghei* sporozoites in **B.** the presence of methionine and **C.** absence of methionine supplemented with SAM, during different time periods. When not incubated with methionine or SAM, infected cells were kept in medium depleted of methionine. Parasite development was measured at 48 hpi, for all conditions, by flow cytometry. N=1. One way ANOVA test: * p<0,05, ** p<0,01, *** p<0,001, ****p<0,0001 and ns - not significant.

Even though it is stated that DNA replication in *Plasmodium* infection has already started at 18hpi [54], preliminary results from our laboratory, using a new tool that enables the detection of DNA replication by fluorescense analysis, have shown that in fact, between 14 -16 hpi, the parasite has already started to perform events of mitosis. As such, we analysed the effect of methionine depletion and SAM supplementation in different intervals post-infection: 2-16hpi; 2-24 hpi; 16-48hpi and 24-48 hpi; in order to determin whether there is an association between methionine and specially SAM with the initiation of DNA replication.

As previously observed, methionine depletion results in the impairment of parasite development. When methionine is provided solely in the beginning of infection (2-16 hpi and 2-24 hpi) parasite development is also significantly lower, however when methionine is provided solely during the replicative phase (16-48hpi and 24-48hpi) parasite development is similar to the control (Figure 12 B). Importantly, the growth profile of parasites analysed is similar when SAM is provided in substitution of methionine (Figure 12 C). When analysing the timepoints 2-16 hpi and 2-24 hpi we observe an increase in parasite development, between the two time periods. The differences observed between 16 and 24 hpi are in line with unpublished evidences from our laboratory that *Plasmodium* starts to replicate its DNA at 16 - 24 hours. These observations also suggest that these metabolites are exerting their functions during the second half of hepatocyte infection, likely during replication and suggest that SAM essenciality during liver stage migh be related to DNA and parasite replication.

Moreover, when comparing the parasite development upon supplementation of SAM, between 16-48hpi and 24-48hpi, it is again evidenced that SAM might be the key player for such an extraordinary multiplication rate, because it is able to boost *Plasmodium* development to levels higher than the control.

1.3. SAM levels inside the parasite depend on exogenous (host) supply

Being SAM a molecule of major importance among all living organisms, its presence can be detected in all types of cells. At 16 hpi parasite DNA is already replicating and at 48 hpi the merozoites containing schizonts are almost fully grown and completely mature. We have previously established that SAM is essential for replication, so in order to understand if this molecule is being uptaken from the host or produced by the parasite it self, we employed an immunofluorescent analysis, to quantify SAM levels inside the EEFs using a specific anti-SAM antibody (Figure 13 A). For this, *P. berghei* infected hepatoma cells, growing in different conditions, were fixed at different time points and processed for staining and microscopy analysis. Quantification of signal intensities was performed on widefield fluorescence Zeiss Cell Observer microscopy images using Fiji software.

The mean intensity values of SAM inside parasites growing under three experimental conditions of methionine sufficiency, methionine depletion or methionine depletion with SAM, were analysed and normalized per parasite area. The analysis was performed at a timepoint of infection (24 hpi) in which, parasite size is similar between the three conditions.

When accessing parasite size, in infected cells growing in medium with or without methionine, fixed at 24 hpi, it is evident that, size does not change, independently of the medium condition (Figure 13 B). This is likely, because replication has not yet achieve its peak and subtle differences cannot be detected.

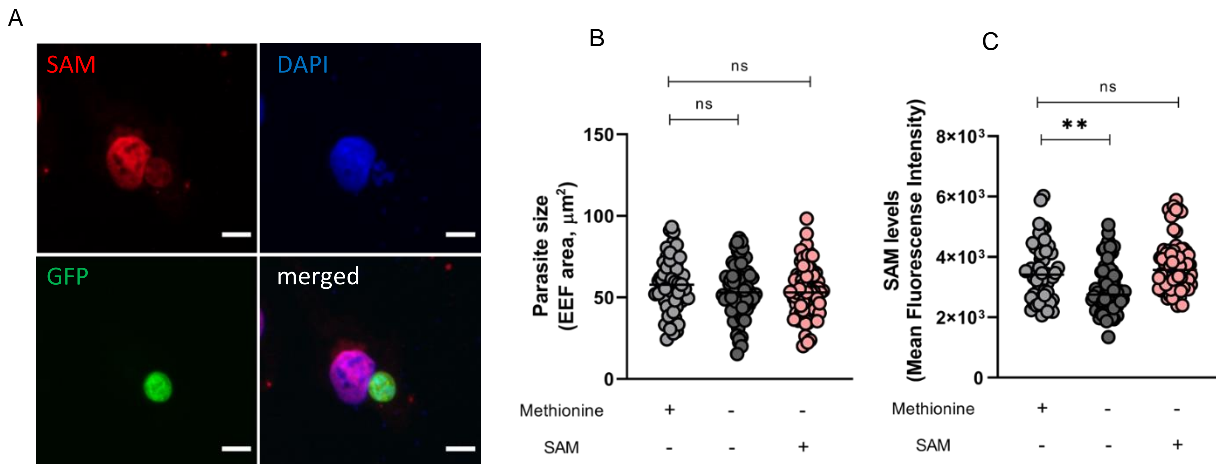

Figure 13 - **SAM levels inside EEF's. A.** Representative image of the immunofluorescense assay, used for quantification of SAM levels. Scale bar 10 µm. **B.** Quantification of the parasite size and **C.** SAM levels inside the EEF's, at 24 hpi measured by immunofluorescense microscopy, for methionine sufficient, methionine depletion and methionine depletion with SAM administration. Both graphs: Mann Whitney test. ns-not significant and ** p<0,01.N=1; +Met, n=54; -Met, n=73; -Met+SAM, n= 71 (representative of 3 independent experiments).

However, when comparing SAM levels quantified in the three conditions, it is evident that, in a medium depleted for methionine, the amount of SAM inside the parasite is lower. The decresed levels of SAM that are verified in methionine deficiency medium, are restored when we administer SAM to the medium (Figure 13 C).

Next, we measured SAM levels in a parasite line deficient for its SAM synthetase enzyme (previoulsy mentioned in Figure 7 as *Pb*Mat iKD). This SAMS inducible knockdown parasite line express the SAMS enzyme in fusion with a destabilizing domain that is stabilized in the presence of the small

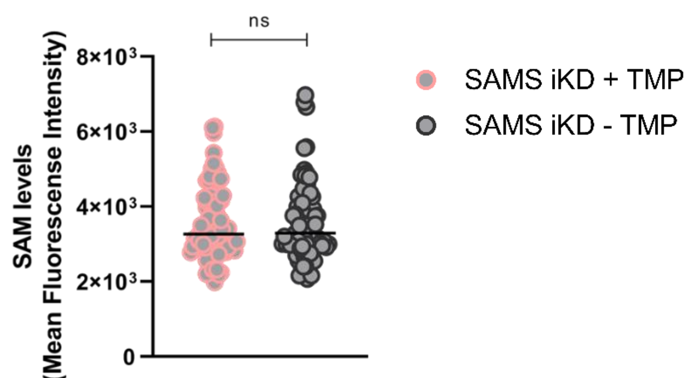

Figure 14 - **SAM levels are the same in wild type and SAMS inducible knockdown parasites.** Quantification of SAM levels inside a SAMS iKD with TMP (phenocopies a wild type) and without TMP (phenocopies a knockout parasite for SAMS ,at 24 hpi, in methionine sufficient condition measured by immunofluorescense microscopy. Both graphs: Mann-Whitney test. ns-not significant. N=1. SAMS iKD + TMP, n=91; SAMS iKD – TMP, n=85.

molecule trimethoprim (TMP)[55,56]. Parasites growing in the presence of TMP phenocopy a *wild type* parasite, with normal levels of SAMS enzyme, and consequently capable of producing SAM. However, in the absence of TMP the parasites don't have SAMS enzyme and therefore cannot produce SAM. The quantification of the levels of SAM in SAMS-WT and SAMS-KD parasites show no differences (Figure 14).

Given that SAMS-KD *P.berghei* parasites cannot synthesize SAM these results suggest that SAM is imported from the host. The evidence that the parasite depends on the exogenous supply of SAM raises the possibility that *Plasmodium* encodes for a SAM transporter that enables the exploitation of this host metabolite by the parasite.

2. *Plasmodium* SAM transporter

2.1. Orthologues in *Plasmodium* genome with homology to known SAM plasma membrane transporters

2.1.1. LH1: a *Leishmania* look alike

Due to its biological input in important metabolic pathways, all organisms have either a SAM producing enzyme, a transporter or carrier that uptakes this compound from the extracellular or intracellular space, respectively, or both systems, that can ensure an efficient supply of this molecule.

While organisms such as some bacterial strains of the genus *Rickettsia* or the fungus *Pneumocystis carinii*, that lack the gene or the activity of a MAT enzyme, responsible for the production of SAM, express transporters that can meet their SAM needs, others that are capable of expressing this enzyme do not need to rely on exogenous supply of this metabolite[57,58]. Other organisms, like most of the fungus and protozoa, have the ability to both synthesize and transport SAM. *Leishmania* spp., an intracellular kinetoplastid, phylogenetically close to Apicomplexan parasites, encodes for a SAM producing enzyme, but also encode for a SAM transporter, AdoMet T1. This high affinity plasma membrane transporter belongs to the family of folate biopterin transporters (FBT), a class of membrane proteins that fits in the major facilitator superfamily (MFS) and is present in most kinetoplastids and Apicomplexan parasites, such as *Toxoplasma* and *Plasmodium* [50].

Bearing in mind the close phylogenetic relation between kinetoplastids, like *Leishmania* and Apicomplexan, like *Plasmodium*, the search for sequences in *Plasmodium* genome with homology to the identified *Leishmania* AdoMet T1 transporter, was the first strategy employed in order to try to identify SAM transporter in malaria parasites.

```
> PF3D7_0828600.1-p1 | transcript=PF3D7_0828600.1 | gene=PF3D7_0828600
| organism=Plasmodium_falciparum_3D7 | gene_product=folate
transporter 1 | transcript_product=folate transporter
1 | location=Pf3D7_08_v3:1229899-1232132(-) | protein_length=505
| sequence_SO=chromosome | SO=protein_coding | is_pseudo=false
Length=505

 Score = 44.3 bits (103),  Expect = 0.001, Method: Compositional matrix adjust.
 Identities = 25/96 (26%), Positives = 46/96 (48%), Gaps = 1/96 (1%)

Query  479  AGLAGVSAFNCIFSKRSYRLTFCVTTFAQVLGGMTDIVIVKRWNLYIGIPDHAMYIWGAA  538
            A    + ++   F+K   R     +T       +  +V++K+ N ++ IP+   +I
Sbjct  307  ASFISIISYMLFFTKIDIRKLLLYSTIIITPFCLLPLVVIKKVNYFLFIPNTLFFITDTV  366

Query  539  VVSEVCYMLGYMPMVVLLSRLCPRGSESVVYALMAG  574
            ++ E       MP++VL SRL P G ES +Y+L+
Sbjct  367  LI-EFIAEFQTMPILVLCSRLIPEGFESTIYSLLLS  401
```

Figure 15 - ***Pf*FT1 folate transporter is *Plasmodium falciparum*'s protein sequence with the highest homology score, to *Leishmania* infantum's AdoMet T1 SAM plasma membrane transporter.** AdoMet T1 protein sequence was aligned with *Plasmodium* genome, using BLAST protein tool alignment.

When aligning AdoMet T1 protein sequence with *Plasmodium* genome, the first hit that resulted from the BLAST tool output, was *Pf*FT1 (accession number PF3D7_0828600), a membrane transporter protein responsible for folate transport and folate salvage in *P. falciparum* (Figure 15). Being already a known, characterized and function associated membrane protein, we excluded the hypothesis of this being a possible candidate for SAM transporter in *Plasmodium*. However, alongside with *Pf*FT1, a previous study on folate salvage in malaria parasites, indicated two other potential folate protein transporters, *Pf*FT2 and an unknown protein with accession number PF3D7_1022200. Thus, when

looking for sequence signatures of the MFS family, in these two other protein sequences, the authors concluded that even though *Pf*FT2 was also a potential folate transporter like *Pf*FT1, for the PF3D7_1022200 unknown protein, were found similarities with the MFS protein members, but not any evidences that this membrane transporter would belong to the biopterin folate transporters family [59].

Another study on the PF3D7_1022200 unknown protein, (here named LH1) excluded LH1 as a potential folate transporter in *Plasmodium* due to its divergence from other known folate transporter proteins. Though, considering some of the actually existing similarities of this protein with members of the MFS family, like *Leishmania* AdoMet T1 transporter, we identified LH1 as being the first possible candidate for *Plasmodium* SAM transporter.

2.1.2. MFR5: homology meets phenotype

A SAM transporter has also been described in the yeast, *Saccharomyces cerevisiae. Sam3* is a plasma membrane transporter protein that belongs to the amino acid permease superfamily. Interestingly, in yeast this transporter is not only responsible for SAM transport, but is also able to uptake sinefungin, a SAM structural analog and an antimicrobial agent, which mode of action is based on the inhibition of methyltransferases that receive the methyl groups from SAM, by competing with SAM for the binding site [60]. Sinefungin resistant yeast strains were shown to have loss of function in *SAM3* membrane protein [61]. A common transporter protein for sinefungin and SAM, has also been reported in *Leishmania* (AdoMet T1 membrane protein) and in *Trypanosoma brucei* (unknown protein) [50,51].

In the *Plasmodium*'s closest relative *Toxoplasma gondii*, resistance to sinefungin was associated with a single nucleotide polymorphism (SNP) mutation in a putative amino acid transporter, although this transporter has never been formally characterized as SAM transporter [52]. Being *Toxoplasma*, a closer relative to the malaria parasite, we sought to determine whether the *Toxoplasma gondii* TGVAND_290860 membrane protein, linked to sinefungin susceptibility, was also able to uptake SAM, and in that case use this sequence to look for putative SAM transporters within the *Plasmodium* genome.

We started by subcloning the sequence of the TGVAND_290860 gene, optimized by GenScript for mammalian expression, in a specific mammalian expression vector pCDNA3 (Figure 16 A). We transfected Hek293T cells with the pcDNA3 construct and performed a transport assay using radioactive labeled [^{3}H]-SAM molecule. The transport assay (Figure 16 B) shows that *T.gondii* membrane protein is able to transport SAM, when comparing to the controls, non-transfected cells (NT) and cells transfected with the empty mammalian vector pCDNA3 (EV). The transport of radioactive labeled methionine in non-transfected cells was used as a control for the technique. These results show that similarly to what happens in other organisms such as *S.cerevisiae*, *Leishmania infantum* and

Trypanosoma brucei, Toxoplasma gondii also encodes for a plasma membrane transporter, that is responsible for the transport of both SAM and sinefungin.

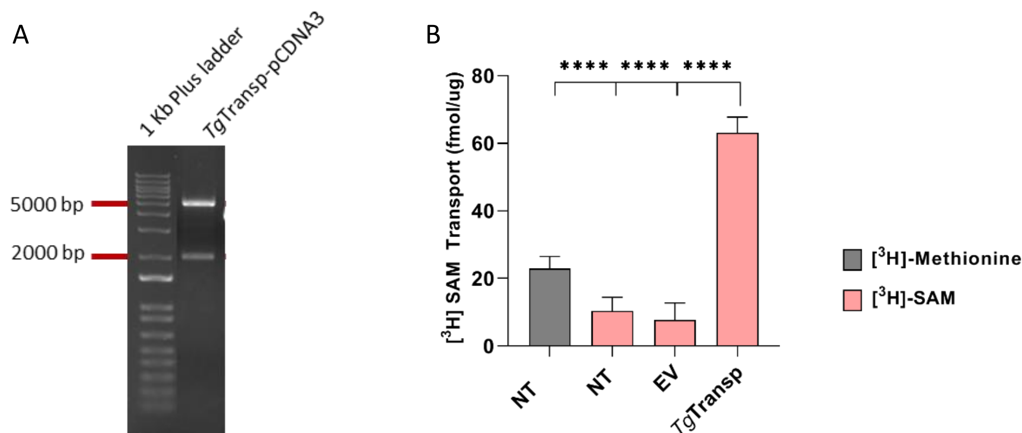

Figure 16 – ***Toxoplasma gondii* TGVAND_290860 (now named *Tg*Transp), associated with sinefungin susceptibility, also transports SAM. A.** Diagnostic restriction reaction for confirmation of *Tg*Transp (2040 bp) purified insert ligation with pCDNA3 (through KpnI and Xho I digestion). Unedited gel image presented in Supplementary Figure 2. **B.** [^{3}H]-methionine uptake by non-transfected (NT) Hek293T cells and [^{3}H]-SAM uptake by Hek293T cells non-transfected (NT), transfected with empy pCDNA3 vector (EV) and TgTransp final construct. N=2. One-way ANOVA. ****, p<0,0001.

Having confirmed that the *Tg*Transp (previously referred to as TGVAND_290860) is a SAM transporter, we used its sequence to align with *Plasmodium falciparum* genome, in order to identify orthologues to *T.gondii* SAM plasma membrane transporter. The results from the alignment identified the MFR5 membrane protein (accession number: PF3D7_1129900) as the closest orthologue (Figure 17). MFR5 is characterized as a member of the major facilitator superfamily-related transporters (MFR). Recently, a study in *P.berghei* pinpointed, from a range of 29 mutant strains of the rodent malaria parasite, MFR5 knockout parasites (mfr5$^-$) as having a slow blood-stage growth . Besides the defect in the erythrocytic stage of infection, mfr5$^-$ *P.berghei* parasites also displayed a severely decreased number of male gametes, exflagellation levels, and did not produce viable sporozoites [62]. Due to the defect in producing sporozoites this parasite line has never been studied in the liver-stage.

Considering the fact that *Pf*MFR5 membrane protein shows significant identity with the *T. gondii* SAM transporter, and that it seems to have an important function across different stages of *P.berghei* lifecycle (interestingly most stages affected depend on replication), we choose MFR5 as the second putative candidate for *Plasmodium* SAM transporter.

So far, the two membrane proteins identified as possible SAM transporters in *Plasmodium*, LH1 and MFR5, were selected based on homology to two known SAM transporters, *Leishmania infantum* AdoMet T1 and *Toxoplasma gondii.* Both plasma membrane transporters uptake the methyl donor SAM and the antimicrobial peptide sinefungin. So, we wanted to explore the possibility that in *Plasmodium*,

```
> PF3D7_1129900.1-p1 | transcript=PF3D7_1129900.1 | gene=PF3D7_1129900
| organism=Plasmodium_falciparum_3D7 | gene_product=major
facilitator superfamily-related transporter, putative
| transcript_product=major facilitator superfamily-related transporter,
putative | location=Pf3D7_11_v3:1156437-1158266(+)
| protein_length=609 | sequence_SO=chromosome | SO=protein_coding
| is_pseudo=false
Length=609

 Score =  155 bits (391),  Expect = 2e-39, Method: Compositional matrix adjust.
 Identities = 136/587 (23%), Positives = 232/587 (40%), Gaps = 94/587 (16%)

Query  17   LKAWIPPSDLP----------GANQPTPCNFNRYALLFFYVISGCLTGVVFFGWPAMASL  66
            LK +  PS L            + Q TP N NR  LLF Y +   LT  +FFGWP +++L
Sbjct  54   LKGYDMPSSLDDLLKKEEIRLSSEQKTPFNINRSVLLFVYFMLIVLTNRLFFGWPNLSNL  113

Query  67   IFYNEGFSTLCARDPATGAFSPDFRQEGQLFICDAQDAAVQKLYTLAGLLCCVMSACGGA  126
            +F  + +   C ++   G +    R + + + CD QD AVQ ++          S   G
Sbjct  114  LFREDTYIWKCQKNEH-GEYD---RFDDKRYSCDEQDKAVQTIFIFGSSAYFAFSFFNGL  169

Query  127  LLDFIGPKYTMCLGQLLSITGWLFLAFSGAAPSTYYAGIAFIGLGADVGFLPTMCVTRLL  186
            ++D++G +++M LG +L++ GW+ +  S      Y  G  F+    D+    T+  + L
Sbjct  170  IVDYLGSRFSMLLGHILNLIGWVLMLMSNEHFDAYVIGGIFMSASIDLASFSTLNASGLF  229

Query  187  PGSAGLVITLLSSASSASSAVPMVLAKVVEHHGASLKTVALWYICCGPIVSLLIALFLLP  246
            PG+  L++ ++S A S S+    +L  ++  +    KT  LWY+C       L+ +FL P
Sbjct  230  PGNENLIVNIISGAGSLSTGTMTILDLIITRYNLPFKTFMLWYMCISVSFFFLLTIFLFP  289

Query  247  RRNYLVGDEFADRGRSLEDHTCTVGDGRSEGDETGRDADMED-DGASRR--DAPAKQNVR  303
            +  Y    EF               D      E     D ED D ++++  D   K++
Sbjct  290  KNRYYRQYEF---------------DNYYNNKEIDL-KDYEDFDNSTKKIYDHDKKKDGH  333

Query  304  QMTNQSSSNWLSRGREKTLDVSRVQEARQKHTPATGAVEPSMPEGASFGCRNVVQATELA  363
            Q  N   S   + G   +L V       +KH       E  +    S   ++V    E
Sbjct  334  QYNNNVHS---TVGVNNSLVV-------KKHEGGINRRELELKNVNS--SKHVFNDLENN  381

Query  364  DGARCEEVRPRTEEVVCLTDSEERWRGGVDDAAGNAATSIPGRTVARERDGPQQSIEAQG  423
            D  + E                             AA++     + + +     S
Sbjct  382  DSKKDEY----------------------------AASTNNSSNLVKSKFNIFNS-----  408

Query  424  EREEESKNKGGERPPAPPPFFKQLFSLRYLLVVVYFVGVACAGAFFQQAPRRMFNDTVVD  483
                          P      K      +L + +Y    A    F+      + +    D
Sbjct  409  --------------PTVKDLIKIFTCAHFLCLWIYGPLNAIYNTFYFSVVENILSKDKND  454

Query  484  FMEMMQPLAFLPCIIFGKCADVFGILKVWMVVNTCGLLMYATSMIHGFHNAFGYTSVLLY  543
             +  + P +F+PC++ G  +D FG++ ++         MYA S I    N   + SV+
Sbjct  455  ILGYILPFSFIPCVLLGNLSDKFGVMLMFTYELIFAFSMYAFSYIKS--NWAQWISVISN  512

Query  544  TLYMSVFSSQVFVYIEETFSPQYFGKMIGLTAMCGGLLSMVSNSLYE  590
             LY +  + Q++ +I  TFS +Y   +IG   +  G++S V  SL+E
Sbjct  513  ALYSACANGQLWTFISFTFSSKYHSTLIGFLNLVCGVVSFVRLSLFE  559
```

Figure 17 - ***Pf*MFR5 is *Plasmodium falciparum*'s protein sequence with the highest identity score to *T.gondii* SAM transporter *Tg*Transp plasma membrane protein.** *Tg*Transp protein sequence was aligned with *Plasmodium falciparum* genome, using BLAST protein tool alignment.

SAM and sinefungin, for which we have evidences that are taken up from the extracellular medium, share a common transporter. For that, we infected HepG2 cells with luciferase-expressing *P.berghei* parasites and added increasing concentrations of sinefungin in the presence or absence of SAM, to determine whether the IC_{50} (concentration of a compound necessary to inhibit half of the normal parasite development) of sinefungin would change, when administrating a possible competitor for the same transporter, SAM.

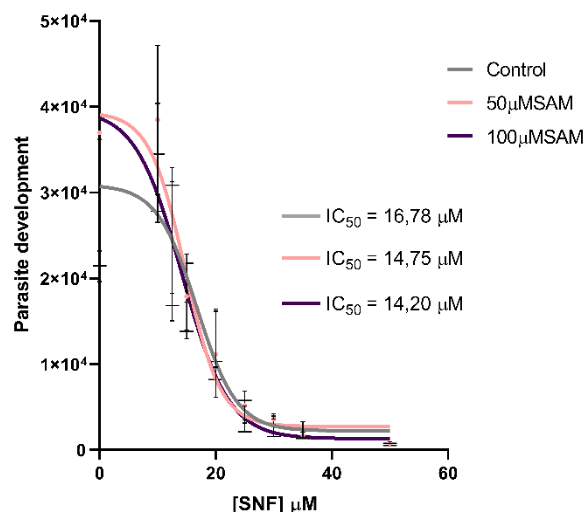

Figure 18 - ***P.berghei's* sinefungin IC_{50} does not change with the administration of SAM.** Dose-response curve generated for P.berghei (in the context of HepG2 cells infection) suscetibility to sinefungin in two differente concentrations of SAM (50 and 100 µM). Parasite development was measured by luciferase reporter assay, at 48 hpi. Control condition: 100 µM of methionine. N=2.

We tested 7 different sinefungin concentrations and established that sinefungin IC_{50} for the liver stage is 16,78 µM. However, in contrast to what happens in *Trypanosoma* and *Leishmania spp.*, sinefungin IC_{50} seems to be unaltered in the presence of higher concentrations of SAM: with 50 µM of SAM, IC_{50}(SNF) is 14,75 µM, and with 100 µM of SAM IC_{50}(SNF) is 14,20 µM (Figure 18). So, adding SAM in the presence of sinefungin, does not block or interfere with sinefungin entering inside the parasite. These data suggest that maybe, in *Plasmodium*, SAM and sinefungin do not share the same transporter, in opposition to its close relatives' protozoan parasites.

2.1.3. MFS6: not all roads lead to the membrane

Even though some living organisms possess SAM transporters localized in the plasma membrane, most organisms have SAM carriers localized in intracellular organelles. For example, *S.cerevisiae*, besides having a SAM plasma membrane transporter also encodes for a mitochondrial SAM carrier, as it also happens with *Arabidopsis thaliana* and in human cells [63,64].

In a recent study that addresses the essentiality of the so called "orphan" transporters in *P.berghei* life cycle the authors study MFS6, a transporter protein that has a protein domain that belongs to the MFS family [62]. Even though mfs6 deficient *P. berghei* parasites showed a slow blood stage growth, these parasites produce viable gametocytes that can be transmitted to the mosquito and produce viable sporozoites that can be transmitted to a new host. However, when infecting hepatocytes these parasites displayed a severely reduced *in vivo* liver load. The careful analysis of liver stage EEFs showed that $mfs6^{-}$ exhibit distorted nuclei with clear evidences of a replication deficit, resulting in the absence of production of liver-stage merozoites [62]. In a later study, the same authors determined that this protein, in blood stage parasites is localized in the apicoplast but was not essential for the survival during erythrocytic development. However, once again, they highlighted the inability of these parasites to successfully infect hepatocytes and form infectious-merozoites due to a replication phenotype [65].

Considering that MFS6 is a transporter protein and that its knockout seems to have a specific phenotype associated with the parasite replication inside the hepatocyte (a phenotype that is not very common), we choose MFS6 (accession number: PF3D7_1440800) as being a third *Plasmodium* SAM candidate protein.

2.2. Heterologous expression of *Plasmodium* SAM transporter candidates in mammalian cells

Having identified three putative *Plasmodium* SAM transporters, LH1, MFR5 and MFS6 that have either homology to known SAM transporters from other organisms or an interesting defective phenotype upon gene deletion, we proceeded to functionally study these proteins using heterologous expression systems. In order to test if any of these candidates can indeed transport SAM, we set up to express the sequences of interest in mammalian cells followed by radioactive SAM transport assays.

Mammalian cells are one of the various models for recombinant protein expression. These cell lines are indicated for expression of eukaryotic proteins because the synthesis, processing and secretion signals of these proteins are extremely well recognized and enable posttranslational modifications, which are essential for the correct folding, leading to functionally relevant and active expressed proteins [66].

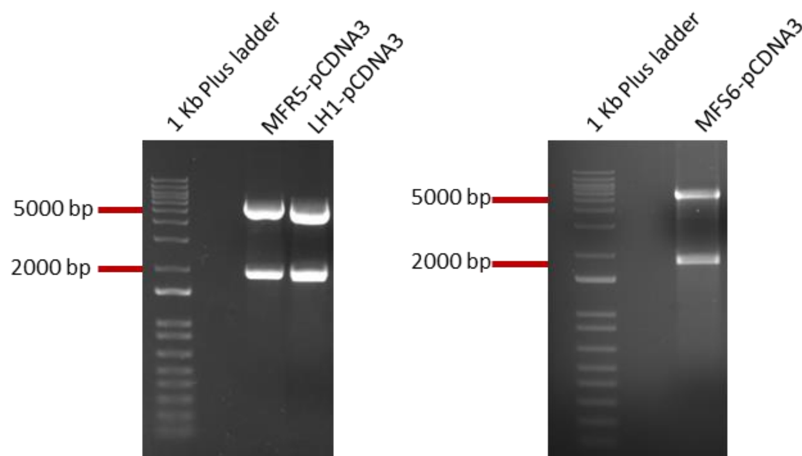

Figure 19 - **Generation of recombinant vectors containing *Plasmodium falciparum* SAM transporter candidates.** Diagnostic restriction reaction for confirmation of MFR5 (1902 bp), LH1 (1962 bp) and MFS6 (1794 bp) purified inserts ligation with pCDNA3, through KpnI and Xho I digestion. Unedited gel images presented in Supplementary Figure 2 and 3.

We started by subcloning codon-optimized versions of the sequences of the *P. falciparum* putative SAM transporter sequences (PF3D7_1022200; PF3D7_1129900 and PF3D7_1440800), optimized by GenScript for mammalian expression, into a specific mammalian expression plasmid, pCDNA3, as previously done for the *Toxoplasma* transporter. The final constructs were transfected in different mammalian cell lines and subsequently processed for transport assays, protein expression and subcellular localization analysis.

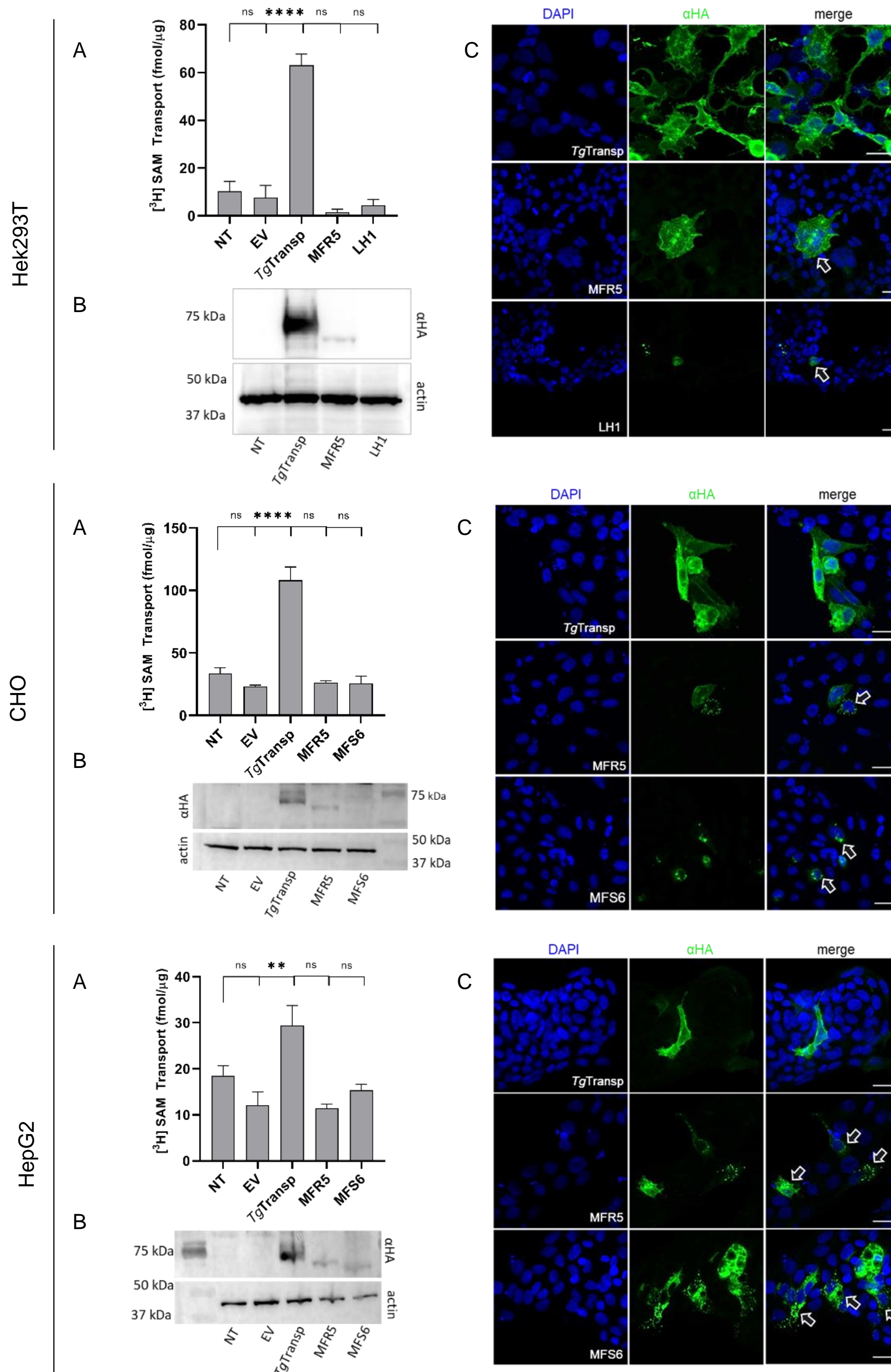

Figure 20 - **Heterologous expression and functional characterization of *Plasmodium* SAM candidate transporters, in Hek293T, CHO and HepG2 mammalian cell lines. A.** [^{3}H] -SAM uptake for the indicated cell lines, non-transfected (NT), transfected with empty vector (EV) or transfected with *Plasmodium* SAM candidate transporters. Intracellular accumulation of radioactive labelled SAM was measured by addition of liquid scintillation solution after transport assay, using MicroBet Trilux. **B.** Lysed cells from the radioactive transport were used for immunoblotting analysis with anti-HA antibody of each one of the HA-tagged protein candidates (*Tg*Transp – 72,429 kDa; MFR5 – 70,137 kDa; LH1 – 74,843 kDa; MFS6 – 68,089 kDa; actin – 42 kDa). **C.** Confocal images of HA-tagged *P.falciparum* proteins in the indicated cell lines. Expressed proteins were detected using the anti-HA antibody, and cell nuclei was stained with DAPI. Scale bar – 25 µm. Arrows pinpoint accumulations of protein around cell nuclei. N=1

Three different cell lines were used for the transfection of the generated constructs: Hek293T, CHO and HepG2 cells. Hek293T are human embryonic epithelial kidney cells, which together with CHO, chinese hamster ovary cells, represent the two main cell lines used and most appropriate for recombinant protein expression. HepG2 cells, derive from a human hepatocellular carcinoma and despite having a low transfection efficacy, is a cell line commonly used to study liver stage development as it sustains infection with rodent infective *Plasmodium* species.

Radioactive labeled SAM transport assays were performed 48 hours after transfection of each cell line. In all experiments non-transfected cells (NT) and cells transfected with the empty vector pCDNA3 (EV) were used as negative control. The *T.gondii* SAM transporter (*Tg*Transp) was used as a positive control and indeed cells transfected with *Tg*Transp exhibit a significant uptake of the radioactive SAM (60, 100 and 30 fentomol of [^{3}H]-SAM/µg of protein, for Hek293T, CHO and HepG2 cells, respectively), when compared to the negative controls (Figure 20 A). However, we could not detect specific SAM transport in any of the cell lines transfected with the *Plasmodium* candidate sequences ([^{3}H]-SAM uptake levels are similar to the negative controls). The analysis of protein expression and subcellular localization, reveal that these proteins were not being expressed, or placed in the plasma membrane to the same extent as *T.gondii* SAM transporter was. Indeed, and in agreement with the transport assay, *T.gondii* expression was detected by Western blot (WB) (Figure 20 B) in all cell lines tested, and the subcellular localization of the HA-tagged proteins, analyzed by immunofluorescence assay (IFA) using an anti-HA antibody, showed that, in all three cell lines *Tg*Transp protein was well placed in the membrane (Figure 20 C). However, LH1 protein expression was never detected in any of the different cell lines, by WB nor by IFA (shown for Hek293T and not shown for CHO and HepG2 cells). Expression of MFR5 protein was low but detectable in all cell lines, by WB as well as by IFA, however a detailed analysis of the subcellular localization of the MRF5 protein revealed that instead of being localized to the plasma membrane, MFR5 protein was retained in vesicle-like organelles around the nuclei suggesting that probably the low amount of protein that was indeed being expressed, was being trapped likely in the endoplasmatic reticulum (ER) or the Golgi apparatus (pointed by arrows in Figure 20 C, for the three cell lines), blocking protein trafficking to the membrane and explaining the absence of radioactive labeled SAM. MFS6 protein expression exhibit a similar behavior to MFR5. Protein expression was detected in all cell lines although expression was slightly higher in HepG2 cells (shown for CHO and HepG2 and not shown for Hek293T cells), however protein was retained intracellularly (pointed by arrows in Figure 20 C).

These observations lead us to conclude that the overall heterologous expression in the mammalian model system did not allow to determine the potential of any of these candidate protein sequences being SAM transporter in malaria parasites.

2.3. Troubleshooting the defective heterologous protein expression in mammalian cells

Even though mammalian cells represent a good system for recombinant protein expression, these cells are especially efficient in the production of eukaryotic proteins of mammalian origin. The accumulation of protein that was verified by immunofluorescence analysis of the HA-tagged *Plasmodium* candidate proteins, likely in the ER or the Golgi apparatus (Figure 20 C), might have been a consequence of a defective protein trafficking through the mammalian cells endomembrane system and secretory pathway.

Protein's amino acid sequences possesse molecular signals that dictate the protein's movement through the system and its eventual location in the cells. Proteins might also move back and forth, depending on the "decision" as to whether the protein is to be retained in that compartment or transported further. The ER membrane is the site of production of all transmembrane proteins, and during translation, proteins which final destiny is the cell plasma membrane (or other parts of the endomembrane system), are conducted to the ER, due to the recognition of specific signalling sequences, by signal recognition particles (SRP) present in the cytosol that performs this co-translational transport from the cytosol to the ER, where the SRP receptors present in the ER membrane recognize and translocate the protein to the ER lumen [67].

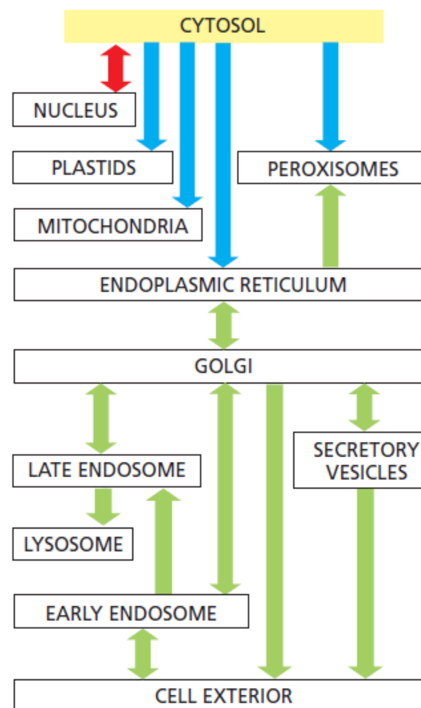

Figure 21 - **Map of protein trafficking in eukaryotic cells.** Except proteins that are synthesized in other organelles like chloroplasts and mitochondria, all cells during translation are transported through the cytosol, in a ribosome while being synthetized. Along the protein amino acid sequence, are present different molecular signals that direct the protein's movement through the system, determining its location in the cell. In each "intermediate station" (boxes), the protein can either be retained in that compartment or transported further, accordingly to the signal present in the protein sequence. Endomembrane system and compartments of the eukaryotic cell. (Red arrows, gated transport; blue arrows, transmembrane transport; green arrows, vesicle transport) (from Bruce Alberts *et al.* 5th ed. 2008).

In the ER, proteins are correctly folded and are then directed to the Golgi apparatus, where they might suffer other modifications, accordingly to their specific function and subcellular localization. From the Golgi, if proteins do not show any other specific tags, they are transported by vesicles that bud from the Golgi and that, in case of membrane proteins, fuse with the cell plasma membrane, incorporating the proteins in their destination.

These candidate proteins have plasmodial origin, as such, we have to take into account the fact that *Plasmodium* genome has long stretches of AT repeats, which might compromise the expression in mammalian cells. In addition, the signals to properly fold and place proteins may be different in *Plasmodium* and as such, proteins are not being correctly processed and trafficked throughout the ER, Golgi and other protein carrying vesicles, within mammalian cells. Finally, there is also the possibility that these proteins have molecular tags, such as signal peptides or specific localization sequences that result in the misplacement of these proteins to other organelles instead of to the cell plasma membrane[67].

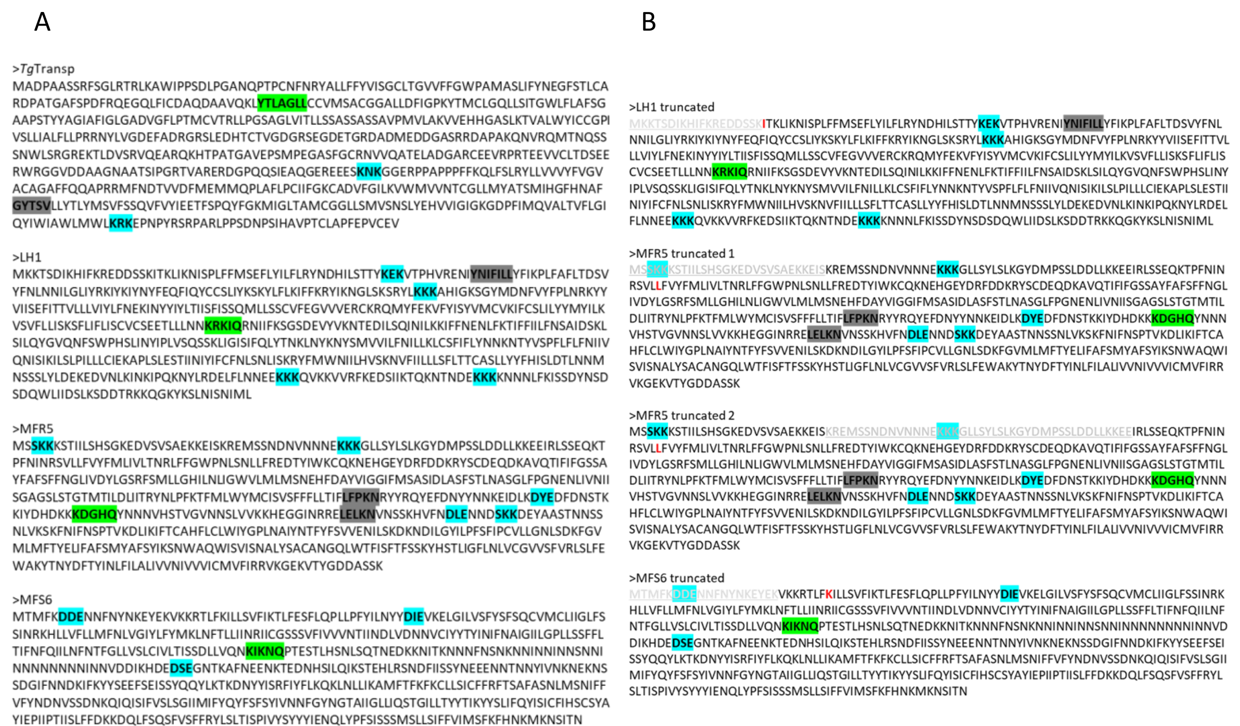

Figure 22 - ***In silico* analysis of *T.gondii* SAM transporter *Tg*Transp (positive control) and *Plasmodium* SAM transporter candidates**, with highlighted signalling sequences for ER (blue), Golgi (green) and lysosomes (grey) trafficking. Begin of the first transmembrane domain is marked in red. **A.** original sequences, retrieved from PlasmoDB and **B.** proposed truncated versions of each protein.

To address this last question we collaborated with Dr. Francisco Enguita, at the IMM, to perform an *in silico* analysis of the *P.falciparum* candidate proteins that we failed to express in mammalian cells, with the purpose of try to identify possible signalling sequences that could explain the redirection or retention of these proteins in specific organelles in the transfected cells.

When analysing the *Plasmodium* sequences in comparison with the sequence of the *Toxoplasma* *Tg*Transp, which was successfully expressed, we observed that all *Plasmodium* sequences, similarly to the *Tg*Transp, have one Golgi associated sequence. However, all *Plasmodium*

sequences have multiple ER trafficking associated signalling sequence, that appear before the Golgi associated sequence, contrarily to what happens in the *Toxoplasma* sequence (Figure 22 A). The presence of these first amino acid sequences, may cause the retention of the candidate proteins in the ER explaining the observed accumulation of MFR5, LH1 and MFS6 proteins around the nucleus. This analysis is in accordance with the model proposed for parasite proteins targeted to the plasma membrane, which involves nucleus – ER – Golgi – membrane/apicoplast path [68]. Being redirected or trapped in the ER and due to the presence of a specific signalling sequence, it is possible that these proteins are not being well processed and folded and are retained in this organelle.

Furthermore, and specially for MFS6 protein, there is an added layer of complexity that is the fact that, at least in the blood stage, this protein is localized in *Plasmodium* apicoplast outer membrane, which may mean that there is yet another specific localization signal that does not have a similar or equivalent in mammalian cells and it is difficult to predict what would happen. Considering this analysis, we proposed truncated versions of each one of the candidate *P.falciparu*m proteins (Figure 22 B) that might allow to overcome this complication and successfully express these proteins and correctly place them in the plasma membrane of mammalian cells. The truncations will target the first stretch of amino acids in order to eliminate the ER signalling sequences without disturbing the transmembrane domains important for the placement of these proteins in membranes.

D. Conclusions and future work perspectives

Malaria is still the king of infectious diseases, killing a child every two minutes and leading to 200 million new cases, each year. Even though in 2017 were reported 20 million less cases than in 2010, during the time period between 2015 and 2017 no significant progress was made towards the reduction of global malaria cases [2]. Malaria control programmes are extremely focused on improving and promoting the delivery of resources and commodities such as insecticide-treated bed nets, rapid diagnostic tests and Artemisin-based Combination Therapies (ACTs), in countries affected by this devastating disease. However, malaria preventing strategies based on the identification of new therapeutic targets, remains an extremely important field to invest in. Being the liver the organ where *Plasmodium* parasites stay silently while undergoing one of the most efficient replication rates amongst eukaryotes to overcome one of the parasite's population bottleneck, it represents the ideal target for new prophylactic therapies.

Intracellular obligate organisms are specialized in exploiting host resources, to ensure their own survival, and their metabolic needs are many times met by the expression of transporter proteins, that facilitate the uptake of important nutrients from the extracellular environment. As it is no exception, in *Plasmodium*, this host resource's exploitation mechanism is highly frequent, and the parasite is able to express different type of transporter systems in its own membrane and organelles or even in the host cell membrane, that allows the uptake of nutrients like cysteine, arginine, folate, glucose, lipids and others [35,36,59,69,70]. SAM is an extremely important compound in all living organisms, being the major methyl group donor in methylations reactions, that are involved in DNA, RNA, protein and lipids expression and regulation, and also in cell growth and redox balance. Because of this, both the mammalian host and the parasite encode for the enzyme responsible for methionine conversion into SAM, methionine adenosyltransferase or SAM synthetase (MAT or SAMS). Previous experiments from the laboratory have demonstrated that during liver stage development, the parasite relies on the host enzyme to thrive. These evidences, allied to the fact that the mammalian liver is the main SAM producer, able to synthesize 6-8 g of this compound daily [46], lead us to hypothesize that the parasite is taking advantage of the host methionine metabolism, to access the host SAM metabolic pool to meet their needs.

During this project we aimed to study the temporal dynamics and dependency on host methionine and SAM, by *Plasmodium* and to identify the *Plasmodium* SAM transporter that is allowing the exploitation of host produced SAM.

Supported by preliminary results from the laboratory evidencing that hepatic methionine metabolism is important for *Plasmodium* liver infection, data herein shows that upon methionine deficiency, parasite ability to survive inside hepatocytes is not affected. Instead it is its development and growth within the host cell that is massively impaired. Time course analysis revealed that methionine, and SAM, are crucial from 16 until 48hpi, which matches with the timeframe in which the parasite is replicating by schizogony. Overall, these results show that methionine, and consequently SAM supply, are fundamental for parasite replication during liver stage of infection, and its restriction would ultimately

result in a lower number of RBC-infectious merozoites formed and released in the blood to cause disease.

Importantly, we also showed that the parasite relies on the exogenous supply of this molecule. Indeed, SAM quantities inside the parasite are dependent on the culture medium condition, being reduced when methionine is depleted, and restored upon the administration of SAM. In addition, even though the parasite possesses the enzymatic machinery to produce SAM from methionine (SAM synthetase enzyme), the quantification of SAM levels inside parasites deficient for the SAM synthetase are similar to that of *wild type* parasite, that can fully perform this metabolic reaction. This data allied to the previous knowledge that *Plasmodium* SAMS enzyme is dispensable during liver stage of infection, highlights once more the possibility of malaria parasites hijacking SAM produced by the host hepatocytes. In the future, the quantification of SAM levels in *wild type* and hepatocytes deficient for the liver specific MAT1 enzyme, would be important to fully confirm that indeed the source of SAM used by *Plasmodium* is the mammalian hepatocyte.

It is not uncommon for obligate intracellular organisms to take advantage of the host SAM pool. Parasites that have evolve side by side with *Plasmodium*, such as the kinetoplastids *Leishmania* and *Trypanosoma* spp., and the apicomplexan *Toxoplasma gondii*, have a SAM transporter (identified and characterized in *Leishmania*, AdoMetT1, still unidentified in *Trypanosoma* spp., and uncharacterized in *Toxoplasma gondii*). A common feature among the three species, is the susceptibility to the antimicrobial compound sinefungin, a structural analogue of SAM. SAM and sinefungin are known to share a plasma membrane transporter in *L.infantum* and *S. cerevisiae*. In this work, we confirm by radioactive labelled SAM transport, that the protein sequence responsible for the uptake of sinefungin in *T. gondii* is also responsible for the uptake of SAM. Using the protein sequence of the transporters of two phylogenetically close organisms, *L. infantum*'s AdoMet T1 and *T. gondii*'s *Tg*Transp, we identified two putative candidates for SAM transporter *in the Plasmodium* genome (LH1 and MFR5). A third candidate protein was chosen due to the specific phenotype associated gene deletion. MFS6 null *P.berghei* parasites exhibit a defective replication process during the liver stage of infection. This phenotype is in line with our hypothesis that SAM uptake is necessary for *Plasmodium* replication, when infecting hepatocytes. The functional assessment of the three *Plasmodium* SAM transporter candidates using the mammalian heterologous expression system, was inconclusive leaving the possibility that one of these candidates is indeed the *Plasmodium* SAM transporter open.

In an attempt to shed some light in the identity of the *Plasmodium* SAM transporter, and considering that in *Leishmania* and *Toxoplasma*, SAM and its structural analogue sinefungin compete for the same plasma membrane transporter, we sought to assess whether the same is true for *Plasmodium.* For this, we determined the sinefungin IC_{50} in the presence and absence of SAM. We concluded the sinefungin IC_{50} is not altered in the presence of SAM, which might suggest that contrarily to what is observed in other organisms, SAM and sinefungin might not share the same transporter protein in *Plasmodium*. In the future, increasing concentrations of SAM should also be tested, in order to confidently affirm that SAM and sinefungin do not compete for the same transporter.

Other heterologous expression, such as yeast cells are available as an alternative expression model, combining the capacity of performing posttranslational modifications (also present in mammalian cells) and the ability to produce eukaryotic proteins in general, including integral membrane proteins. *SAM3* encodes for a high affinity SAM transporter in yeast, and its deletion confers resistance to sinefungin. In the future, we will transform the *SAM3* null strain, *sam3* mutant, with plasmids containing our candidate sequences (full length and truncated versions), and access if any of the expressed proteins leads to sinefungin suscetibility in this mutant strain. This system can be further used to assess if the same candidate transporter proteins are also responsible for SAM uptake, by performing [^{3}H]-SAM transport assays in normal *wild type S. cerevisiae* strain and comparing with the *sam3* null strain and the mutant strain complemented with our candidate proteins. Like this we would not only identify the SAM transporter, we would also assess whether is a shared transporter between SAM and sinefungin.

Overall, the results obtained during this project identify SAM as an essential nutrient for *Plasmodium* replication during liver stage, probably due to its key functions in processes such as cell growth, DNA and protein methylation. Interestingly, this SAM dependency during the pre-erythrocytic stage of infection seems to be an evolutionary advantageous and “well-thought decision” by *Plasmodium.* The dependency on this host molecule occurs during the time frame where it achieves one of the highest replication rates reported in eukaryotes, and while it is developing in a location where the surrounding environment can fully provide this metabolite in unlimited amounts, the hepatocyte. Interestingly, this differences in SAM bioavailability in liver stage comparing to blood stage and to other cell types that non-mammalian infective *Plasmodium* species infect, might be the one of the reasons behind the differences of replication observed in these different infection contexts.

Furthermore, *Plasmodium* dependency of exogenously supplied SAM, independently of expressing or not its own SAM synthetase enzyme, highlights the possibility of the existence of a SAM transporter protein like in other organisms. Interestingly, these organisms are also able to synthesize this compound suggesting that the parasites can “choose” to synthesize or uptake SAM, maybe depending on the environmental conditions, or stages of the lifecycle.

The identification of a *Plasmodium* SAM transporter, and the understanding of how and when is essential may open the door to a new prophylactic strategy, that allows the reduction or possible elimination of liver stage parasites, and consequently preventing malaria disease and parasite transmission.

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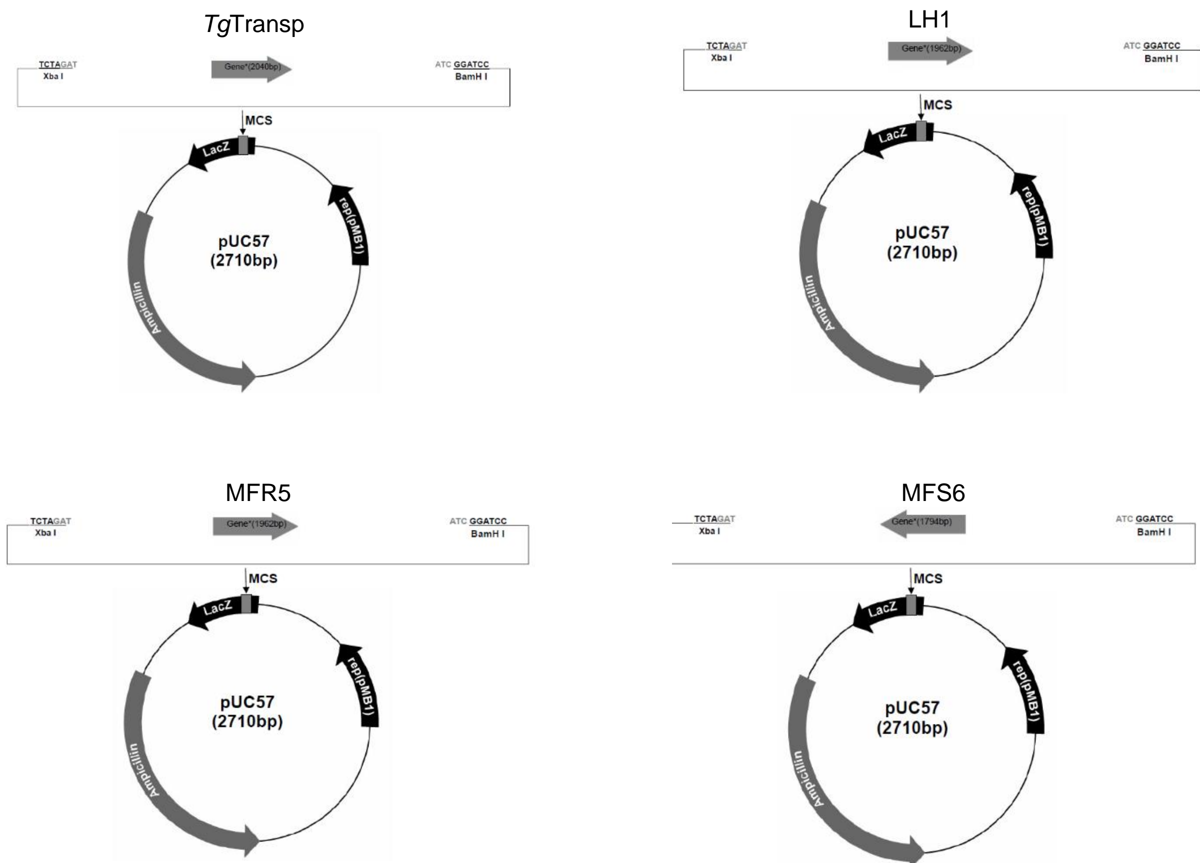
F. Supplementary data

1. Transporter sequences retrieved from ToxoDB and PlasmoDB

Table 1 - *Tg*Transp and *Plasmodium* SAM transporter proteins information.

Name	Accession number	Species	Annotated protein function	Transcript length	Protein length	Protein sequence
*Tg*Transp	TGVAND_290860	*Toxoplasma gondii*	Putative amino acid transporter	1995 bp	664 aa	MADPAASSRFSGLRTRLKAWIPPSDLPGANQPTPCNFNRYALLFFYVISGCLTGVVFFGWPAMASLIFYNEGFSTLCARDPATGAFSPDFRQEGQLFICDAQDAAVQKLYTLAGLLCCVMSACGGALLDFIGPKYTMCLGQLLSITGWLFLAFSGAAPSTYYAGIAFIGLGADVGFLPTMCVTRLLPGSAGLVITLLSSASSASSAVPMVLAKVVEHHGASLKTVALWYICCGPIVSLLIALFLLPRRNYLVGDEFADRGRSLEDHTCTVGDGRSEGDETGRDADMEDDGASRRDAPAKQNRQMTNQSSSNWLSRGREKTLDVSRVQEARQKHTPATGAVEPSMPEGASFGCRNVVQATELADGARCEEVRPRTEEVVCLTDSEERWRGGVDDAAGNAATSIPGRTVARERDGPQQSIEAQGEREEESKNKGGERPPAPPPFFKQLFSLRYLLVVVYFVGVACAGAFFQQAPRRMFNDTVVDFMEMMQPLAFLPCIIFGKCADVFGILKVWMVVNTCGLLMYATSMIHGFHNAFGYTVLLYTLYMSVFSSQVFVYIEETFSPQYFGKMIGLTAMCGGLLSMVSNSLYEHVVIGIGKGDPFIMQVALTVFLGIQYIWIAWMWLKRKEPNPYRSRPARLPPSDNPSIHAVPTCLAPFEPVCEV
LH1	PF3D7_1022200		Conserved *Plasmodium* membrane protein, unknown function	1890 bp	629 aa	MKKTSDIKHIFKREDDSSKITKLIKNISPLFFMSEFLYILFLRYNDHILSTTYKEKVTPHVRENIYNIFILLYFIKPLFAFLTDSVYFNLNNILGLIYRKIYKIYNYFEQFIQYCCSLIYKSKYLFLKIFFKRYIKNGLSKSRYLKKKAHIGKSGYMDNFVYFPLNRKYYVIISEFITTVLLLVIYLFNEKINYYIYLTIISFISSQMLLSSCVFEGVVVERCKRQMYFEKVFYISYVMCVKIFCSLILYYMYILKVSVFLLISKSFLIFLISCVCSEETLLLNNKRKIQRNIIFKSGSDEVYVKNTEDILSQINILKKIFFNENLFKTIFFIILFNSAIDSKLSILQYGVQNFSWPHSLINYIPLVSQSSKLIGISIFQLYTNKLNYKNYSMVVILFNILLKLCSFIFLYNNKNTYVSPFLFLFNIIVQNISIKILSLPILLLCIEKAPLSLESTIINIYIFCFNLSNLISKRYFMWNIILHVSKNVFIILLLSFLTTCASLLYYFHISLDTLNNMNSSSLYLDEKEDVNLKINKIPQKNYLRDELFLNNEEKKKQVKKVVRFKEDSIIKTQKNTNDEKKKKNNNLFKISSDYNSDSDQWLIIDSLKSDDTRKKQGKYKSLNISNIML
MFR5	PF3D7_1129900	*Plasmodium falciparum*	Major facilitator superfamily-related transporter, putative	1830 bp	609 aa	MSSKKKSTIILSHSGKEDVSVSAEKKEISKREMSSNDNVNNNEKKKGLLSYLSLKGYDMPSSLDDLLKKEEIRLSSEQKTPFNINRSVLLFVYFMLIVLTNRLFFGWPNLSNLLFREDTYIWKCQKNEHGEYDRFDDKRYSCDEQDKAVQTIFIFGSSAYFAFSFFNGLIVDYLGSRFSMLLGHILNLIGWVLMLMSNEHFDAYVIGGIFMSASIDLASFSTLNASGLFPGNENLIVNIISGAGSLSTGTMTILDLIITRYNLPFKTFMLWYMCISVSFFFLLTIFLFPKNRYYRQYEFDNYYNNKEIDLKDYEDFDNSTKKIYDHDKKKDGHQYNNNVHSTVGVNNSLVVKKHEGGINRRELELKNVNSSKHVFNDLENNDSKKDEYAASTNNSSNLVKSKFNIFNSPTVKDLIKIFTCAHFLCLWIYGPLNAIYNTFYFSVVENILSKDKNDILGYILPFSFIPCVLLGNLSDKFGVMLMFTYELIFAFSMYAFSYIKSNWAQWISVISNALYSACANGQLWTFISFTFSSKYHSTLIGFLNLVCGVVSFVRLSLFEWAKYTNYDFTYINLFILALIVVNIVVVICMVFIRRVKGEKVTYGDDASSK
MFS6	PF3D7_1440800		Putative major facilitator superfamily domain-containing protein	1749 bp	582 aa	MTMFKDDENNFNYNKEYEKVKKRTLFKILLSVFIKTLFESFLQPLLPFYILNYYDIEVKELGILVSFYSFSQCVMCLIIGLFSSINRKHLLVFLLMFNLVGIYLFYMKLNFTLLIINRIICGSSSVFIVVVNTIINDLVDNNVCIYYTYINIFNAIGIILGPLLSSFFLTIFNFQIILNFNTFGLLVSLCIVLTISSDLLVQNKIKNQPTESTLHSNLSQTNEDKKNITKNNNFNSNKNNINNINNSNNINNNNNNNNINNVDDIKHDEDSEGNTKAFNEENKTEDNHSILQIKSTEHLRSNDFIISSYNEEENNTNNYIVNKNEKNSSDGIFNNDKIFKYYSEEFSEISSYQQYLKTKDNYYISRFIYFLKQKLNLLIKAMFTFKFKCLLSICFFRFTSAFASNLMSNIFFVFYNDNVSSDNKQIQISIFVSLSGIIMIFYQYFSFSYIVNNFGYNGTAIIGLLIQSTGILLTYYTIKYYSLIFQYISICFIHSCSYAYIEPIIPTIISLFFDKKDQLFSQSFVSFFRYLSLTISPIVYSYYYIENQLYPFSISSSMSLLSIFFVIMSFKFHNKMKNSITN

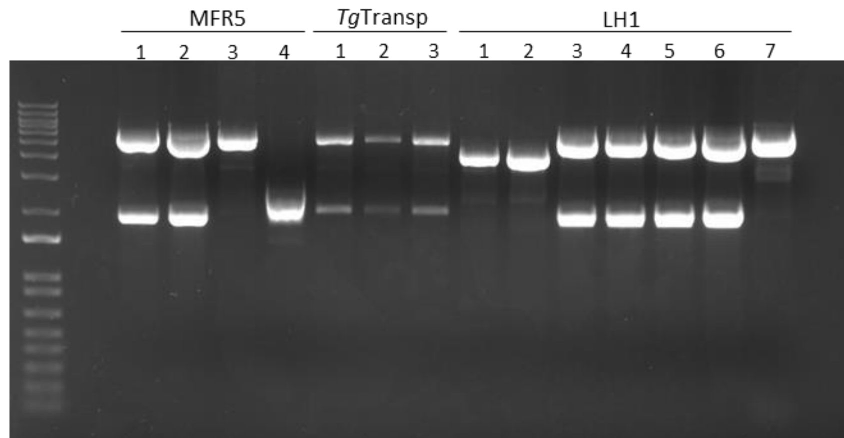
2. GenScript codon optimized for mammalian expression proteins



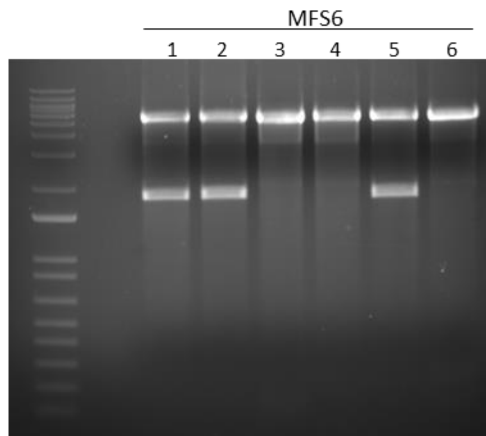
Supplementary Figure 1 – Original plasmids containing *Tg*Transp, LH1, MFR5 and MFS6 coding sequences, optimized for mammalian expression, from GenScript.

B

3. Unedited agarose gel images



Supplementary Figure 2 – Unedited image of agarose gel correspondent to the diagnostic restriction reaction for confirmation of MFR5, *Tg*Transp and LH1 purified inserts ligation in pCDNA3 vector from different transformant colonies. In Figure 16 is represented lane 3 for TgTransp, and in Figure 19 is represented lane 1 for MFR5 and lane 3 for LH1.



Supplementary Figure 3 - Unedited image of agarose gel correspondent to the diagnostic restriction reaction for confirmation of MFS6 purified insert in pCDNA3 vector, from different transformant colonies. In Figure 19 is represented lane 5 for MFS6.