Plasmodium exploitation of host methionine metabolism

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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

Introduction

Malaria has moved scientists all over the world to join efforts, with the mission of eradicating this 435 000 annual deaths-causing disease, through the unveiling of the biology behind the infectious agent, *Plasmodium* parasites [1]. During the bite of an infected female *Anopheles* mosquito, approximately 10 to 100 sporozoites are injected under the skin of the vertebrate host that will migrate through blood vessels until reaching the liver, where they will infect hepatocytes, and develop into erythrocyte-infectious merozoites. This replication occurs trough schizogony inside a parasitophorous vacuole (PV) at a rapid, intense and efficient rate, originating tens of thousands of erythrocyte-infective merozoites, that will after being released into the blood stream initiate the erythrocytic stage of the infection [2]. As soon as merozoites get to the bloodstream, they start to infect red blood cells, where they start the asexual replication cycle. In this developmental process, each merozoite replicates into 16 to 32 new merozoites, that when released, infect other erythrocytes. This red blood cell infection phase is associated with the symptoms of the disease. To continue *Plasmodium* life cycle, some merozoites, when leaving the erythrocyte, develop into male and/or female gametocytes, that will be later ingested by the mosquito, when a new blood meal takes place, initiating the

sexual reproduction of the parasite. Inside the mosquito midgut, the gametocytes will develop into gametes and fertilization will occur originating a zygote, that will develop into ookinetes and sporozoites-containing oocysts. When fully matured, the oocysts burst and release sporozoites, which migrate to the salivary glands of the insect, ready to be injected in a new vertebrate host during the next blood meal, closing *Plasmodium* life cycle [3]. Throughout its life cycle, malaria parasites suffer dramatic losses when it comes to parasite numbers, upon traveling from the mosquito midgut, to reach the mammalian liver. This "bottleneck" in malaria parasites transmission has to do with the parasite exposure to host components, including host immune system, that submits parasite population to aggressive environmental conditions and impose a natural selection in *Plasmodium* sporozoites [4]. However, upon the small proportion of sporozoites that are able to reach the mammalian liver, somehow, from one single sporozoite-infected hepatocyte, tens of thousands of merozoites are born, a remarkable replication rate that is only achieved in mammalian-infective malaria parasites, in opposition to avian and reptile infective *Plasmodium* species, that when infecting macrophages at the mosquito bite site, only generate dozens of new parasites [5]. During infection, mammalian-infective *Plasmodium* sporozoites, undergo the first and obligatory phase in the developmental journey of the parasite within the host hepatocytes, and that is essential to guarantee the progression of infection by overcoming the bottleneck of malaria transmission [6]. The mammalian liver represents a special and unique organ, due to the efficient metabolism of methionine, an essential amino acid that can only be obtained through the diet. In the liver 50% of dietary methionine is metabolized, and the major pool of S-adenosylmethionine (SAM or SAMe) is generated [7]. The hepatocytes are able to produce unlimited amounts of SAM due to the expression of liver-specific methionine adenosyltransferase I (MATI/MATIII), which unlike the extra-hepatic enzyme MATII, it is not inhibited by physiological concentrations of SAM. SAM represents an important metabolic intermediate, in methylation reactions, being the most important methyl group donor, that engages in DNA repair and gene expression, but also

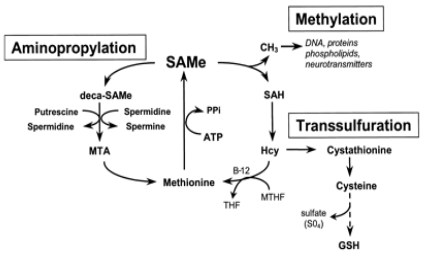

Figure 1 - **Liver methionine and s-adenosylmethionine metabolism**: SAMe – s-adenosylmethionine; SAH - s-adenosylhomocysteine; Hcy - homocysteine; MTHF - methyltetrahydrofolate; THF - tetrahydrofolate; GSH - glutathione; deca-SAMe - decarboxylated S-adenosyl-L-methionine; MTA - , methylthioadenosine; ATP - adenosine triphosphate; PPi - pyrophosphate (Bottiglieri, T., 2002).

participates in polyamines formation, essential to cell growth and development and synthesis of the major cellular antioxidant, glutathione.[8] In resemblance to most eukaryotes, *Plasmodium* cannot synthetize methionine *de novo*, and in the absence of this essential amino acid, parasite development is severely impaired. However, what previous data from the laboratory have showed is that, when trying to compensate methionine depletion with the byproducts of methionine metabolism, SAM, polyamines and glutathione, only SAM is able to rescue the entire parasite load. Still it is important to highlight that, both *Plasmodium* and its mammalian host express the MAT enzyme. Preliminary data also showed that *Plasmodium* MAT enzyme during liver stage of infection is dispensable, since *wild type* and *P.berghei* parasites knocked down for its MAT enzyme, display the same infection load. Importantly, *Plasmodium* development in the liver stage depends on the host MAT enzyme, evidenced by the decreased parasite load when infecting MAT1 knockout mice or primary hepatocytes. Given the evidences that the parasite needs SAM and depends on the host to synthesize it, in this project we aim to unravel the dynamics of *Plasmodium* dependency on SAM hepatocyte metabolic pool and to identify the SAM transporter in *Plasmodium* parasites, that may contribute to the high rate of replication observed during liver infection. Our results revealed a crucial function for SAM, during the second half of *Plasmodium* infection in the hepatocytes, that coincides with the peak of parasite replication. Furthermore, SAM levels detected inside the parasite are dependent on exogenous (host) supply, suggesting the existence of a *Plasmodium* SAM transporter, that

can sustain the uptake of this important molecule from the mammalian hepatocyte.

Materials and Methods

In vitro P. berghei infection and measure by luminescence and flow cytometry

Hepatic infection was assessed in HepG2 cells, infected with either GFP or luciferase-expressing *P. berghei* parasites. HepG2 cells were seeded in 24 (for flow cytometry, to access parasite development and number of infected cells) or 96 (for luciferase assay, to determine infection load) well-plate, the day before infection, in DMEM. At the day of infection, freshly dissected *P.berghei* sporozoites were added to the cells, followed by a centrifugation step at 3000×g for 5 min. Medium was replaced 2hpi to RPMI with no methionine, containing 1 % PenStrep, 1 % HEPES, 1 % Glutamine, 10 % FBS, 1:300 Fungizone, and varying concentrations of methionine or SAM. At 48 hpi, parasite infection was determined, either by flow cytometry (preceded by trypsinization and collection of infected cells from the plate, for following data acquisition on BD Accuri C6 and analysis using FlowJo software) or luciferase assay (accompanied by measure of cell viability by Cell Titer Blue assay Promega). Cells were maintained in a 5% CO_2 humidified incubator at 37ºC, throughout the entire infection.

SAM quantification by immunofluorescence assay

HepG2 cells were inoculated on coverslips or 96-well black imaging plates, infected with GFP-expressing *P.berghei* parasites, either *wild type* or deficient for the *Pb*Mat enzyme (Pb-SAMS-DD-HA), that expresses the MAT enzyme in fusion with a destabilizing domain (DD), that is stabilized in the presence of trimethoprim (TMP). Infected cells were 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 for nuclei staining 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 a widefield Zeiss Cell Observer Microscope.

Cloning of *Plasmodium* homologues to AdoMet T1 and *Tg*Transp SAM transporters in mammalian cells

AdoMet T1 (accession number LINF_100008900-T1) and TGVAND_290860 (here referred as *Tg*Transp) protein sequences were obtained from *Leishmania* and *Toxoplasma* genome database (TritTryDB and ToxoDB, respectively) and aligned with *Plasmodium* genome in PlasmoDB using the bio-informatic tool BLAST, for homology search. Mammalian expression codon-optimized version of the sequences of *Toxoplasma gondii* transporter TGVAND_290860 and *Plasmodium falciparum* candidates for SAM transporter proteins, LH1, MFR5 and MFS6 synthesized by GenScript, were digested from the initial carrying vector (pUC57) with Bgl-II and Not I - HF restriction endonucleases (New England Biolabs®). Ligation of the purified gene coding 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. Transformed bacteria with plasmid constructs were then plated on LB Agar plates supplemented with Ampicilin (1:1000), incubated overnight at 37ºC and colony DNA extracted using NZYMiniprep kits.

Mammalian cell transfection

Final vectors containing cloned transporter sequences were transfected into Hek293T, HepG2 or CHO cells, following specific manufacturer instructions, using FuGENE® HD Transfection Reagent protocol. Cells were plated in 24 (for protein expression analysis) or 96 (for immunofluorescense analysis) well-plates 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 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 37ºC.

[^{3}H]-SAM uptake assay, immunofluorescence and immunoblotting analysis

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. For protein expression analysis, the 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, Cell Signaling) 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, Cell Signaling), 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®). For immunofluorescence analysis, to access subcellular localization, 48 hours post transfection, cells were fixed, permeabilized and blocked, as previously explained. Cells were then incubated with Rabbit anti-HA primary antibody (1:300, Cell Signaling) for 1 hour at room temperature. After primary incubation, cells were rinsed in 1x PBS and incubated with Donkey anti-Rabbit conjugated to Alexa Fluor 488 (1:400, Thermo Fisher Scientific), for HA-tagged protein and DAPI (1:1000, Sigma), for 1 hour at room temperature. Samples were mounted as mentioned before and imaging was performed by fluorescence microscopy using LSM-710 Zeiss Microscope.

Statistical analysis

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

Results

Administration of SAM rescues the impairment in infection caused by methionine deficiency

The previous results obtained in the laboratory show that in the context of a deficient methionine supply, whether by administration of methionine deficient diet to mice or a depletion in the culture medium methionine concentration, *Plasmodium* infection is impaired. To replicate these results, hepatoma HepG2 cells were infected with firefly luciferase or GFP - expressing *P.berghei* sporozoites and the outcome of infection was analyzed either by bioluminescence assay or flow cytometry . Two hours post infection, cell medium was exchanged to mimic the methionine deficient (10 μM) and methionine sufficient (100 μM) conditions. A third experimental condition was also employed where methionine deficient medium was supplemented with SAM (500 Mm). At 48 hours post infection, luciferase expression 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. However, the parasite load in methionine deficient medium was reestablished to values close to the ones observed in methionine

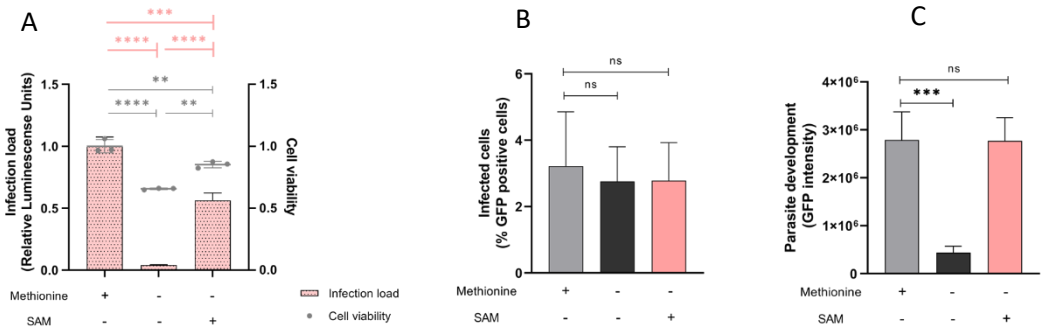

Figure 2-***Plasmodium*** **infection impairment in the absence of methionine, is reflected in the parasite replication and not in the number of infected cells.** *P. berghei* infection in HepG2 cells cultured in the presence or absence of methionine and supplemented with SAM between 2-48 hpi, measured by bioluminescence (A) and flow cytometry at 48 hpi. (B,C) **Graph A**: N=1 One-way ANOVA test. ** p<0,01, *** p<0,001, **** p<0,0001. **Graphs B and C**: Mann-Whitney test. ns – not significant and **** p<0,0001

sufficient culture medium with the supplementation of SAM (60%) (Figure 2A), as had been observed in previous experiments from the laboratory. In this experiment, cell viability was also slightly affected, although to a lower extent when compared to the effect in infection. On the other hand, the analysis of the infection outcome by flow cytometry, measuring frequency of infected cells and the geometric mean of the GFP signal, confirmed that the number of infected cells was similar for the three conditions (Figure 2 B), while parasite development, and consequently parasite growth and replication, upon methionine depletion was reduced (Figure 2 C). However, the compensation of methionine depletion with the addition of SAM, restored parasite development to levels similar to the ones in methionine sufficient medium, meaning that the absence of methionine, and therefore SAM, has direct consequences not on parasite ability to survive inside hepatocytes, but instead in the development and growth of the EEFs.

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

Plasmodium berghei development lasts between 48 to 56 hours, before releasing infective merozoites into the blood stream. This development inside hepatocytes can be divided into three main stages: invasion, which takes approximately 2 hours; transformation, that comprises the rounding of the sporozoite, the beginning of growth and branching of both the mitochondrion and apicoplast, and the preparation of the parasite for the replication processes, until 16-20 hpi; and the replication by schizogony stage, where the parasite gives rise to the thousands of merozoites that will be released between 48-56 hpi, to infect RBCs.[9] Considering that SAM appears to be critical for a successful intrahepatic development, it was important to understand at what specific stage of the infection, and therefore, for what specific developmental process, was this metabolite being essential. For this, HepG2 cells were infected with GFP-expressing *P. berghei* parasites and kept on methionine sufficient or methionine deficient medium, with or without the administration of SAM, for either the first 2- 16/2-24 hpi or for the 16-48/24-48 hpi. Even though it is stated that DNA replication in *Plasmodium* infection starts at 18hpi [10], preliminary results from the laboratory, using a tool that detects the incorporation of a synthetic nucleoside troughout DNA replication by fluorescense analysis, have shown that in fact, between 14-16 hpi, the parasite has already started to perform events of mitosis that will lead to the replication of DNA and the development of the parasite into a multinucleated schizont. So, besides the timecourses of 2-24 hpi and 24-48 hpi, a third and forth timepoints were created (2-16hpi and 16-48hpi), with the purpose of determining whether there is some association of methionine and specially SAM availability with the initiation of DNA replication. When acessing parasite development for incubation with methionine or SAM, solely for the first two timepoints (2-16/2-24hpi) it is very evident that parasite development is significantly lower, when compared to the control conditions of having methionine or SAM throughout the whole 48 hours of infection. Furthermore, having these metabolites only during these first hours, results in a similar development as the one observed for

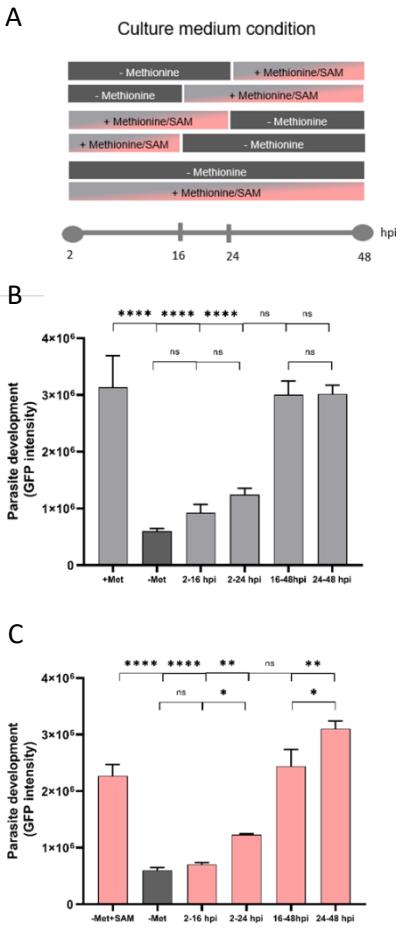

Figure 3 - **SAM is essential for *Plasmodium* replication inside the hepatocyte, in the late stage of liver infection**. **A.** Different medium conditions for the different timepoints. HepG2 cells infected with GFP-expressing P. berghei sporozoites in **B.** the presence of methionine and **C.** absence of methionine supplemented with SAM, during different time periods. 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.

the condition of depletion of methionine. In contrast, for the time courses of 16-48 and 24-48 hpi, parasite development is very similar between the supply of methionine or SAM and between time frames, but is also the same as the one showned for the positive controls with methionine or with SAM, during the entire period of 48 hours (Figure 3 B and C). Furthermore, the period of time between 16 and 24 hpi is already a sufficient amount of time, with SAM available in the medium, for the parasite to develop signicantly more from the first time point to the second (Figure 3 C). Considering previous evidences that *Plasmodium* starts to replicate its DNA, to form the multinucleated schizont at already 16 hpi, and the replication it self starts between 20-24 hpi, this data suggests that indeed SAM input and essenciality during liver stage might be associated to DNA and parasite replication, that is especially intensified during the second half of hepatocyte infection. It is also noteworthy, that when comparing the parasite development between the 16-48 and 24-48 hpi, upon supplementation of SAM, from the first to the second time course, this metabolite is able to potentiate *Plasmodium* development, increasing it to levels superior than the ones observed for the control condition (Figure 3 C), evidencing once more that SAM might be the key player for such an extraordinary multiplication rate.

<u>SAM levels inside the parasite depend on exogenous (host) supply</u>

Knowing that SAM plays an essential role during *Plasmodium* liver stage of infection, but that both the parasite and the host are able to produce this metabolite, because it's biological importance is universal to all living organisms, it was still necessary to accurately determine the source of this compound, inside the parasite. For this purpose, Hepg2 cells were infected with GFP-expressing *P. berghei* sporozoites. In order to quantify the amount of this molecule inside *Plasmodium* EEFs, the HepG2 cells infected with *P.berghei* parasites were used to perform immunofluorescent assay, to detect both the GFP and SAM signal, by fluorescence microscopy (Figure 4 A). To correctly determine how SAM levels inside the parasite would change accordingly to different medium conditions, the timepoint of 24 hpi was selected for this analysis, since at this time of infection, independently of having methionine sufficiency, methionine deficiency or SAM, *Plasmodium* parasites have the same size, because replication has not yet achieve its peak and subtle differences cannot be detected. When accessing SAM levels inside *Plasmodium* parasites that replicated equally at 24 hpi there is a significant reduction of SAM levels from the condition of methionine sufficiency to the condition of methionine depletion. Still, this decrease in SAM levels verified in methionine depletion, is restored upon the administration of SAM (Figure 4 B). To assess confirm that indeed the parasite does not rely on its own enzyme to synthesize SAM we used a parasite line where the knockdown of the MAT enzyme (SAMS) can be induced. The

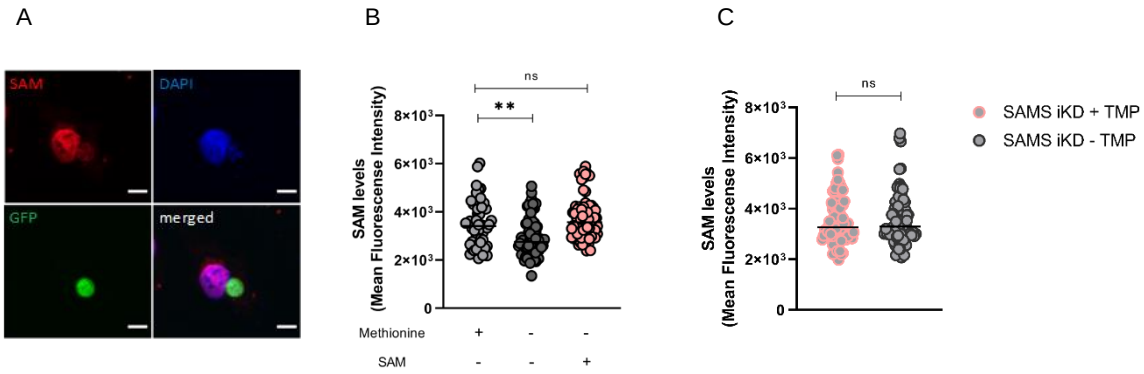

Figure 4 - **SAM levels inside EEF's depend on exogenous SAM supply. A.** Quantification of the parasite size and **B.** SAM levels inside the EEF's, at 24 hpi measured by immunofluorescence microscopy, for methionine sufficient, methionine depletion and methionine depletion with SAM administration. **C.** Quantification of SAM levels inside a SAMS iKD with TMP (phenocopies a wild type) and without TMP (phenocopies a knockout parasite for SAM synthetase enzyme at 24 hpi, measured by immunofluorescence microscopy. All graphs: Mann Whitney test. ns-not significant and ** p<0,01.

P.berghei SAMS-DD parasite line from the laboratory, phenocopies a *wild type* parasite upon the administration of trimethoprim (TMP, a small molecule that stabilizes the destabilizing domain, DD, that is in phusion with the protein) and in this condition is capable of producing SAM. In the absence of TMP, the DD domain is destabilized and the protein degraded, as a consequence the parasite is not able to synthetize SAM. The quantification SAM in both parasite strains showed no difference in SAM levels between parasites that are competent or not for SAM synthesis (Figure 4 C). These data suggest that the amount of SAM that seems to be essential for the parasite replication inside hepatocytes, is dependent on the exogenous supply of SAM. Importantly, the fact that parasites that cannot produce SAM display the exact same quantities of this compound as those that have a fully functional SAMS enzyme, suggests that *Plasmodium* encodes for a SAM transporter, that enables the exploitation of this host metabolite.

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

Due to its biological input in important metabolic pathways, all organisms have either a SAM producing enzyme, a transporter that uptakes this compound from the extracellular space, or both systems, that can ensure an efficient supply of this molecule. *Leishmania* spp. are intracellular kinetoplastids, phylogenetically close to the Apicomplexan phylum. These parasites encode for a SAM producing enzyme but also encode for a SAM transporter, AdoMet T1, which belongs to the family of folate biopterin transporters, a class of membrane proteins that fits in the major facilitator superfamily (MFS) [11]. When searching for sequences in *Plasmodium* genome, with homology to *Leishmania*'s AdoMet T1, using the BLAST tool, the sequence with highest similarity is *Pf*FT1, which in malaria parasites encodes for a folate transporter. Due to its already known and annotated function, we discarded the hypothesis of *Pf*FT1 being *Plasmodium* SAM transporter. However, alongside with *Pf*FT1, *Pf*FT2 and a third unknown protein PF3D7_1022200 were reported in a study associated with folate salvage in malaria parasites, with potential capacity of also being a folate transporte.[12] Because of its divergence from other known folate transporter proteins, these unknown protein was excluded as potential folate transporter, unlike FT1 and FT2. However, this result allied to the fact that these unknown protein (from now on referred to as LH1) belonged to the MFS family, like AdoMet T1, reflected the possibility of LH1 being a possible candidate for *Plasmodium* SAM transporter. On the other hand, for *Plasmodium*'s close relative Apicomplexa parasite *Toxoplasma gondii*, was showed that one of the virulence mechanisms of these parasites, resistance to sinefungin, was associated with a single nucleotide polymorphism (SNP) mutation, in a putative amino acid transporter.[13] In organisms such *Saccharomyces cerevisiae*, *Leishmania infantum* and *Trypanosoma brucei*, has been reported that, SAM and sinefungin a structural analog of SAM share, and therefore compete for the same transporter [14,11,15]. However, upon identifying the amino acid transporter in *T. gondii* responsible for the uptake of sinefungin,

the authors did not access the possibility of this same protein also transporting SAM. Our results show that indeed this *Tg*Transp protein transports SAM (data not shown), and therefore we used *Tg*Transp sequence to align with *Plasmodium* genome and identify possible candidate sequences for malaria parasites SAM transporter. The results from the BLAST alignment process of *Toxoplasma* SAM transporter with *Plasmodium* genome, identified MFR5 as the sequence with the highest identity score. *Pf*MFR5 is a membrane protein in malaria parasites, belonging to the family of major facilitator superfamily-related transporters (MFR). Furthermore, this MFR5 has been already studied and associated with a slow growing rate in blood stage, in *P.berghei* parasites deficient for this gene and a severely decreased number of male gametes exflagellation levels, that led to the inability of producing viable sporozoites. Interestingly, besides MFR5, this study also pinpointed a slow growing blood-stage behavior for MFS6 knockout parasites. *Pf*MFS6 is classified as having a protein domain that also belongs to the MFS family, and during blood stage, it is localized within the apicoplast outer membrane. Even though mfs6^{-} *P.berghei* parasites showed a very slow blood-stage growth, this parasite strain was still efficient in the production of viable sporozoites in the mosquito, to be transmitted to a new host. However, when infecting hepatocytes these parasites displayed distorted nuclei with clear evidences of a replication deficiency, resulting in the absence of production of liver-stage merozoites[16]. This phenotype is compatible with our hypothesis that SAM is important for liver stage replication. To test if LH1, MFR5 or MFS6 could potentially be *Plasmodium* SAM transporter, Hek293T, CHO and HepG2 mammalian cell lines were used to transfect the candidate protein sequences cloned in the mammalian expression vector pCDNA3, and to access [^{3}H]-SAM transport, protein expression and cellular localization, of the HA-tagged proteins. Radioactive labeled SAM transport assay was performed 48 hours after transfection of each cell line (Figure 5 A). For the three transport assays, *Tg*Transp was used as a positive control for SAM transport and cells transfected with the empty vector pCDNA3 (EV) and non-transfected cells (NT) were used as negative control. In all three transport analysis, for the

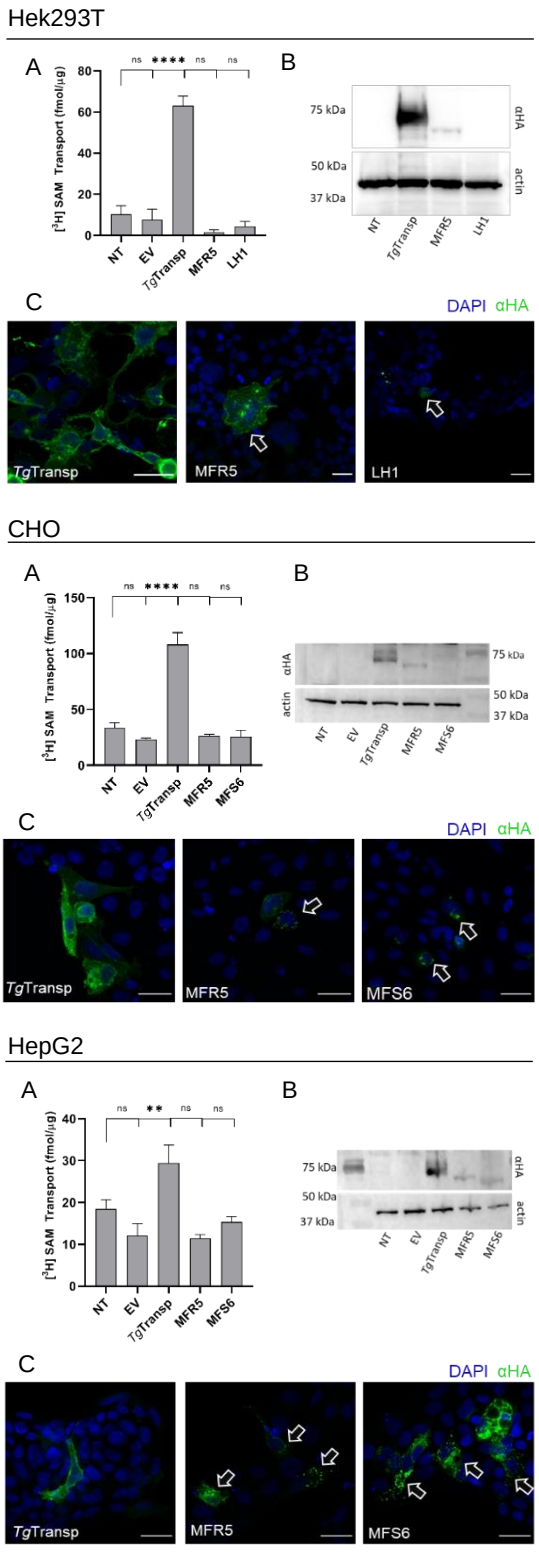

Figure 5 - **Heterologous expression and functional characaterization of *Plasmodium* SAM candidate transporters, in Hek293T, CHO and HepG2 mammalian cell lines. A.** [^{3}H] -SAM uptake for the indicated cell lines, non-transfected (NT), transfected with empty vector (EV) or transfected with *Plasmodium* SAM candidate transporters. **B.** Lysed cells from the radioactive transport were used for immunoblotting analysis with anti-HA antibody (*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, detected by immunofluorescence analysis.

positive control of *T.gondii* SAM transporter was verified a significant amount of uptake of the radioactive molecule (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. Though, when accessing the amount of SAM transported by the candidate sequences, for the three cell lines, we could not detect any 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). When examining protein expression by Western Blot (WB), the results were also indicative that in fact these proteins were not being expressed at the same extent as *T.gondii* SAM transporter (Figure 5 B). Similarly, and in agreement with the transport assay and protein expression results, the subcellular localization of the HA-tagged proteins, analyzed by immunofluorescence assay (IFA), showed that, in the three cell lines, apart from *Tg*Transp, that was clearly well placed within the cellular membrane, none of the candidate transporters seemed to be localized in the membrane of the transfected cells (Figure *5* C). In fact, LH1 protein expression was never detected in any of the three cell lines, by WB or IFA (shown for Hek293T and not shown for CHO and HepG2 cells), while MFR5 protein expression was low but detectable in all cell lines, by WB and IFA. MFS6 also display a similar pattern of protein expression, even though in HepG2 cells, expression was slightly higher (shown for CHO and HepG2 and not shown for Hek293T). Still, both MFR5 and MFS6 exhibit a similar intracellularly protein retention, in vesicle like-organelles, around cell nuclei (pointed by arrows in Figure 5 C), suggesting that these proteins were being trapped likely in the endoplasmatic reticulum (ER) or the Golgi apparatus, blocking protein trafficking to the membrane and explaining the absence of radioactive labeled SAM. These observations lead us to conclude that the heterologous expression using the mammalian model system, did not allow for the determination of the potential of any of these candidate proteins being SAM transporter in malaria parasites.

Conclusion

SAM and its biological role in transmethylation reactions and gene regulation, polyamines synthesis and cell proliferation or cell redox homeostasis, is transversal to all living organisms. In protozoan parasites such as *Leishmania* spp., *Trypanosoma* spp., *Toxoplasma gondii* and even bacteria or yeast, has been recognized the importance of S-adenosylmethionine and respective identified transporter proteins, that assure the supply of this essential molecule, from the surrounding environment [11,13,15]. During blood stage development, *Plasmodium* parasites are known to induce import of methionine from the plasma into the RBC, a low abundant amino acid in the erythrocytes. The parasite then uses methionine to synthesize SAM, a metabolite which is also present in low concentrations in the plasma and RBCs, through the activity of its own SAMS enzyme[17]. However, during liver stage of infection the parasite's SAMS enzyme is dispensable, although the parasite is dependent on this metabolite for intrahepatic development. These data imply that, in contrast to the erythrocytic stage, during hepatocyte infection, given the abundance of both methionine and SAM due to the liver efficient and specific methionine metabolism, *Plasmodium* might have adapted to fully rely on the host production of SAM. In this project, we showed that in fact, in the context of depletion of methionine, parasite development and growth is significantly impaired. However, this reduction in parasite load, is restored when administrating exclusively SAM, and not any other of the methionine metabolism byproducts. Building on the relevant function of SAM for the successful outcome of infection, we show that, SAM plays a crucial role during the DNA and parasite replication processes. In fact, SAM is only necessary in the second half of the infection period, during the massive events of nuclei multiplication. When trying to access the source of the amount of SAM that is required for intrahepatic replication by quantification of SAM levels inside parasites, we show that not only this metabolite concentration inside the parasite is dependent on the exogenous supply, but also that a parasite that has its SAMS enzyme inactivated, has the exact same amount of SAM that a *wild type* parasite, suggesting that indeed *Plasmodium* is hijacking this compound

from the best supplier possible, the mammalian hepatocytes. These set of results point to the existence of a mechanism of exploitation of the host metabolite. Considering *Plasmodium's* close protozoan relatives, for which SAM transporter proteins have been identified, we pinpointed three possible candidates that either have homology to these confirmed SAM transporters or had phenotypic and functional features, that fit with our hypothesis of host SAM essentiality during liver development. Using mammalian cell lines (Hek293T, CHO and HepG2 cells) as heterologous protein expression systems, we analyzed [^{3}H]-SAM transport, protein expression and cellular localization, upon transfection with the HA-tagged proteins LH1, MFR5 and MFS6. However, in contrast to what was observed in the positive control of *Toxoplasma gondii Tg*Transp, for neither of these candidate proteins was possible to confirm if they have the potential to be *Plasmodium* SAM transporter, due to the defective protein expression and trafficking, confirmed by immunoblotting and immunofluorescence analysis. These obstacle upon heterologous expression and functional characterization of these *Plasmodium* SAM transporter candidates in a mammalian cell line model, might be justified by the existence of possible molecular tags in *P.falciparum* candidate sequences, that could be redirecting or retaining these proteins in what seems to be the ER of the cell or endomembrane system vesicles. To try to surpass these adversities, the same heterologous expression strategy must be employed in other heterologous systems, like yeast or bacteria, that represent much simpler systems for protein production. Furthermore, an *in silico* analysis of these protein sequences must be consider, in order to identify possible signaling sequences, that could eventually be responsible for the accumulations of these recombinant proteins inside the cells. Overall, with this work, we clarified the dependency on host produced SAM by *Plasmodium*, specifically to achieve a high replication rate and generate a high number of RBCs-infective merozoites. Furthermore, the identification of a possible SAM transporter in *Plasmodium* parasites, that is enabling this exploitation of host methionine metabolism and SAM metabolic pool, embodies a future target for prophylactic treatments, that might act upon the symptomatically silent stage of the infection, preventing the formation of new parasites, blocking the arising of the disease and allowing for progress towards malaria eradication.

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