SORAYA SOLEDAD BOSCH

Análise da catálise da Aspartato Carbamoyltransferase dentro do metabolismo de aminoácidos do parasita efetor da malária humana *Plasmodium falciparum*

Tese apresentada ao programa de Pós-graduação em biologia da relação patógeno Hospedeiro do Departamento de Parasitologia do Instituto de Ciências Biomédicas da Universidade de São Paulo e ao programa de doutorado do departamento Drug Design da Universidade de Groningen para obtenção da dupla Titularidade de Doutor em Ciências.

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Orientador: Prof. Dr. Carsten Wrenger

Co-Orientador: Dr. Mathew R. Groves

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Analysis of the ATCase catalysis within the amino acid metabolism of the human malaria parasite *Plasmodium falciparum*

Ph. D. thesis presented to the Post-graduation Program Biology of Host-Pathogen Interactions at the Institute of Biomedical Sciences of the University of Sao Paulo and at the Drug Design department of the University of Groningen, to obtain the double degree of Doctor in Science.

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Supervisor: Prof. Dr. Carsten Wrenger

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CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB Nº **615/13** referente ao projeto intitulado: "*Análise da catalise da ATCase no metabolismo de Plasmodium Falciparum e Trypanosoma cruzi*" sob a responsabilidade de **Soraya Soledad Bosch,** foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSH- COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº196 de 1996.

São Paulo, 05 de setembro de 2013.

p/ Dimes

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Esta tesis está dedicada a mi familia, por todo el esfuerzo que hicieron desde el día 1 en traerme a San Pablo para construir este camino, por dejarme abrir las alas y volar lejos de casa, por todas las viajes para visitarme alrededor del mundo, por todas las despedidas, por todo el cariño y amor que les tengo, pero sobre todo por lo mucho que los extraño todos los días.

This thesis is dedicated to my family, for all the effort they give me since day 1 when they bring me, literally, to Sao Paulo to start this new life, for let me open my wings, for all the trips to visit me all around the world, for the goodbyes, for all the love I have for them, and most important for all I miss them every day.

Abstract (Dutch)

BOSCH, S.S. **Analyse van de ATCase katalyse binnen het aminozuurmetabolisme van de *Plasmodium falciparum* malariaparasiet.** 2018. 103p. PhD (Parasitology) −Institute of Biomedical Sciences, University of São Paulo and University of Groningen, São Paulo, 2018.

Malaria, veroorzaakt door de *Plasmodium* parasiet, blijft met over 600,000 doden per jaar één van de meest verwoestende ziektes van onze tijd. *Plasmodium falciparum,* die de tropische variant van malaria veroorzaakt, is de meest gevaarlijke soort binnen het *genus.* Het doel van dit proefschrift is het evalueren van het belang van het enzym aspartaat carbamoyltransferase (ATCase) binnen het aspartaatmetabolisme van de *P. falciparum* parasiet. Het Open Reading Frame dat voor het eiwit codeert is geïdentificeerd en gekloneerd. Na het gecodeerde eiwit recombinant tot expressie te brengen konden we conformationeel en kinetisch inzicht verkrijgen met behulp van kristallisatie-experimenten, en konden we de kristalstructuur van het eiwit ophelderen in “T” (tense, gespannen) en “R” (relaxed, ontspannen) vorm. Daarnaast laten we het belang van *Pf*ATCase zien voor de proliferatie van de malariaparasiet aan de hand van mutagene studies en eiwit-interferentie experimenten. Zoals voorspeld door bio-informatica instrumenten heeft het eiwit een apicoplast-targeting sequence, een aantal aminozuren die ervoor zorgen dat het eiwit in de apicoplast belandt. Hiermee is de lokalisatie van het eiwit in de apicoplast bewezen.

Voorts richt dit werk zich op het onderzoeken van ATCase als geneesmiddeltarget. De resultaten van de dosis-response studies en *in vivo* eiwitinterferentie experimenten bewijzen dat het eiwit een goede kandidaat is als geneesmiddeltarget.

Kernwoorden: *Plasmodium falciparum,* Kristalstructuur, Pyrimidine, Geneesmiddeltarget-validatie.

Abstract (English)

BOSCH, S.S. **Analysis of the ATCase catalysis within the amino acid metabolism of the human malaria parasite *Plasmodium falciparum*** 2018. 103p. Ph.D. (Parasitology) −Institute of Biomedical Sciences, University of São Paulo and University of Groningen, São Paulo, 2018.

Malaria, caused by *Plasmodium* spp., remains with more than 400.000 deaths per year one of the devastating diseases of our time. *Plasmodium falciparum*, which causes tropical malaria, is the most dangerous one leading to severe malaria. The aim of this thesis was to evaluate the necessity of the aspartate carbamoyltransferase (ATCase) within the aspartate metabolism of the human malaria parasite *Plasmodium falciparum*. The respective open reading frame has been identified and was cloned; with the encoded enzyme recombinantly expressed we could get conformational and kinetic insights by crystallization experiments, we could resolve the crystal structure of the enzyme, in "T' (tense) and "R' (relaxed) states. Moreover, in this work, we show the importance of the *Pf*ATCase for the proliferation of the malaria parasite by mutagenic studies and protein interference experiments.

As predicted by bioinformatic tools the protein bears an apicoplast-targeting sequence and therefore its localization was determined here. Furthermore, this work is focusing on the ATCase as a drug target, dose-response experiments and protein interference studies with *in vivo* parasites, proves our hypothesis and the drugability of the enzyme.

Keywords: *Plasmodium falciparum*, Crystal structure, Pyrimidine, Drug target validation.

Resumo

BOSCH, S.S. **Análise da catalises da Aspartato Carbamoyltransferase dentro do metabolismo de amino ácidos do parasita efetor da malária humana *Plasmodium falciparum*.** 2018. 103f. Tese (Doutorado em Parasitologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo e Universidade de Groningen, São Paulo, 2018.

A malária, causada por *Plasmodium* spp., continua sendo uma das doenças mais devastadoras do nosso tempo, com mais de 600.000 mortes por ano. O *Plasmodium falciparum*, é o parasita mais perigoso que produze à malária severa. O objetivo desta tese foi avaliar a necessidade da aspartato carbamoiltransferase (ATCase) no metabolismo do aspartato do parasita da malária humana *Plasmodium falciparum*. O respectivo ORF foi identificado e clonado; com a enzima recombinante expressa, conseguiu-se obter informações conformacionais e cinéticas. Por meio de experimentos de cristalização obteve-se a estrutura tridimensional da enzima, nos estados "T" (tenso) e "R" (relaxado). Além disso, neste trabalho, mostramos a importância do *Pf*ATCase para a proliferação do parasita da malária através de estudos mutagênicos e experimentos de interferência de proteínas. Como previsto por ferramentas bioinformáticas, a proteína possui uma seqüência de direcionamento de apicoplasto e, portanto, sua localização foi determinada em este trabalho.

Os ensaios de drogas, assim como, os ensaios de proliferação dos parasitas *in vivo*, demonstrou que a ATCase é um alvo terapêutico no parasita.

Palavras−chave: *Plasmodium falciparum*. Pirimidinas. Sínteses de pirimidinas. Estrutura cristalográfica. Alvo terapêutico.

CHAPTER 1

INTRODUCTION

1.1. History of Malaria

Malaria is one of the oldest and most devastating parasitic diseases in humans. Hippocrates (460 BC–370 BC) was the first to describe clearly the different types of malaria depending upon the periodicity of the fever patterns. The Romans recognized the relationship of stagnate water in the swamps surrounding Rome and the presence of fevers during the summer months. Initially, these fevers were attributed to bad air —*mal aria* (*mal* = bad, *aria* = air) — since they thought that the foul vapors emanating from the stagnate water and swamps were the cause of the disease. Though this explanation was incorrect, at least it represented an appreciation of the importance of stagnate water somehow being related to the summer fall febrile illnesses among the Romans [1]. 300 years later, the Italian term *mal' aria* was introduced into England by Horace Walpole in a letter he wrote on 5 July 1740 [2].

In 1800s, malaria was endemic in all of Central Europe; to this point it was well-known that patients who died of malaria had black deposits in their organs. Heinrich Meckel conducted an autopsy of a patient with mental illness and found the brain to be dark brown, but he did not associate the pigment with malaria. Only a few years later did Virchow and Frerichs establish the causal relationship of this brown pigment to malaria, and malaria was recognized to be a disease of the blood.

Charles Alphonse Laveran, the first scientist to see the malarial organism in blood in 1880, intensely disliked the name malaria. He considered the term unscientific and vulgar, preferring the name “paludisme” (Latin: *palus* = swamp) which is still used in France today [3].

In 1897, motivated by his mentor Manson, Ronald Ross started to research whether mosquitoes could transmit malaria. He detected characteristic pigmented bodies in the stomach wall of mosquitoes, now known to be *Anopheles* species. After several years, Ross would prove the complete life cycle of the parasite [4].

In 1898, Giovanni Battista Grassi, an Italian zoologist, unequivocally identified *Anopheles claviger* (Greek *anofelís* = good-for-nothing) as the sole vector of malaria in Italy.

Between 1885 and 1892, Bartolomeo Camillo Golgi studied the asexual cycle of the malaria parasite. He observed that the febrile bouts coincided with segmentation. In addition, Golgi found that the two types of intermittent malarial fevers (tertian, 48 hour periodicity, and quartan, 72 hour periodicity) were caused by different species of *Plasmodium* [5].

In 1900, Manson provided convincing experimental proof of the mosquito's role in propagating malarial fevers. He imported *Anopheles* mosquitoes from Rome, which were allowed to bite the hand of his son, and after 14 days the son had a severe attack of fever [6].

In 1947, Henry Shortt and Cyril Garnham were finally able to show a primary division of the parasite in liver cells [7]. Subsequently, Krotoski and colleagues discovered that some *P. vivax* strains, which are called hipnozoites today, could remain in this liver stage for several months [8].

Until the 19th century, malaria was spread throughout the north of Europe, North America and Russia; in the south of Europe the transmission was intense. However, it has since been eradicated from these areas, dropping the number of cases and deaths in these regions. However, in the tropics there was an increase in malaria cases because of the selection of resistant mosquitoes to insecticides and parasites against the drugs.

Today, there are more than 200 known species of the genus *Plasmodium*, but just five of these are agents of human malaria: *P. vivax, P. ovale, P. malariae, P. knowlesi* and the most virulent, *P. falciparum* [9]. The genus *Plasmodium* belongs to the phylum Apicomplexa, which consists of a large group of unicellular eukaryotes sharing the same invasion machinery, the apical complex.

1.1.1. Distribution

Malaria infections were responsible for an estimated 216 million clinical cases in 2016, most were in Africa (90%), next was South-East Asia region (7%) and the Eastern Mediterranean region (2%). The population at risk is distributed in tropical and sub-tropical areas, where of the 91 countries reporting endogenous malaria cases in 2016, 15 countries (all in sub-Saharan Africa, except India) carried 80% of the global malaria burden (Fig. 1) [10].

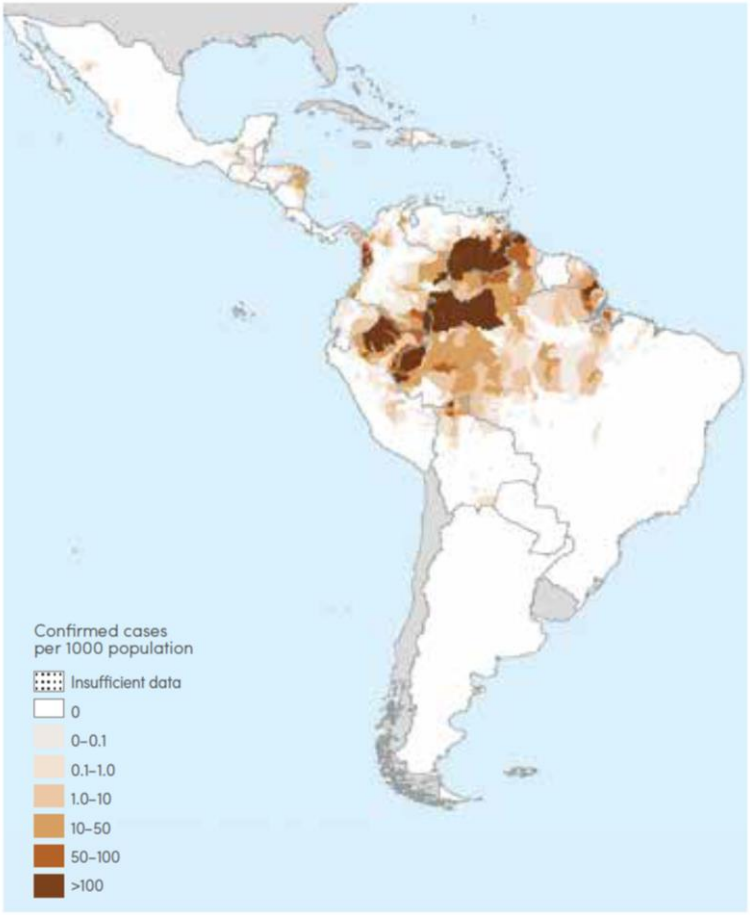
The incidence rate of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk, between 2010 and 2016. The South-East Asian region recorded the most significant decline (48%), followed by the Americas (22%) and the African region (20%). Despite these reductions, between 2014 and 2016 substantial increases in case incidence occurred in the Americas, and marginally in the South-East Asian, Western Pacific, and African regions.

Figure 1-World distribution and cases of death through malaria.

Spots demonstrate the global distribution of *P. falciparum* and *P. vivax* infections correlating with the number of deaths caused by malaria in 2016 (indicated in blue) Data were available at http://www.who.int/en/. 29.01.2019.

Plasmodium falciparum is the most prevalent malaria parasite in sub-Saharan Africa, accounting for 99% of estimated malaria cases in 2016. Outside of Africa, *P. vivax* is the predominant parasite in the Americas, representing 64% of malaria cases. Indeed, Brazil reported a 72% decline of local *P. falciparum* cases between 2010 and 2016. Furthermore, the transmission of the disease is focalized: nearly 45% of cases in Brazil come from 15 municipalities in Acre and Amazonas (Figure 2) [10].

Figure 2- Distribution of cases in Latin America.



The map shows the confirmed malaria cases per 1000 population of 2016. The population at risk is estimated to be 126.8 million. The confirmed cases decreased from 678.200 in 2010 to 562.800 in 2016 (17% decrease) and deaths decreased from 190 in 2010 to 110 in 2016 (42% decrease) [10].

1.1.2. Control and Resistance

Two of the mechanical barriers used to control the vector are insecticide-treated mosquito nets (ITNs) and indoor residual spraying in areas of high risks. Between 2014 and 2016, manufacturers reported they had delivered 582 million ITNs globally. Of these, 505 million ITNs were delivered in sub-Saharan Africa. Another type of net also in use is the long-lasting insecticidal net that contains pyrethroids, which protects for up to 3 years and is highly recommended, especially for young children and pregnant women, in endemic areas. However, mosquito resistance to pyrethroids is already reported and, in some areas, even all four classes of insecticides have already shown a decreased effect [10].

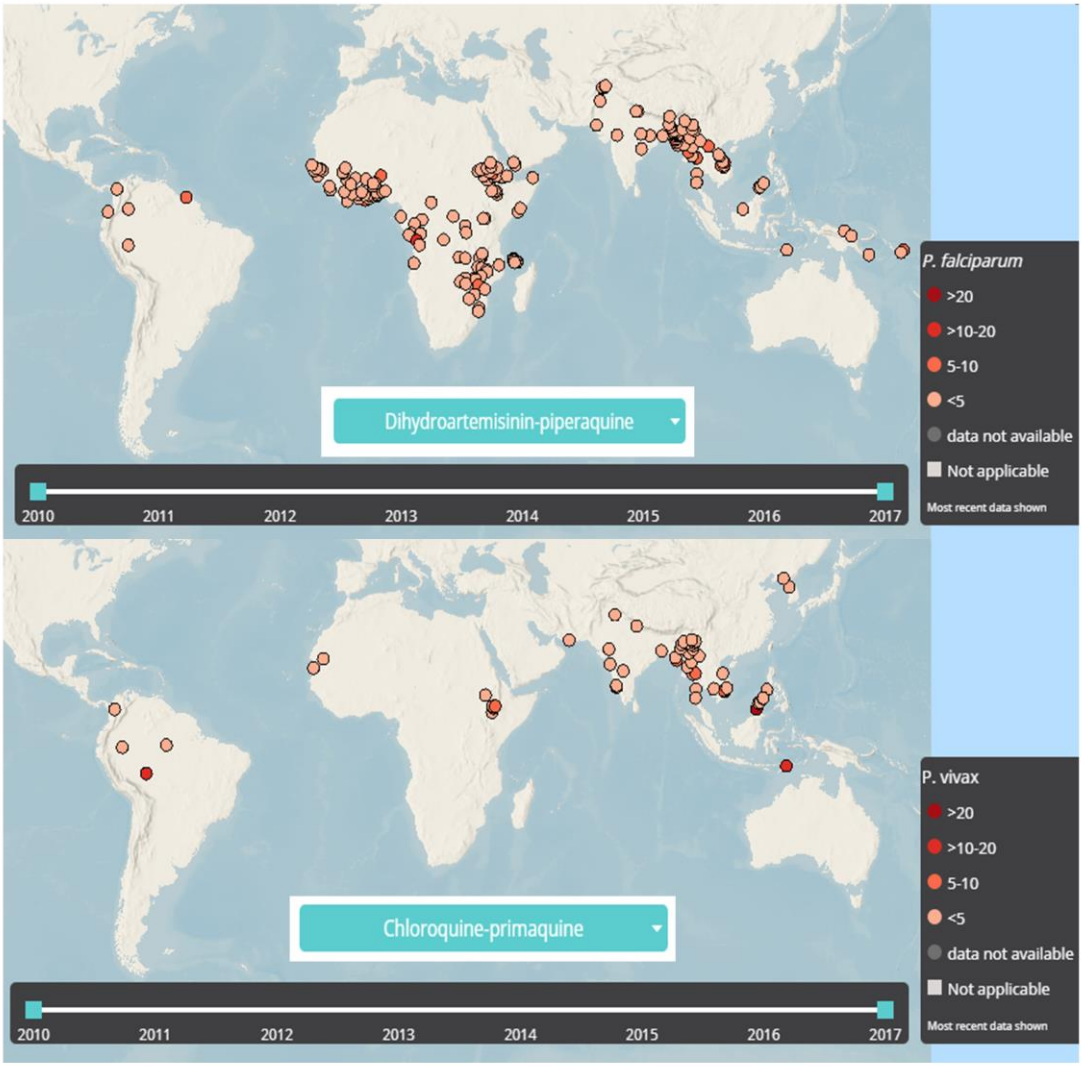
There are several vaccines in develop, which inhibit the proliferation of the parasite in different points, from sporozoite to sexual stage development. The most tested vaccine candidate for the prevention of *P. falciparum* is RTS,S/AS01. The RTS,S was implemented on a pilot scale, nonetheless, the major limitation of this candidate appears to be maintaining the high antibody levels [11].

One of the oldest known antimalarials is quinine, an alkaloid derived from the bark of the cinchona tree. It was brought in the 17th century from Peru to Europe and it was first isolated in the 19th century by French researchers Pierre Joseph Pelletier and Joseph Bienaimé Caventou. It remained the antimalarial drug of choice until the 1940s when chloroquine (CQ) took over [12]. Already in 1934, Hans Andersag had discovered the quinine-related antimalarial [13], which was used massively worldwide. All 4-aminoquinolines—quinine, CQ, mefloquine, amodiaquine, and quinoline-methanols—are supposed to interfere with the plasmodial haem detoxification inside the digestive vacuole (DV), thereby killing the parasite [14, 15]. CQ had several advantages such as high efficacy, low production costs, and low toxicity; nonetheless after the selection of CQ-resistant *Plasmodium* strains in the late 1950s it was necessary to find new drugs [3]. The resistance is mediated through mutations in the *P. falciparum* chloroquine resistance transporter (*Pf*CRT) located on the DV membrane allowing the efflux of CQ [16, 17].

Antifolates were discovered as an alternative, acting through the inhibition of the biosynthesis of tetrahydrofolate (the active form of folate, vitamin B9), solely present in the parasite. Antibiotics like sulfadoxine (a sulfonamide antibiotic) inhibit the enzyme dihydropteroate synthetase, while pyrimethamine inhibites the dihydrofolate reductase and dihydropteroate synthase [18, 19]. To enhance their effect, both drugs were used in combination to inhibit two different steps in the same biosynthetic pathway. Nevertheless, in 1970 the selection of resistant strains was noted in Thailand; it spread rapidly through Asia and to the African continent. Indeed, selected strains resistant against all known antimalarials were spreading fast, which consequently required the development of new drugs. At this point artemisinin was discovered and isolated from a Chinese herb, *Artemisia annua*. Artemisinin was effective against all multi-drug resistant parasites [20]. Several artemisinin derivates exist that all reduce blood parasitaemia very rapidly. However, the drug´s half-life is very short, and for that reason the drug is given only in combination with other antimalarials, and is known as artemisinin combination therapies (ATCs) [21]. These ACTs are part of the recent success in global malaria control, and protect their efficacy for the treatment of

malaria, which is a global health priority. The main advantage of ACTs is that the artemisinin quickly kills most of the malaria parasites and the partner drug clears the remaining ones. However, the efficacy of ACTs is threatened by the emergence of both artemisinin and partner drug resistance. Today ATCs are the treatment of choice for uncomplicated malaria. Nevertheless, parasites with resistance to artemisinin have already been identified in 5 countries of South East Asia (Cambodia, Laos, Myanmar, Thailand, and Vietnam) [10].

Figure 3- Distribution of the resistant strains of *P. vivax* and *P. falciparum*



In this interactive map, it is possible to select the drug and see the where the resistant strains are localized. The image above shows the strain *P. falciparum* resistance strain to dual treatment Dihydroartemisinin with Piperaquine. The

image below shows the distribution of *P. vivax* resistant strain to Chloroquine with Primaquine. The map is available in http://apps.who.int/malaria/maps/threats/[10].

Another strategy of new antimalarials is to block the parasite transmission by targeting the liver or the sexual stages of the parasite. Till today there was just one drug family available attacking also hypnozoites. The 8-aminoquinolines like as primaquine is the only available drug to use for relapsing malaria caused by *P. vivax* or *P. ovale*. The mechanism of action is still unclear but probably involves cytochrome P450s and monoamine oxidase, as well as the formation of reactive intermediates [22]. However, this drug is not recommended for glucose-6-phosphate-deficient patients as well as for pregnant woman since the drug could produce haemolytic anaemia.

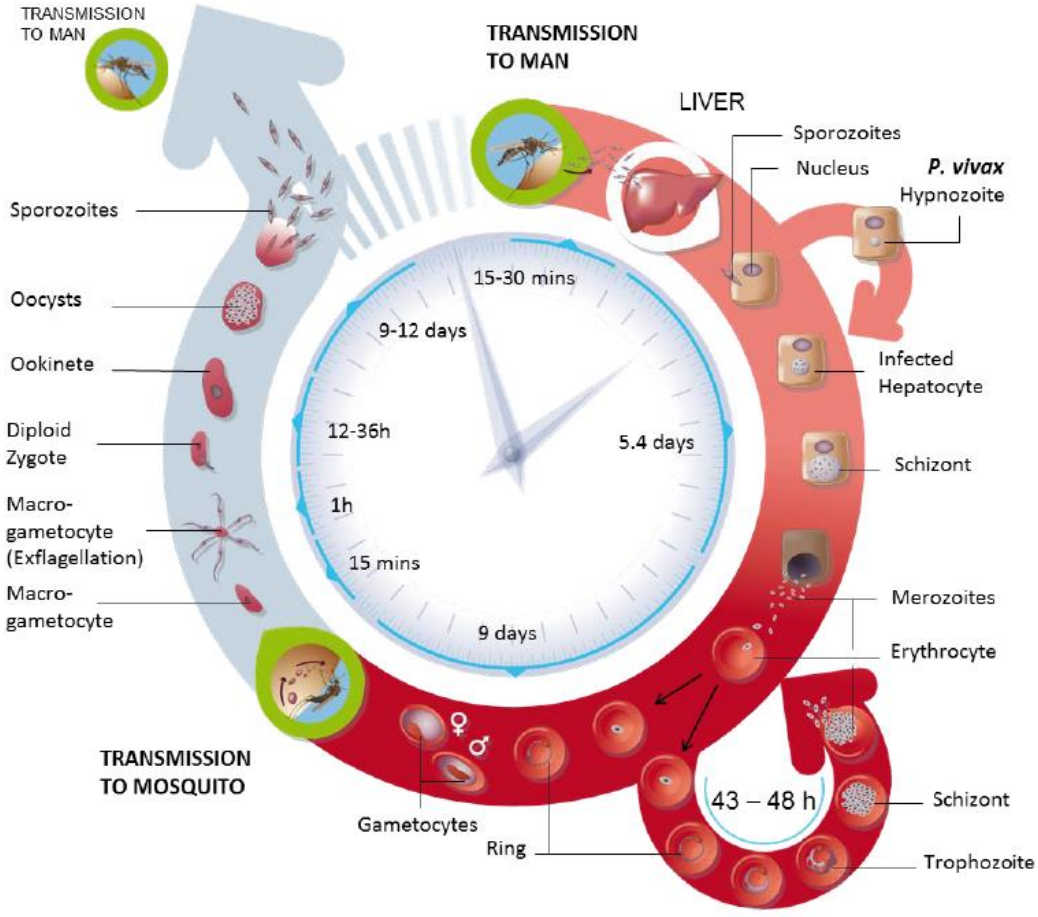
For *P. vivax*, chloroquine remains an effective first-line treatment in many countries. Actually, the first-line treatment policy is Artemether-lumefantrine (AL) in Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama and Suriname; artesunate-mefloquine (AS MQ) in Brazil, Peru and Venezuela; and chloroquine together with primaquine in Costa Rica, Dominican Republic, Guatemala, Haiti, Honduras and Nicaragua. Apart from one small study conducted in Suriname in 2011 (which detected a 9% treatment failure rate of AL), studies in the period 2010–2016 showed effective first-line treatment for *P. falciparum*. Artemisinin resistance was suspected in French Guiana, Guyana and Suriname, but molecular markers of artemisinin resistance (*Pf*K13 C580Y) were only detected in a retrospective study of Guyanese samples from 2010, and a more extensive survey in 2016 confirmed the emergence of artemisinin resistance with a genetic profile compatible with a South American origin [10].

The inevitable emergence of antimalarial drug resistance [23, 24] forces continuous efforts toward the discovery and development of new antimalarial drugs [25–30]. Recently, the Food and Drug Administration has approved the drug Krintafel (tafenoquine) as a single dose medication for the treatment of hypnozoites caused by *Plasmodium vivax*. Nonetheless, the need is urgent for novel chemotherapeutic targets. New drugs should be created [31–35] to target solely the parasite with minimal (or no) toxicity to the human host. Therefore, good drug targets should be sufficiently different from those of the host, or ideally be absent from the host altogether.

1.2. Comprehensive life cycle of *Plasmodium*

All *Plasmodium* spp. share a complex life cycle within the insect and the vertebrate host. Human malaria is transmitted via the female *Anopheles* mosquito, which injects sporozoites during a blood meal. After invading liver cells, each sporozoite can mature into up to 40,000 merozoites, which will then be released into the bloodstream via merosomes [36]. However, *P. vivax* and *P. ovale* are able to form hypnozoites, an attenuated form of liver schizont, which can remain in the liver for several months before proceeding to the blood stage. The released merozoites can infect red blood cells (RBCs), causing these cells to remodel in order to facilitate their proliferation and differentiation from ring to trophozoite and then into schizont.

Figure 4- Comprehensive lifecycle *of Plasmodium falciparum*



The life cycle of *Plasmodium* spp. is occurring in two hosts. After a blood meal of the female of *Anopheles* mosquito, the parasite infects human hepatocytes and proliferates into merozoites. While an infection with *P. vivax* or *P. ovale* can lead to a sporozoite differentiation into hypnozoites in all other cases merozoites will directly infect RBC and replicate via schizogony. This asexual replication can be repeated infinitely. Other merozoites develop into

male and female gametocytes that infect mosquitoes when taken up by the next blood meal. The sexual stages mature into the mosquito gut where they fuse and form an ookinete. The ookinete develops into the oocyst which releases new sporozoites that migrate to the insect's salivary glands (Modified from [37]).

One of the reasons for the high virulence of *P. falciparum* is the export of *Pf*EMP1 (*Plasmodium falciparum* infected erythrocyte membrane protein 1) to the infected RBC (iRBC) surface. *Pf*EMP1 allows the iRBC to bind to the endothelium, avoiding its clearance by the spleen and leading to a disrupted blood flow which can cause cerebral or placental malaria when they occur in the brain or placenta [38]. The asexual blood cycle is responsible for anemia and periodic fevers characteristic of the disease as it ends with the haemolysis and release of new merozoite forms into the bloodstream. While most of the merozoites will reinfect other erythrocytes, some differentiate into male and female gametocytes. These gametocytes differentiate into gametes within the mid-gut of a female *Anopheles* mosquito after the next blood meal, and sexual proliferation takes place. After the diploid zygote forms, the zygote differentiates to the ookinete and later oocyst, and subsequently new sporozoites are formed. The released sporozoites migrate to the mosquito's salivary gland, where they will be transmitted during its next blood meal [37].

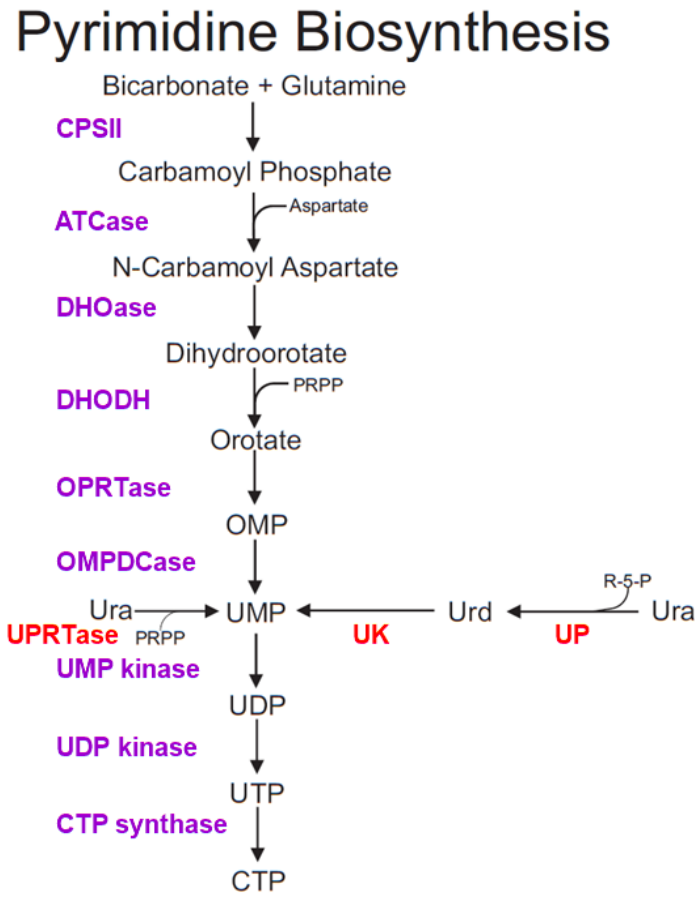
1.3. Pyrimidines Biosynthesis

Our group has focused on the need for certain nutrient requirements for the the malaria parasite to proliferate [39–42], such as vitamins, sugars, and amino acid metabolites [43–45]. In the latter nutrient, we focused on aspartate metabolism. This metabolite is not only important for the maintenance of the unique tri-carbon-acid cycle in *P. falciparum* [39, 46]–[48] as well as a constituent within the protein biosynthesis, but it is also involved in a variety of metabolic reactions [49–52] such as the initiation of pyrimidine biosynthesis, which has fundamental importance in the survival of the malaria parasite [44], [53], [54].

Furthermore, in *Plasmodium* species, besides the DNA, the pyrimidine nucleotide is also involved in the biosynthesis of RNA, phospholipids, and glycoproteins [55–57]. *Plasmodium* parasites rely on the *de novo* pyrimidine–biosynthesis pathway for their proliferation (Fig. 5). All the genes encoding for the enzymes of the *de novo* synthesis, as well as the uracil pyrimidine salvage enzymes, were found in the genome database of the parasite. The enzyme activities include inter-converting uracil, uridine, and UMP of the pyrimidine salvage pathway (uracil phosphoribosyltransferase, UPRT; uridine phosphorylase, UP; uridine kinase, UK) were demonstrated in *P. falciparum* [58]. Despite the presence of the salvage pathway, the parasites

depend exclusively on the *de novo* pathway as a source of pyrimidines for their survival, which may relate to the fact that mature mammalian erythrocytes lose their ability to synthesize pyrimidines. [56], [59], [60].

Figure 5- Biosynthesis of Pyrimidines.

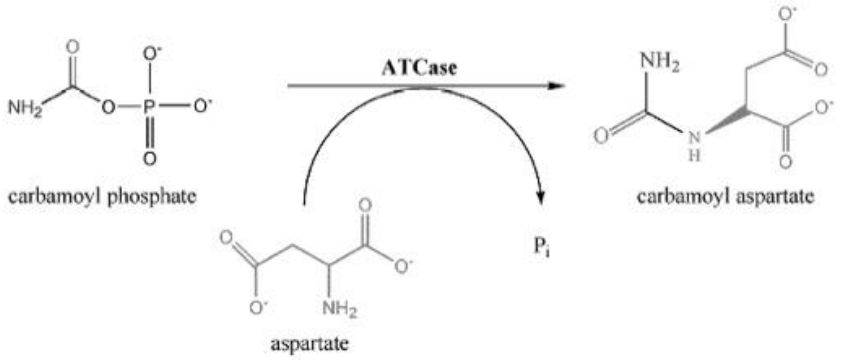


Pyrimidine biosynthesis in *Plasmodium falciparum*. In purple, there are the enzymes present in the parasite and in red, there are the salvage enzymes.

The first enzyme of the pathway is the carbamoyl phosphate synthetase II (CPSII) that is responsible for the formation of carbamoyl phosphate from bicarbonate, glutamine, and ATP [61]. This enzyme with the molecular mass of 275kDa is one of the biggest genes in the genome of *P. falciparum*. Flores et al. show that this enzyme is a control point, being inhibited by UTP and activated by α-D-phosphoribosyl pyrophosphate (PRPP) [61].

The second enzyme, aspartate transcarbamoylase (PF3D7_1344800, ATCase) catalyzes the condensation of aspartate and carbamoyl phosphate to form N-carbamoyl-l-aspartate and inorganic phosphate.

Figure 6 - Reaction of Aspartate Carbamoyltransferase



The substrates of the reaction are carbamoyl phosphate and aspartate, the ATCase catalyse the transference of carbamoyl group to the aspartate, releasing inorganic phosphate and carbamoyl aspartate.

From this step, the pathway follows basically the same steps found in the human host and in other eukaryotes: orotate is formed by dihydroorotase (DHOase) and dihydroorotate dehydrogenase (DHODH). The enzyme orotate phosphoribosyl transferase (OPRTase) catalyzes the formation of orotidine 5′-monophosphate (OMP) from PRPP and orotate, the fifth step within the pyrimidine biosynthesis. The metabolite PRPP is synthesized by an enzyme from outside of the pathway, namely phosphoribosylpyrophosphate synthetase (PRSase), which will be described later. Orotidine 5′-monophosphate decarboxylase (OMPDCase) catalyzes the final step of the pathway: the decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP), which is the precursor of all other pyrimidine nucleotides and deoxynucleotides needed for nucleic acid synthesis [62]. These last two steps of the pyrimidine biosynthesis in *P. falciparum* are catalyzed by a heteromeric complex that consists of two homodimers of *Pf*OPRTase and *Pf*OMPDCase encoded by two separate genes [63, 64].

In prokaryotes and plants, the first three enzymes of the pathway–CPSase, ATCase, and DHOase–are encoded as separate proteins that either act independently or are associated into complexes. In contrast, in animals the **C**PSase, **A**TCase, and **D**HOase activities are assembled as different domains within a single multifunctional polypeptide of approximately 240 kDa named

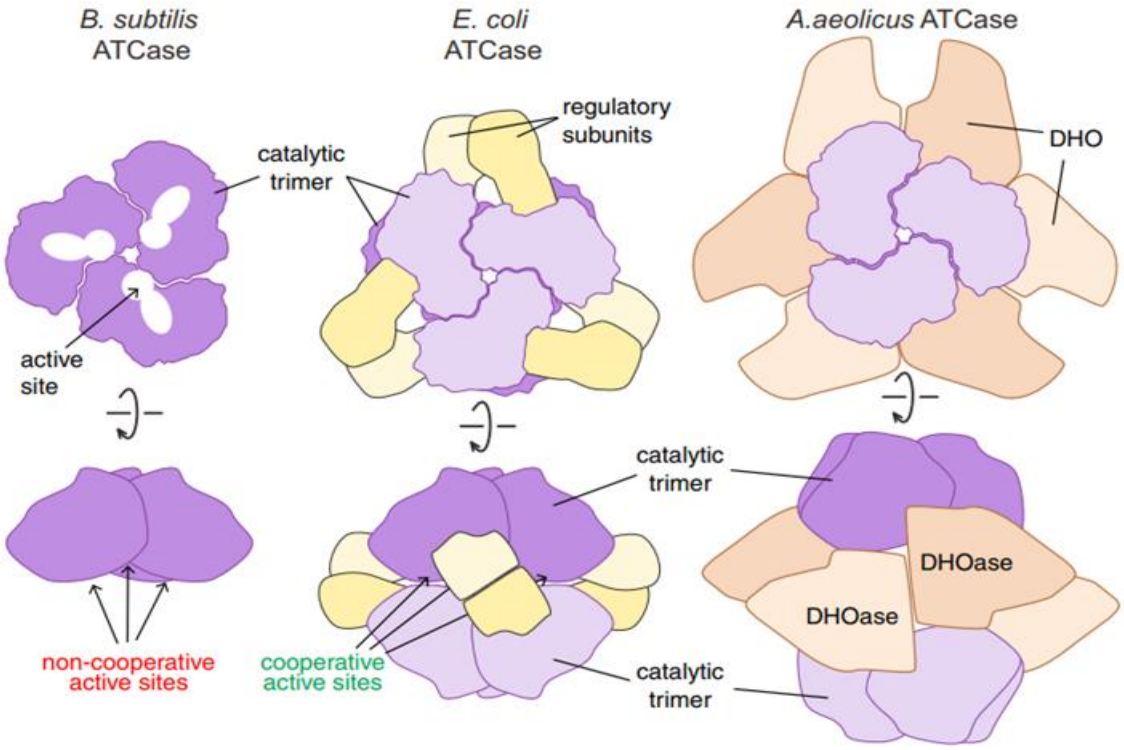
CAD (an acronym for the three catalytic activities), in which the ATCase occupies the most C-terminal position [65]. As well as CAD, in humans OPRTase and OMP decarboxylase are fused together in one gene. In fungi, CPSase and ATCase are fused into a CAD-like polypeptide that contains a catalytically inactive DHOase-like domain, and this activity is provided by a separate protein.

In *Plasmodium*, the ORFs of the first six enzymes – *Pf*CPSII (chromosome 13), *Pf*ATCase (chromosome 13), *Pf*DHOase (chromosome 14), *Pf*DHOD (chromosomes 7 & 9), *Pf*OPRTase (chromosomes 5&7), *Pf*OMPDCase (chromosome 10), including *Pf*CA (chromosome 11) and *Pf*UP (chromosomes 5&7) – were identified and located on various chromosomes. Krungkrai et al. show that the malarial CPS, DHOase, and OPRTase genes were conserved to bacterial counterparts, whereas ATCase, DHODH, and OMPDCase were mosaic variations that were homologous to both bacterial and eukaryotic counterparts, including human [58].

1.3.1. Aspartate transcarbamoylase (ATCase)

Since the 50s the aspartate transcarbamoylase (EC 2.1.3.2) from *Escherichia coli* has been studied intensively, being a paradigm of feedback inhibition and a model of cooperativity and allosteric regulation. Today, it is present in most textbooks on kinetics [49, 66, 67]. In prokaryotes, ATCases are organized into three major groups (Fig. 7) depending on whether the catalytic trimers function independently (e.g., *Bacillus subtilis*) or are associated face to face through DHOase (*Aquifex aeolicus*) or regulatory dimers (*Escherichia coli*) [68]. This last one was fully characterized by William Lipscomb and colleagues. *Ec*ATCase is known to be a highly regulated enzyme: it controls the rate of pyrimidine biosynthesis in response to cellular levels of both purines and pyrimidines [69]. As an allosteric enzyme, ATP and CTP, end products of purine and pyrimidine pathways respectively, stimulate and inhibit the ATC catalytic activity. These two allosteric effectors can bind to the regulatory subunits, producing conformational changes in the structure, which relies on the regulation of the entire pathway [70, 71]. Indeed, the dissociation of the ecATCase holoenzyme results in isolated catalytic trimers that, similar to *B. subtilis* ATCase (Fig. 7), lack cooperativity and allosteric regulation.

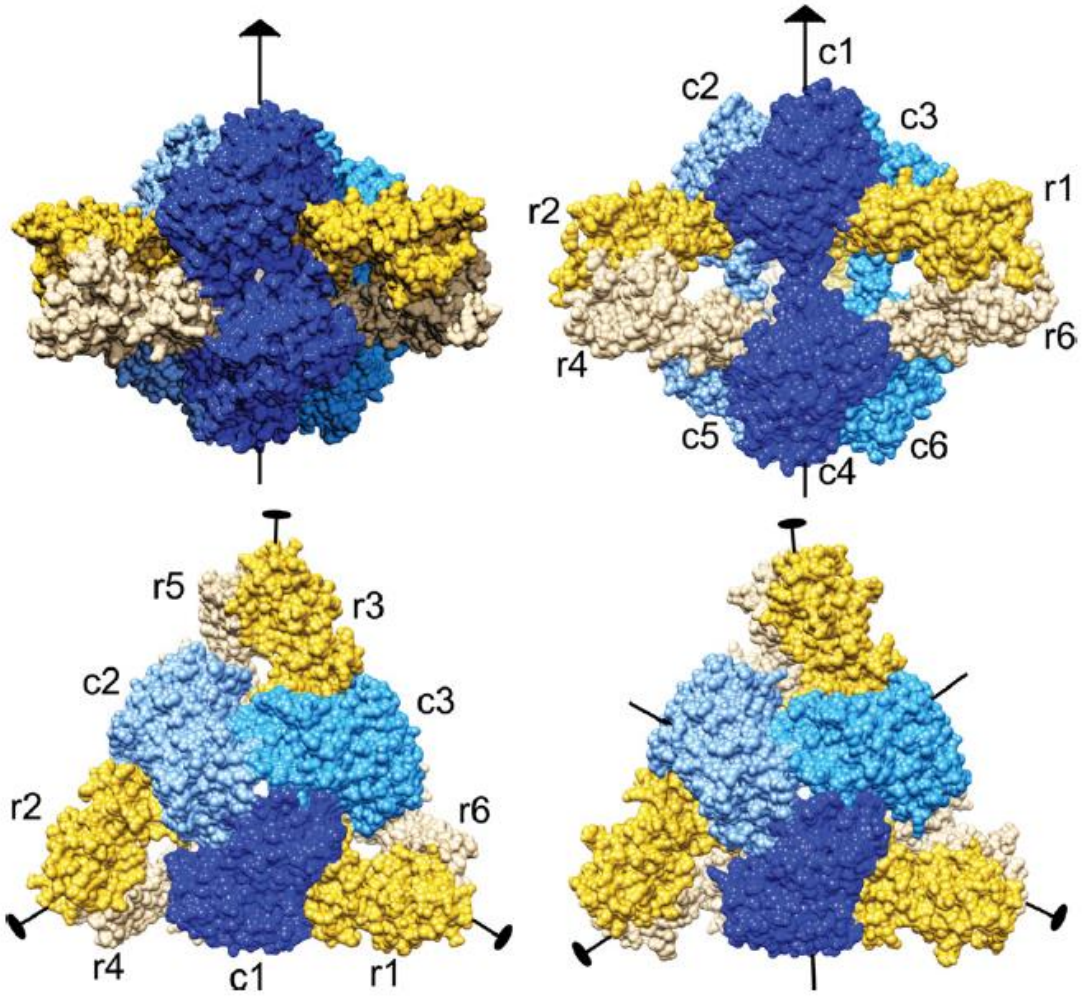
Figure 7- Different quaternary organizations of prokaryotic ATCases.



Scheme of quaternary structures of different ATCases. In *B. subtilis* the native structure of the protein is a homotrimer, in *E. coli* there is two trimers attach with three dimers of regulatory subunits and finally, in *A. aeolicus* the two trimers are also anchored with three dimers of DHOase. Modified from [72].

The interesting characteristic among these enzymes is that the active site is formed in the interphase of two subunits, both polypeptide chains contribute to the cavity of the active site. Noteworthy is that the basic catalytic conformation of this enzyme is three subunits that could lead to the formation of a trimer or a hexamer; this pattern is repeated in most of the species [73].

Figure 8- Three dimensional structure of *Escherichia coli* ATCase



Quaternary structure of ATCase from *E. coli* in the T (left) and R (right) states with the molecular 3-fold axis vertical (top) and viewed down the molecular 3-fold axis (bottom). The molecule expands 11 Å along the 3-fold axis during the allosteric transition. The catalytic chains are shown in shades of blue and the regulatory chains are shown in yellow and nude. Modified from [74].

Regarding the kinetics, the mechanism of the enzyme was reported to be an ordered-binding, where carbamoyl phosphate (CP) must bind before aspartate (Asp) and carbamoyl aspartate (CA) departing before inorganic phosphate (Pi) [49].

Structural studies by stop flow techniques, made from *B. subtillis* ATCase, revealed an extensive conformational change induced by CP binding, reducing the volume of the active site cavity by one-half. This binding is responsible for the creation of active sites that have high-activity and high-affinity for aspartate. Thus, CP binding is responsible for the induction of a positive cooperativity effect. The binding of CP not only creates a physical pocket to bind Asp but also

changes considerably the electrostatic environment of the active site. The binding of aspartate induces a closure in the domains that assists in lowering the activation energy, producing catalysis [75].

In contrast, in the ATCase holoenzyme of *E. coli*, the aspartate produces this conformational movement that causes the necessary loop movements inducing cooperativity [75]. This phenomenon is explained by an Asp-induced conversion of the holoenzyme from a "T" (tense) state, where active sites are constrained in an open conformation with low activity and low affinity for Asp, to a relaxed "R" state with increased affinity and activity.

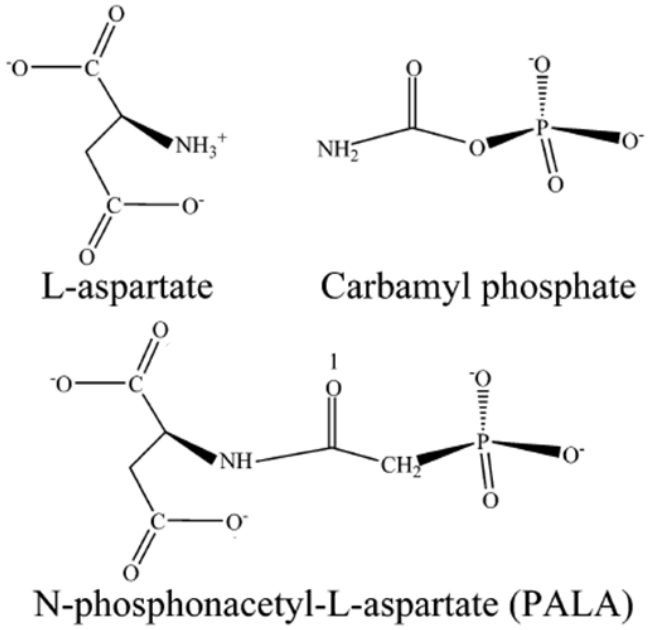
A common characteristic in the kinetics of many ATCases is the strong substrate inhibition by aspartate [76]; it appears that aspartate has the ability to bind to the same site as CP in the non-liganded form.

1.3.1.1. Inhibitors of Aspartate transcarbamoylase

In the literature, N-phosphonacetyl-L-aspartate (PALA) is a well-known inhibitor among the ATCases. A potent inhibitor, PALA combines the features of the two substrates and resembles the transition state of the reaction (Fig. 9). Inhibition is competitive in CP and non-competitive in aspartate, proving again the ordered binding mechanism, with CP binding to the enzyme prior to aspartate for catalysis to occur. Moreover, the molecule was also used to perform co-crystallization experiments with ATCases of several organisms, such as the *E. coli* and human protein [65, 75–77].

In the 1970s several studies demonstrated that PALA is able to inhibit CAD, the human complex [13] and stop the proliferation of cancer cells in culture [78]. Indeed, PALA demonstrated a broad spectrum of activity against experimental tumor models, and its biochemical and pharmacological effects are well characterized. Phase I trials were followed by broad Phase II screening for antitumor activity. Unfortunately, PALA was inactive as a single agent [79].

Figure 9- Three dimensional structure of substrates of ATCase and PALA.

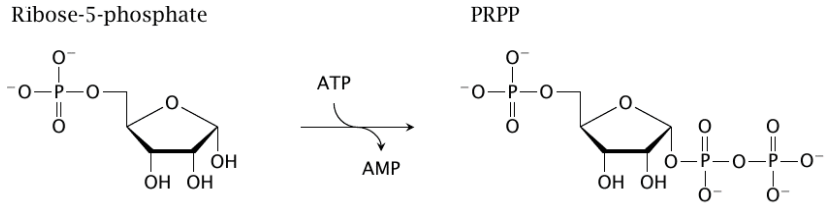


Structure of two substrates of ATCases, L-Aspartate and Carbamoyl Phosphate, as well as N-phosphonacetyl-L-aspartate (PALA), a well known inhibitor of the protein. In this image, it is easy to see the similarities between the structures.

1.3.2. Phosphoribosylpyrophosphate synthetase (PRSase)

Phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) is an enzyme that catalyzes ribose 5-phosphate into phosphoribosyl pyrophosphate (PRPP), using ATP as a donor of pyrophosphate and releasing AMP (Fig. 10). The product of the reaction, PRPP, is a key metabolite of several important pathways, such as synthesis of nucleotides (Fig. 5), pentose phosphate pathway, among others, inside the cell.

Figure 10 - Schematic illustration of catalysis of the phosphoribosylpyrophosphate synthetase (PRSase)



The enzyme catalyzed the transference of pyrophosphate from ATP to the carbon number 1 of the ribose-5 phosphate, forming the Phosphoribosyl pyrophosphate, which plays a role in transferring phospho-ribose groups in several reactions.

This enzyme came to be known after the publications of Sun et al. and Hanson et al. These authors reported a potent antimalarial drug called Torin2, which has an EC_{50} for asexual blood stages to be 1.4 nM, as well as being highly potent against early gametocytes, with a slightly lower EC_{50} of 6.62 nM. Additionally, they reported that Torin2 attached to a specific matrix and could bind 3 proteins after passing a lysate of gametocytes of *P. falciparum*. These 3 proteins were aspartate carbamoyltransferase (PF3D7_1344800, ATCase), phosphoribosylpyrophosphate synthetase (PF3D7_1325100, PRSase), and a putative transporter (PF3D7_0914700). As our work is focusing on aspartate carbamoyltransferase and to continue with our research line, we decided to set up the characterization and preliminary crystallization experiments of plasmodial Phosphoribosylpyrophosphate synthetase (*Pf*PRSase) [80, 81].

CHAPTER 6

CONCLUSION

In this work the enzyme aspartate carbamoyltransferase from *P. falciparum* has been characterize which is the second enzyme of the pyrimidine biosynthesis. This enzyme is essential for the parasite, have a trimer/hexamer conformation in solution and the active sites reside in the interphases between two subunits. Active site mutations do not have an influence on the protein assembly and enables Protein Interference Assays (PIA) in the parasite. Applied PIA show that a deficiency of *Pf*ATCase has a knock-down phenotype on the proliferation of the parasite which proves again that PIA are an excellent method to test essentiality in in vivo experiments.

Furthermore, we demonstrated the mechanism of action of the potent drug Torin2, which opens several options for the design of new compounds against *Pf*ATCase and *Pf*PRSase.

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APPENDIX

List of publications

1. Batista FA*, **Bosch SS***, Lunev S, Meissner KA, Butzloff S, Linzke M, Romero A, Wang C, Muller I, Dömling I, Groves MR and Wrenger C. (2018) Oligomeric Protein Interference Validates Druggability of Aspartate Interconversion in *Plasmodium falciparum*. MicrobiologyOpen. (2018) DOI: 10.1002/mbo3.779 *Authors contributed equally.

2. Lunev S*, **Bosch SS***, Batista FA, Wang C, Li J, Linzke M, Kruithof P, Chamoun G, Dömling A, Wrenger C, Groves MR. (2018) Identification of a non-competitive inhibitor of *Plasmodium falciparum* aspartate transcarbamoylase. Biochemical and Biophysical Research Communications (BBRC). *Authors contributed equally. DOI: 10.1016/j.bbrc.2018.02.112

3. Lunev S, Batista FA, **Bosch SS**, Wrenger C and Groves MR. (2016) Identification and Validation of Novel Drug Targets for the Treatment of *Plasmodium falciparum* Malaria: New Insight. Book Chapter. DOI: 10.5772/65659

4. Lunev S*, **Bosch SS** *, Batista FA, Wrenger C and Groves MR. (2016) Crystal structure of truncated Aspartate Transcarbamoylase from *Plasmodium falciparum*. Acta Crystallogr F Struct Biol Commun. DOI: 10.1107/S2053230X16008475. *Authors contributed equally.

5. **Bosch SS***, Kronenberger T*, Meissner KA*, Zimbres FM*, Stegehake D, Izui NM, Schettert I, Liebau E, Wrenger C. (2015) Oxidative Stress Control by Apicomplexan Parasites. BioMed Research International. *Authors contributed equally.

6. Batista FA, Gyau B, **Bosch SS***, Lunev S, Chao W, Vilacha JF, Wrenger C and Groves MR. (2019) Protein Interference Assay (PIA) offers a novel venue towards the validation of antimalarial target candidates. *Authors contributed equally. (SUBMITTED)

7. **Bosch SS***, Lunev S*, Batista FA, Wrenger C, Dömling A, and Groves MR. (2019) Molecular target validation of Aspartate Transcarbamoylase from Plasmodium falciparum by Torin 2. *Authors contributed equally. (SUBMITTED)

About the author

Soraya S. Bosch was born on the 18th of February 1988 in Reconquista, Argentina. After finishing the school in 2005, she started a Bachelor and Master degree in Biotechnology in March of 2006, at the University of Rosario (UNR). In 2010, in association with the biophysics department, she initiated her Master studies under supervision of Dr Pablo E. Tomatis and Prof. Dr Alejandro J. Vila as co-advisor. The title of the master dissertation was "Optimization of the hydrolytic capacity of the serine β-lactamase KPC by directed molecular evolution". The objective of the project was to study the Serin-beta-lactamase, KPC-2. This protein has a high relevance nowadays in the clinic, because of its ability to hydrolyse carbapenems (antibiotics of the last defence line).

After defending the Master thesis in September of 2012, she worked as a technician in the Laboratory of Prof. Vila until she moved to São Paulo, Brazil to start a PhD with Prof. Carsten Wrenger, in August of 2013. The Soraya' doctoral studies were focused on a protein called Aspartate Carbamoyltransferase of the malaria parasite *Plasmodium falciparum* which she validated as a drug target by *in vitro* characterization of the protein as well by *in vivo* experiments using the protein interference technique with transgenic parasites. Subsequently, thank to several negotiations and networking between the Universities of São Paulo and the Groningen, she initiated the PhD double degree program between São Paulo and Groningen and became the first Brazilian student who participated in the program.