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UNCOVERING THE ROLE OF HOST PEROXISOMAL FUNCTIONS IN *PLASMODIUM* LIVER STAGE INFECTION

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Publications

Part of the present work was presented in poster format at “1º Encontro de Biologia Molecular em Saúde” held at Escola Superior de Saúde Egas Moniz in Monte da Caparica, and at “Young Researchers in Life Sciences 2011”, held at Institut Pasteur in Paris.

Carrelha, J., Cabal, G. G., Real, E., Mota, M.M. Role of host peroxisomal proteins in malaria liver stage. *1º Encontro de Biologia Molecular em Saúde*. Lisbon, Portugal (2011)

Carrelha, J., Cabal, G. G., Real, E., Hannus, M., Mota, M.M. Role of host peroxisomal proteins in malaria liver stage. *Young Researchers In Life Sciences*. Paris, France (2011)

ACKNOWLEDGEMENTS

To my supervisor **Ghislain** for all that he taught me about lab work and work ethics and for his patience and calmness in desperate times. Merci! À minha supervisora honorária **Eliana** pelo seu apoio e por me ter ensinado a trabalhar com praticidade e propósito. E, claro, por todos os filmes que não me deixou ir ver sozinha! Devo muito a ambos pela sua dedicação e por me terem ajudado a tornar esta dissertação no que merecia ser.

Big thanks to **Maria** and everybody at **UMA**, from MSc student to staff scientist, for welcoming me into their lab and their lives. Also to all of our friends spread out throughout **IMM**. I truly enjoyed working in such a fun and diverse environment and learning something new from someone different every day. Obrigada a todos!

Às Professoras **Margarida Ramos**, **Rita Zilhão** e **Filomena Caeiro** da FCUL por terem sempre mantido um olho aberto na minha direcção, mesmo nos longos períodos de silêncio, e me ajudarem através do percurso do Mestrado BMG.

À minha querida Mãe, meu porto de abrigo. Não há nem nunca haverá ninguém tão forte.

Ao meu Pai, o grande Tretas, por me ter ensinado Física, criatividade e a sublime arte do desenrascanço em toda e qualquer situação.

À minha irmã, por me ter dado a conhecer o fabuloso mundo da Biologia e partilhar comigo tantas referências inúteis da cultura pop. *CQ, CQ, this is W9GFO. Is anybody out there?*

À minha avó Lídia, por todo o apoio e orgulho com que sempre me honrou e pelo seu stock infinito de bolachas corintias e creamy kiss.

Aos meus tios, por sempre acreditarem que eu podia ir até ao infinito e mais além.

A todos os meus amigos do **CBD** e da **FCUL**. Um abraço especial à **Joana Filipa** e à **Sílvia** que, coitadas, nunca se livraram de mim desde que nos conhecemos na pré-primária. Ao **Rui** e à **Ana** e a todos os nossos amigos metaleiros espalhados pelo mundo inteiro. Viva a música, os festivais, os concertos, o headbanging, as t-shirts estampadas, os autógrafos e a cerveja! *Let him who hath understanding reckon the number of the Beast, for it is a human number. Its number is six hundred and sixty six!*

ABSTRACT

Malaria, the world's leading tropical parasitic disease, is caused by protozoan parasites of the genus *Plasmodium*. During its life cycle, *Plasmodium* inhabits an insect vector and a vertebrate host. Liver infection in the vertebrate host is the asymptomatic obligatory step before the onset of malaria disease. Cellular and molecular interactions between host and parasite play a key role in the establishment of susceptibility to malaria infection, and so the identification of relevant host factors is crucial for the rational development of new antimalarial strategies. We hypothesized that peroxisomes-less *Plasmodium* may have acquired host-dependency at the level of liver peroxisomes, and that it can take advantage of host cell peroxisomal functions and metabolites during liver stage. The myriad pathways in which peroxisomes are involved and their abundance in mammalian livers seems to place these organelles in a privileged position to be exploited in the context of intracellular parasitism. Live fluorescence microscopy and flow cytometry of DsRed-labeled peroxisomes revealed that the intracellular presence of *Plasmodium* can alter the dynamic properties of the host peroxisomal population. We then focused on the two major mammalian peroxisomal functions, fatty acid β-oxidation and detoxification of reactive oxygen species. Impairment of fatty acid β-oxidation by a drug inhibitor, knockdown of β-oxidation enzymes and overexpression of a key peroxisomal thiolase showed that a host-factor dependency does exist and that it is important for both cell invasion and subsequent parasite development. This is probably tied to the parasite's metabolic requirements for membrane biosynthesis during these processes. Catalase inhibition and knockdown of other peroxisomal peroxidases showed that this antioxidant network does not play a strong role in *Plasmodium* infection, but fluorescence microscopy revealed that the peroxisomal marker enzyme catalase may be recruited by the parasite to complement the functions of its own antioxidant systems in the maintenance of redox homeostasis during liver stage.

Keywords: malaria, host-parasite interactions, peroxisomes, β-oxidation, peroxidases

ABSTRACT

A malária constitui a principal doença parasitária tropical no mundo, sendo causada por protozoários do género *Plasmodium*. O ciclo de vida deste parasita inclui dois hospedeiros: um insecto vector e um vertebrado. A infecção do fígado do hospedeiro vertebrado é uma etapa obrigatória e precede a manifestação clínica da doença. As interacções celulares e moleculares entre parasita e hospedeiro têm um papel determinante no estabelecimento da susceptibilidade à infecção e, portanto, a identificação de factores do hospedeiro relevantes para o desenrolar da infecção é essencial numa perspectiva de desenvolvimento de novas estratégias anti-maláricas. No âmbito deste trabalho formulámos a hipótese de que o parasita causador da malária, o qual é desprovido de peroxissomas, poderá, ao longo da evolução, ter adquirido a capacidade de subverter as funções e/ou metabolitos peroxissomais do hospedeiro vertebrado. De facto, a diversidade de vias metabólicas em que os peroxissomas estão envolvidos, bem como a sua abundância no fígado, levantam a questão da importância destes organelos num contexto de parasitismo intracelular. Começámos por mostrar que a presença de *Plasmodium* pode alterar as propriedades dinâmicas da população peroxissomal da célula hospedeira. Focámo-nos, então, nas duas principais funções dos peroxissomas, a β-oxidação de ácidos gordos e a degradação de espécies reactivas de oxigénio. Bloqueio da β-oxidação através de um inibidor ou por silenciamento da expressão de enzimas-chave desta via metabólica, bem como sobre-expressão de uma importante tiolase peroxissomal, permitiu-nos demonstrar que existe de facto uma dependência entre parasita e hospedeiro e que a β-oxidação peroxissomal é importante tanto para a invasão da célula hospedeira como para o subsequente desenvolvimento do parasita. Este efeito está provavelmente associado às necessidades lipídicas do parasita, nomeadamente para a síntese de membranas durante ambos os processos. Por outro lado, inibição da catalase e silenciamento da expressão de outras peroxidases peroxissomais revelou que esta rede antioxidante não tem um papel crucial na infecção por *Plasmodium*. Curiosamente, experiências de microscopia de fluorescência sugerem que a catalase do hospedeiro poderá ser recrutada pelo parasita, o que poderá constituir um mecanismo de homeostase durante a infecção hepática.

Palavras-chave: malária, interacções parasita-hospedeiro, peroxissomas, β-oxidação, peroxidases

SUMMARY / RESUMO

A malária é a doença parasitária com maior impacto no mundo, sendo responsável por cerca de 780.000 mortes por ano, 85% das quais correspondem a crianças com idade inferior a cinco anos. A área com maior incidência de malária é, sem dúvida, a África subsariana, mas esta doença é também endémica do sudeste da Ásia, da América central, da América do sul, do Mediterrâneo leste e de regiões do Pacífico.

Os agentes causadores de malária são protozoários do género *Plasmodium*, filo Apicomplexa. Entre as cinco espécies que podem transmitir malária aos humanos, *P. falciparum* é o principal contribuidor para a morbidade e mortalidade associadas à malária. *Plasmodium* possui um ciclo de vida complexo, o qual inclui fases distintas de desenvolvimento em dois hospedeiros. O desenvolvimento sexual do parasita ocorre num insecto vector enquanto que o desenvolvimento asexual ocorre num hospedeiro vertebrado. A manisfestação clínica da malária só se dá quando o parasita inicia a infecção cíclica dos eritrócitos em circulação, o que só pode ocorrer após ter completado a primeira fase do seu ciclo asexual no fígado do hospedeiro vertebrado. Apesar desta etapa obrigatória no fígado ser assimptomática, decorrem durante ela inúmeras interacções moleculares e celulares entre parasita e hospedeiro que condicionam fortemente o progresso da infecção. Este forte tropismo e associação muito próxima entre o metabolismo do hepatócito hospedeiro e o metabolismo do parasita tornam a fase pré-eritrocítica do ciclo de vida de *Plasmodium* de particular interesse para o desenvolvimento de novas estratégias para o combate contra a progressão da infecção e o surgimento dos sintomas de malária através do bloqueio do estabelecimento do parasita no fígado. Infelizmente, a acessibilidade experimental à fase hepática de *Plasmodium* é ainda bastante limitada, e o estudo da biologia celular e molecular das formas hepáticas do parasita da malária ainda se encontra na infância. Porém, uma quantidade substancial de investigação *in vitro* e *in vivo* tornou-se possível devido ao uso de parasitas-modelo que causam malária apenas em roedores, nomeadamente *P. berghei* e *P. yoelii*. Sistemas *in vitro* em que linhas celulares de hepatoma humano suportam o desenvolvimento de parasitas de roedores são modelos práticos e ferramentas excepcionalmente valiosas para o estudo experimental da fase hepática da malária nos mamíferos. Recentemente, um perfil da expressão de genes de células de hepatoma infectadas por *P. berghei* revelou que a infecção por *Plasmodium* induz uma sequência de eventos biológicos coordenados que podem ser divididos em três categorias gerais: resposta fisiológica ao stress causado pela presença do parasita, recrutamento dos processos metabólicos do hospedeiro e manutenção da viabilidade da célula hospedeira ao longo da infecção.

A taxa de crescimento de *Plasmodium* no fígado é uma das mais rápidas alguma vez registada entre eucariotas, e como tal, o parasita tem elevadas exigências metabólicas, particularmente ao nível da abundância e variedade de recursos lipídicos. Apesar de ter capacidade para sintetizar determinadas classes e determinadas quantidades de ácidos gordos, sendo um parasita intracelular obrigatório, muitos dos seus recursos são também derivados da célula hospedeira. Ao longo da evolução, a selecção natural tem vindo a moldar o metabolismo dos parasitas intracelulares no sentido do desenvolvimento de mecanismos eficientes na subversão dos recursos da célula hospedeira. Adicionalmente, a grande variedade e disponibilidade de metabolitos no citoplasma da célula hospedeira levou à redução ou mesmo perda de vias metabólicas centrais dos parasitas. As diferentes estratégias metabólicas praticadas pelos patogénios de humanos são influenciadas pelo nicho, ou nichos, que cada um ocupa. Enquanto que o refinamento e adaptação do metabolismo mitocondrial é um processo evolutivo comum entre os patogénios eucariotas, várias linhas de protozoários, incluindo *Plasmodium*, perderam os seus peroxissomas. Apesar de ainda não ser completamente claro se *Plasmodium* não possui de todo um compartimento celular estruturalmente semelhante ao peroxissoma, dados que demonstram a inexistência de peroxissomas canónicos são fornecidos por diversos estudos bioinformáticos, citológicos e enzimáticos. A ausência dos genes conservados que codificam para os factores de biogénese dos peroxissomas (genes *PEX*) e também a ausência da enzima catalase, considerada a enzima representativa dos peroxissomas, é deveras conspícua.

No presente trabalho procurou-se testar a hipótese de que o parasita da malária evoluiu no sentido de uma parcial dependência metabólica em relação ao seu hospedeiro, tendo adquirido a capacidade de subverter certas vias metabólicas da célula hospedeira para suportar o seu desenvolvimento no fígado. Devido às suas propriedades dinâmicas, ao seu envolvimento em variadas vias metabólicas importantes, à sua abundância no fígado e ao facto de que *Plasmodium* não os possui, os peroxissomas apresentaram-se como bons candidatos para o estudo de interacções funcionais entre componentes da célula hospedeira e o parasita, as quais têm o potencial de providenciar alvos para novas estratégias quimioterapêuticas contra a fase hepática da infecção malárica.

Os peroxissomas são organelos ubíquos que partilham determinadas vias e mecanismos com as mitocôndrias, mas cada um destes organelos tem especificidade para diferentes substratos e, portanto, estão envolvidos em funções fisiológicas distintas. Os peroxissomas são providos de um grande número de enzimas, as quais constituem redes interactivas que se estendem além do compartimento do organelo e que são em grande parte reguladas por factores exteriores ao organelo. Tendo já sido considerados, durante um longo período de tempo, nada mais do que “organelos-fóssil”, os peroxissomas são hoje

em dia reconhecidos como componentes celulares dinâmicos e activos e cujas funções fisiológicas são indespensáveis à saúde humana. Começámos, então, por avaliar o impacto da presença intracelular de *Plasmodium* na população peroxissomal da célula hospedeira, pois sabe-se que a morfologia e propriedades dinâmicas destes organelos podem ser reguladas por diversos estímulos intra- e extracelulares, incluindo condições criadas por infecção de parasitas, como é o caso de *Leishmania donovani*. Por meio de microscopia e citometria de fluxo de peroxissomas marcados com uma proteína fluorescente, observámos que o tamanho da população peroxissomal e/ou de peroxissomas individuais diminui nas células infectadas, um efeito que poderá ser directa ou indirectamente regulado pelo parasita.

Prosseguimos o estudo focando-nos nas duas principais funções dos peroxissomas nos mamíferos: β-oxidação de ácidos gordos e degradação de espécies reactivas de oxigénio (ROS). O núcleo da via de β-oxidação consiste em quatro etapas sequenciais: desidrogenação, hidratação, desidrogenação e clivagem tiolítica. Para a maioria dos substratos estas estapas são catalizadas pela oxidase ACOX1, pela desidrogenase/hidratase D-BP e pela tiolase SCP-x. Inibição específica da β-oxidação peroxissomal por tioridazina e o silenciamento da expressão das enzimas-chave já mencionadas, bem como sobre-expressão da tiolase que catalisa a última etapa da β-oxidação, permitiu-nos demonstrar que existe de facto uma dependência entre parasita e hospedeiro ao nível dos peroxissomas. A β-oxidação peroxissomal de ácidos gordos aparenta ser importante tanto para a invasão da célula hospedeira como para o subsequente desenvolvimento do parasita, um efeito que está provavelmente associado às necessidades lipídicas do parasita para a síntese de membranas durante ambos os processos.

ROS são um grupo de moléculas altamente reactivas que são naturalmente geradas pelo metabolismo celular, mas também por exposição a factores ambientais como choques térmicos e radiação ultra-violeta. O termo ROS é um termo geral que inclui radicais livres como o anião superóxido e o hidróxido, mas também não-radicais como o peróxido de hidrogénio (H_2O_2). A importância de H_2O_2 reside na sua capacidade de penetrar facilmente membranas biológicas, no seu papel na produção de moléculas ROS mais reactivas, e a sua função como molécula de sinalização celular. H_2O_2 é degradado por três tipos de enzimas: catalases, glutationa peroxidases e peroxirredoxinas. A inibição da catalase por 3-aminotriazole e o silenciamento da expressão de catalase e de duas peroxirredoxinas revelaram que a rede antioxidante peroxissomal não parece ter um papel crucial na infecção por *Plasmodium*. Porém, experiências de microscopia de fluorescência sugerem que a catalase do hospedeiro poderá ser recrutada pelo parasita, o que poderá constituir um mecanismo de homeostase oxidativa durante a infecção hepática.

ABBREVIATIONS

3-AT: 3-amino-1,2,4-triazole

C400: 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (general ROS indicator)

AdGFP / Ad-SRBI-GFP / AdGFP-SCP-x: Adenoviral constructs overexpressing GFP alone, C-terminally GFP-tagged SR-BI, and N-terminally GFP-tagged SCP-x

cDNA: Complementary DNA

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DsRed: Discosoma red fluorescent protein

EEF: Exoerythrocytic form

FA: Fatty acid

FCM: Flow cytometry

GFP: Green fluorescent protein

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HUH7: Human hepatoma cell line

H_2O_2 : Hydrogen peroxide

***Pb*-GFP$_{con}$ / *Pb*GFP-LUC$_{con}$ / Pb-RFP$_{con}$**: Transgenic *Plasmodium berghei* that constitutively expresses GFP, GFP-LUC fusion or RFP, respectively

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PRDX: Peroxiredoxin

PTS1: Peroxisomal targeting signal 1

PVM: Parasitophorus vacuole membrane

qRT-PCR: Quantitative real-time reverse transcription PCR

RFP: Red fluorescent protein

RPMI: Roswell Park Memorial Institute medium

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SCP-x/2: Sterol carrier protein X/2

siRNA: Small interfering RNA

VLCFA: Very-long-chain fatty acid

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INTRODUCTION

Malaria burden worldwide

In 2009, over 3% of the world's population suffered from malaria. The estimated 225 million clinical cases resulted in 781.000 deaths, 85% of which corresponded to children under the age of five (WHO 2010a). Half of the world's population is at risk of contracting malaria, and although a decrease in incidence has been witnessed since 2005 (WHO 2010a), it is still considered to be the fifth leading cause of death in low-income countries (WHO 2011). The main impact area is sub-Saharan Africa, where a staggering 90% of all malaria deaths occur, but malaria is also endemic to South-East Asia, Central and South America, Eastern Mediterranean and Western Pacific regions (**Fig. 1**) (WHO 2009; WHO 2010a). Thus, over a century since Alphonse Laveran identified its causative agent (Laveran 1881), malaria remains by far the world's leading tropical parasitic disease.

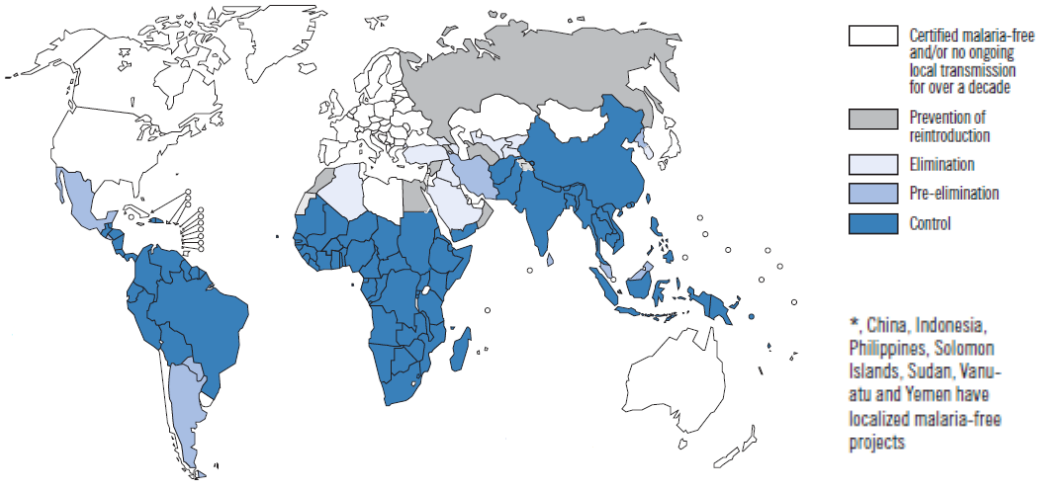

Figure 1. **Malaria incidence worldwide.** Malaria-free and malaria-endemic countries in phases of control, pre-elimination, elimination, or prevention at the end of 2008 (Adapted from WHO 2009).

The fight against malaria

Increased drug resistance in malaria endemic countries is currently causing cheap and widely used antimalarials to fail, and yet, the high financial costs associated with newly recommended therapeutic strategies has greatly hindered their wide implementation (WHO 2010b). Fortunately, the international community has become increasingly aware of the unacceptable burden that malaria represents in large parts of the world. Together with strong financial support, efforts to better understand the complex biology of the parasite and the immunity it induces in the host, to find novel targets and to design new drugs and vaccines, will hopefully lead to new trends in the management of malaria and improved global health (Greenwood & Mutabingwa 2002; Kappe *et al*. 2010).

Life cycle of the malaria parasite

Malaria is caused by obligatory intracellular protozoan parasites of the genus *Plasmodium*, phylum Apicomplexa. The species that cause malaria in humans are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Among them, *P. falciparum* is the top contributor to severe malaria morbidity and mortality (WHO 2009), but there is growing evidence that *P. vivax* may also be responsible for a significant malaria burden worldwide (reviewed in Mendis *et al*. 2001 & Price *et al*. 2008).

The life cycle of *Plasmodium* consists of sexual development in an insect vector (**Fig.2A**) and asexual development in a vertebrate host, which includes a liver stage (**Fig.2B**) and a blood stage (**Fig. 2C**). Clinical manifestation of malaria arises from blood infection (Laveran 1881), but before the actual onset of malaria disease in the host, the parasite must undergo an intricate developmental program involving a series of molecular and cellular interactions (reviewed in Silvie *et al*. 2008 & Aly *et al*. 2009). Mosquito species of the genus *Anopheles* are the vectors that transmit malaria to humans (Grassi *et al*. 1899; Ross 1923).

Plasmodium asexual development in humans starts with the injection of elongated parasite forms called sporozoites by a female *Anopheles* mosquito under the human host's skin during a blood meal (Ponnudurai *et al*. 1990). The inoculated sporozoites initiate vigorous gliding motility until they enter a dermal blood capillary, but since these forms are not competent to directly infect erythrocytes, they are simply transported by circulation (Amino *et al*. 2006). Through interaction of circumsporozoite protein (CSP), which covers the surface of sporozoites, with heparin sulfate proteoglycans (HSPGs) on liver cells, sporozoites in circulation quickly accumulate in the liver (reviewed in Prudêncio *et al.* 2006a & Silvie *et al*. 2008), and then cross the liver sinusoidal cell layer through membrane disruption and transmigration (Mota *et al*. 2001). Sporozoites eventually switch to productive invasion, which occurs without host cell plasma membrane rupture and culminates in the production of a specialized compartment in the cytosol of the invaded hepatocyte, the parasitophorus vacuole (PV) (reviewed in Prudêncio *et al*. 2006a). Inside its own PV each sporozoite develops into an exoerythrocitic form (EEF) that grows exponentially and replicates by schizogony into thousands of infective merozoites over the course of several days (reviewed in Mikolajczak & Kappe 2006 & Silvie *et al*. 2008). The asymptomatic liver stage concludes with the release into the bloodstream of merozomes containing thousands of merozoites (Sturm *et al*. 2006). Once released from merosomes, merozoites infect erythrocytes and blood stage begins. The minimum time elapsed between sporozoite infection and the first detectable wave of merozoites that reaches the bloodstream (i.e. the prepatent period), as well as the number of merozoites produced per invading sporozoite, are species-dependent (Boyd & Stratman-Thomas 1934; Boyd & Kitchen 1937). *P. vivax* and *P. ovale* can also exist

as dormant forms in the liver that do not undergo asexual replication, called hypnozoites. Malaria caused by these *Plasmodium* species is characterized by disease relapses, for which these latent non-merozoite-like hepatic forms are responsible (Krotoski 1985; reviewed in Markus 2011).

Asexual development of merozoites in erythrocytes consists of three successive morphological stages: ring, trophozoite, and schizont stage, each being accompanied by specific host cell modifications (Bannister *et al*. 2000; Grüring *et al*. 2011). During the erythrocytic schizont stage each parasite generates dozens of daughter merozoites that, after rupture of the host cell, invade new erythrocytes. Eventually, a few merozoites exit the asexual self-propagating cycle and develop into male and female gamete precursors, called gametocytes (reviewed in Baker 2010). These sexually reproductive parasite forms are responsible for the infection of mosquitoes during blood meals from human hosts (reviewed in Sinden 2009).

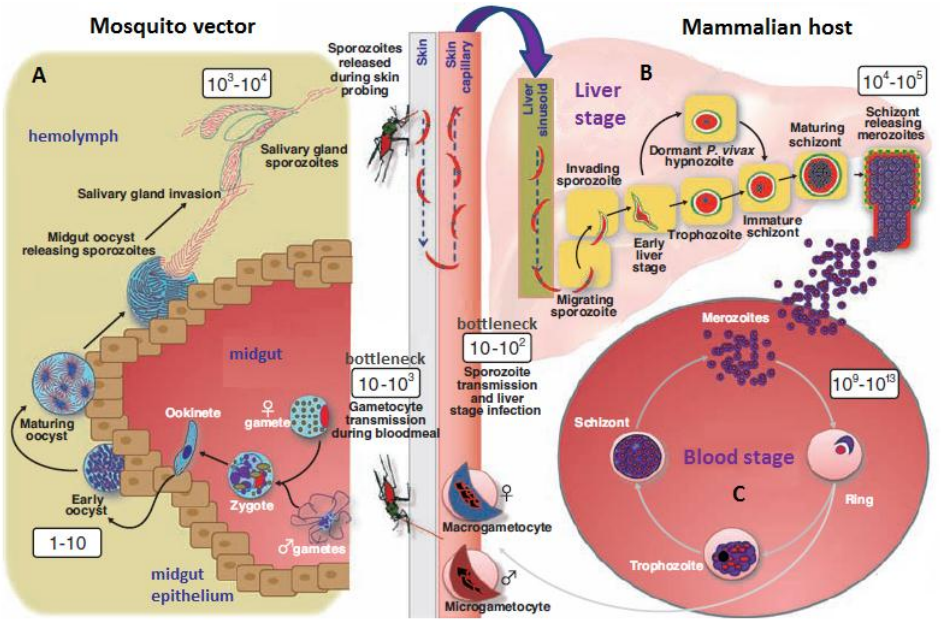

Figure 2. **Life cycle of *Plasmodium*. (A)** A female *Anopheles* mosquito ingests *Plasmodium* gametocytes with the blood of an infected mammalian host, which develop to gametes in the mosquito's midgut. Gamete fertilization generates a zygote, which differentiates into an ookinete that embeds itself in the basal lamina of the midgut epithelium. The resulting oocyst produces sporozoites, which migrate to the salivary glands. **(B)** Sporozoites in the salivary glands are injected into a mammalian host and transported to the liver by circulation. Each sporozoite invades a hepatocyte, within which it grows and replicates into thousands of merozoites that are released into the blood stream. **(C)** Merozoites infect erythrocytes and blood stage proceeds through cyclic infection, replication and merozoite release. Some merozoites form sexual-stage gametocytes, which can be ingested by a new mosquito. The two transmission events between hosts are considered bottlenecks of the parasite life cycle. Boxed numbers indicate parasite population size during life cycle progression (Adapted from Kappe *et al*. 2010).

Gametocytes ingested by *Anopheles* mosquitoes during an infected blood meal develop into gametes in the mosquito midgut lumen. The female gametes are subsequently fertilized

by the male gametes, giving rise to motile diploid zygotes called ookinetes, the only extracellular developmental stage of the malaria parasite life cycle (Aly *et al*. 2009). Ookinetes migrate to the gut periphery and cross the midgut epithelium, embed beneath the basal lamina and further differentiate into oocysts. Through several synchronous endomitotic divisions, each oocyst produces thousands of sporozoites, which migrate to the mosquito salivary glands for subsequent delivery to the human host (reviewed in Aly *et al*. 2009; Ghosh *et al*. 2000; Matuschewiski 2006).

Uncovering host factors in malaria liver stage

Cellular and molecular interactions between host and parasite play a crucial role in the establishment of susceptibility to malaria infection. Every stage relies, to different extents, on the presence of host molecules that enable parasite development. Thus, the identification and characterization of these host factors is crucial for the rational development of effective antimalarial drugs and vaccines (Prudêncio *et al.* 2006b). Due to its strong tropism, unique features and close association between host cell and parasite metabolism, the pre-erythrocytic stage of *Plasmodium* is of particular interest for the development of new strategies that completely prevent infection by impairing parasite development in the liver. Unfortunately, the liver stage has limited experimental accessibility, and the study of cellular and molecular biology of malaria liver stages is still in its infancy (Kappe & Duffy 2006). Nevertheless, a significant amount of *in vitro* and *in vivo* research has been conducted on this stage by taking advantage of model rodent malaria parasites, most notably *P. berghei* and *P. yoelii* (Prudêncio *et al*. 2006b; Bano *et al*. 2007), which do not pose direct danger to man. *In vitro* systems in which human hepatoma cell lines support the development of rodent *P. berghei* parasites (Hollingdale *et al*. 1983) are practical models and exceptionally valuable tools for the experimental study of mammalian malaria liver stage. Most importantly, these parasites are analogous to human and primate malarias in the essential aspects of biology, physiology and life cycle (Carter & Diggs 1977). Secondly, there is a wide availability of susceptible, genetically-defined and knockout mouse strains and some rodent life cycle stages can also be grown *in vitro*, allowing for direct comparison between *in vivo* and *in vitro* data. Efficient methodologies for genetic modification of the parasite are already established (Janse *et al*. 2006), and there is an extensive range of well-characterized clones with relevant biological phenotypes and also transgenic mutant lines, including several that express useful reporter genes (Franke-Fayard *et al*. 2004; Janse *et al*. 2006; Sturm *et al*. 2009). Finally, an analysis of *P. berghei* partial genome at 3x coverage has been published (Hall *et al*. 2005) and is publicly accessible in online databases. It is known that genome organization and housekeeping genes are conserved between rodent and human parasites.

It is relevant to mention a few recent studies that took advantage of rodent malaria *in vitro* systems to gain insight into host-parasite interactions during malaria liver stage. A microarray-based transcriptional profile of *P. berghei*-infected hepatoma cells revealed that *Plasmodium* infection leads to a coordinated and sequential set of biological events in the host cell, which can be broadly divided into three categories: initial stress response to the presence of the parasite, engagement of host cell metabolic processes, and maintenance of host cell viability throughout infection (Albuquerque *et al*. 2009). A search for interactions between host factors and a small transmembrane protein up-regulated in infective *Plasmodium* sporozoites, called UIS3, uncovered an important interaction with mouse liver-fatty acid binding protein (L-FABP) (Mikolajczak *et al*. 2007). This suggested that a direct pathway for fatty acid (FA) acquisition by liver stage parasites from the host cell is necessary for there to be enough membrane synthesis to sustain massive intracellular parasite growth, even though the parasite itself is capable of FA synthesis. Additionally, two independent studies concluded that host cell class B, type I scavenger receptor (SR-BI), a lipoprotein receptor, is a strong regulator of *Plasmodium* infection. SR-BI significantly boosts host cell permissiveness to invasion and intracellular parasite development by being a major provider of lipoprotein-derived cholesterol (Rodrigues *et al*. 2008; Yalaoui *et al*. 2008). Both of these studies point to an important role of host lipid metabolism during *Plasmodium* infection.

Host peroxisomes in malaria liver stage: hypothesis

Adaptive evolution has shaped the metabolism of parasites to the point of emergence of novel pathways for the subversion of host defenses. Additionally, the large availability of host metabolites has often led to abandonment of standard core metabolic pathways by the parasite. Unsurprisingly, the loss of some pathways is a driving force in the evolution of obligate, as opposed to opportunistic, parasitism (Ginger 2006).

The different metabolic strategies employed by human pathogens are influenced by the environmental niche, or niches, that each parasite occupies. While the adaptation and refinement of mitochondrial functions appears to be commonplace among microbial eukaryotes, peroxisomes have been lost from several protozoan lineages, including the Apicomplexa, with the possible exception of *Toxoplasma* spp (Ding *et al*. 2000; Kasch & Joiner 2000). Although it is not yet clear if the apicomplexan *Plasmodium* possesses any peroxisome-like structures, evidence that it lacks canonical peroxisomes comes from cell cytology studies (McIntosh *et al*. 2005), the absence of the hallmark peroxisomal enzyme catalase (Becker *et al*. 2005; Ding *et al*. 2000; Gardner *et al*. 2002), and also the lack of conserved peroxisome biogenesis genes (*PEX* genes) in the known genome sequences of *Plasmodium* species (Ding *et al*. 2000; Gardner *et al*. 2002). Peroxisomes appeared early in

eukaryotic evolution and free-living members of *Plasmodium*'s superphylum have peroxisomes (Baldauf 2003). It is, therefore, reasonable to hypothesize that peroxisomes-less *Plasmodium* may have acquired a host-dependency at the level of peroxisomes, and that it can take advantage of host cell peroxisomal functions and metabolites during its development. The myriad pathways in which peroxisomes are involved and their abundance in mammalian livers seems to place these organelles in a privileged position to be exploited in the context of intracellular parasitism.

Structure, biogenesis, and dynamics of mammalian peroxisomes

Peroxisomes (**Fig. 3**) were discovered in 1954 by electron microscopy of mouse kidney tissue (Rhodin 1954). Having once been considered to be nothing more than fossil organelles, they are now acknowledged as dynamic and metabolically active cellular compartments whose physiological role is indispensable for human health (Schader & Fahimi 2008). This is clear by the severe consequences of mutations that impair peroxisomal protein import or that inactivate peroxisomal enzymes, conditions that are known as peroxisome biogenesis disorders (PBDs) (reviewed in Steinberg *et al*. 2006) and single peroxisomal enzyme deficiencies (PEDs) (reviewed in Wanders & Waterham 2006b), respectively.

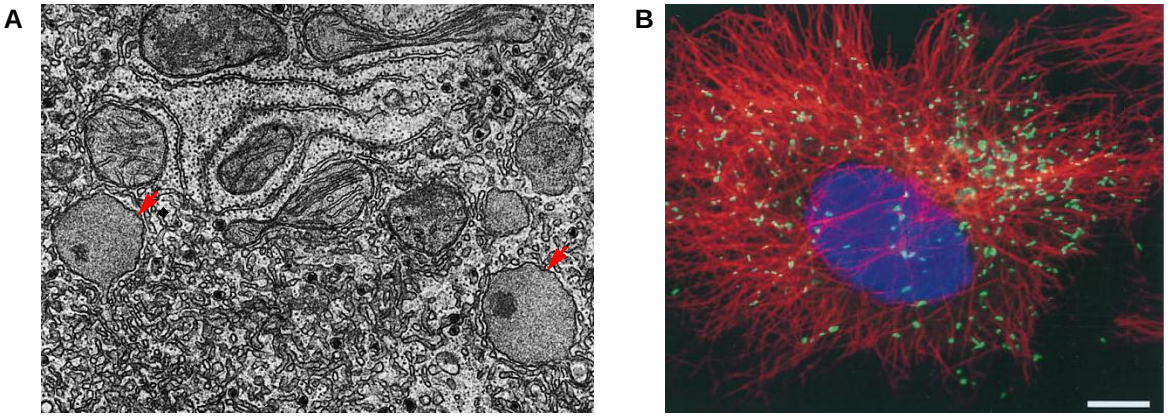

Figure 3. Mammalian peroxisome morphology. (A) Electron micrograph of rat liver peroxisomes (red arrows) with a urate oxidase crystalloid core that is not found in human peroxisomes (Adapted from Fawcett 1981) **(B)** Fluorescence imaging of African green monkey kidney fibroblasts. Peroxisomes in green (GFP-PTS1), nucleus in blue (propidium iodide) and microtubules (β-tubulin) in red. Scale bar, 10μm. (Adapted from Wiemer *et al*. 1997)

The liver is the mammalian organ with the most peroxisomes, with approximately 200 per hepatocyte, and these organelles are also much larger in the liver (Pavelka & Roth 2010; Khan *et al*. 2007). The behavior of peroxisomal structures is very dynamic. They exist both in the form of roughly spherical individual microperoxisomes and, at a moderate frequency, as networks of interconnected tubules called peroxisomal reticulum (Schrader *et al*. 2000). Peroxisomes divide and segregate to daughter cells during cell division, but they can also

divide independently of this process, increasing or decreasing in size and number in response to environmental cues (reviewed in Yan *et al*. 2005). Peroxisome number is regulated by three main pathways: division, proliferation and turnover. New peroxisomes can arise through growth and fission of preexisting peroxisomes (reviewed in Smith & Aitchison 2009) or by *de novo* formation from the endoplasmic reticulum (ER) (Tabak *et al*. 2003). Selective degradation of superfluous peroxisomes is also a major pathway of population regulation. Peroxisomal degradation occurs by macropexophagy – sequestration by the autophagosome and subsequent delivery to the lysosome; by micropexophagy – direct sequestration by the lysosomal membrane; and by 15-lipoxygenase-mediated autolysis – peroxisomal membrane is disrupted by 15-lipoxygenase and the organelle contents are exposed to cytosolic proteases (reviewed in Platta *et al*. 2007 & Huybrechts *et al*. 2009).

Since peroxisomes are devoid of DNA and transcription/translation machineries, all peroxisomal proteins are encoded by the nuclear genome and post-translationally imported (Lazarow & Fujiki 1985). Peroxisome biogenesis and division are complex processes that involve a network of at least 18 different proteins (in humans), collectively called peroxins or Pex proteins. This network controls assembly of peroxisomal membrane proteins, recognition of peroxisomal targeting sequences by specific receptors, receptor docking, protein import and translocation to the peroxisomal matrix, and receptor recycling (reviewed in Ma *et al*. 2011 & Rucktäschel *et al*. 2011). The targeting of matrix peroxisomal proteins depends on amino acid sequences termed peroxisomal targeting signals (PTS). PTS1, the signal that most matrix proteins possess, is a C-terminal, non-cleavable tripeptide – serine-lysine-leucine (SKL) or conserved variants (Gould *et al*. 1989). A smaller subset of peroxisomal matrix proteins are targeted by PTS2, an often cleavable N-terminal or internal nonapeptide (Swinkels *et al*. 1991).

About 60 peroxisomal matrix enzymes and 45 integral or peripheral membrane proteins have been documented so far (Subramani 2004). Several enzymes of the same pathway are enclosed within the granular matrix by the peroxisomal single lipid bilayer membrane (Pavelka & Roth 2010). The name “peroxisome” is a functional term derived from the fact that metabolic enzymes that generate hydrogen peroxide (H_2O_2) as a by-product of their activity co-localize in these organelles with the H_2O_2-degrading enzyme catalase. In this way, toxic peroxides remain sequestered in the same compartment as the enzymes that can detoxify them (de Duve 1965). In addition to the detoxification of reactive oxygen species (ROS), the other major peroxisomal function is the β-oxidation of long-chain and very-long-chain fatty acids (reviewed in Wanders & Waterham 2006a).

Peroxisomal fatty acid β-oxidation

Fatty acids (FAs) and their derivatives can originate from exogenous sources or from the intracellular breakdown of lipids. The major degradative pathway for FAs is β-oxidation, and in mammals it occurs both in peroxisomes and mitochondria. Although the mechanism and participating enzymes are similar in both organelles (**Fig.4A**), mitochondrial and peroxisomal FA β-oxidation fulfill distinct physiological functions (Poirier *et al*. 2006).

The bulk dietary intake of FAs is metabolized by mitochondria, some FAs are metabolized by both organelles, and others, such as very-long-chain FAs (VLCFAs) are solely metabolized by peroxisomes (Singh *et al*. 1984; Wanders & Waterham 2006a; Poirier *et al*. 2006). Peroxisomal β-oxidation, unlike what occurs in mitochondria, is not a complete process of FA degradation. Peroxisomes can only chain-shorten FAs but cannot degrade them into acetyl-CoA units. Chain-shortened products must be exported to mitochondria (Bieber *et al*. 1981; Vamecq 1987) in order to be degraded to carbon dioxide (CO_2) and water (H_2O) in the citric acid cycle, which peroxisomes lack (review in Wanders *et al*. 2000 & Wanders *et al*. 2001).

FAs destined for β-oxidation must be activated in order to enter peroxisomes as acyl-CoA esters. The core pathway of peroxisomal β-oxidation of activated FAs consists of four sequential steps: dehydrogenation, hydration, dehydrogenation and thiolysis (**Fig4B**). The first reaction is catalyzed by an acyl-CoA oxidase (ACOX) and is considered to be the rate-limiting enzymatic step (Infante *et al*. 2002). Humans have two functional ACOX proteins. Palmitoyl-CoA/straight-chain acyl-CoA oxidase (ACOX1) catalyzes the oxidation of straight chain FAs, and branched-chain acyl-CoA oxidase (ACOX2) participates in the degradation of branched substrates. Unlike mitochondrial dehydrogenases, which transfer electrons from $FADH_2$ and NADH to the electron transport chain in order to generate chemical energy in the form adenosine triphosphate (ATP), ACOX proteins transfer electrons from the $FADH_2$ that is produced during β-oxidation directly to molecular oxygen (O_2), thus producing H_2O_2 that must be detoxified by peroxisomal peroxidases (Poirier *et al*. 2006). Unlike mitochondria, peroxisomes lack an electron transport chain and a citric acid cycle. Consequently, peroxisomal β-oxidation by itself does not yield ATP (**Fig.4A**). The second and third reactions of the pathway are catalyzed by two multifunctional enzymes (MFEs), MFE-1 or L-bifunctional protein (LBP) and MFE-2 or D-bifunctional protein (DBP), each of which displays both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. Although both MFEs show broad substrate specificity (Poirier *et al*. 2006), it is well established that D-BP is the main enzyme involved in the β-oxidation of peroxisome-specific FA substrates (Wanders & Waterham 2006a). The last step of peroxisomal β-oxidation is the thiolityc cleavage of 3-ketoacyl-CoA to acetyl-CoA and acyl-CoA shortened by two carbons. Human peroxisomes

have two 3-ketoacyl-CoA thiolases, the straight-chain thiolase ACAA1 and the branched-chain thiolase SCP-x (Wanders *et al*. 1997). SCP-x protein houses an N-terminal thiolase domain and a C-terminal non-specific lipid transfer protein domain (SCP-2) (Seedorf *et al*. 2000). Approximately half of SCP-x proteins are cleaved to yield separate thiolase and SCP-2 proteins after import into peroxisomes (Gallegos *et al*. 2001). SCP-2 can also arise by transcription from an alternative promoter of the SCP2 gene which encodes SCP-x. While SCP-x appears to be exclusively peroxisomal, over half of total SCP-2 is extraperoxisomal and diffusely distributed in the cytoplasm (Schroeder *et al*. 2000).

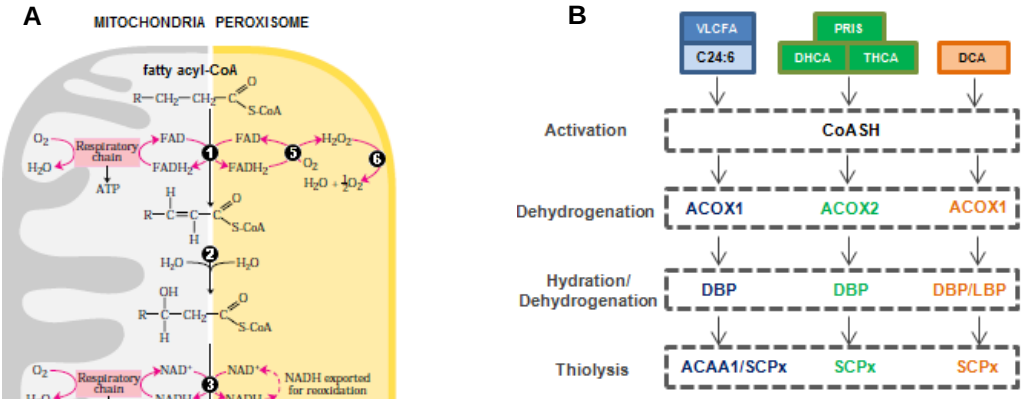

Figure 4. Fatty acid β-oxidation. **(A)** In each cycle of β-oxidation four enzymatic reactions convert a fatty acyl-CoA molecule to acetyl-CoA and a fatty acyl-CoA shortened by two carbon atoms: dehydrogenation (1), hydration (2), dehydrogenation (3), and thiolysis (4). In mitochondria, ATP is generated through the electron transport chain and the citric acid cycle. In peroxisomes, acetyl-CoA oxidases (5) transfer electrons directly to O_2 and the resulting H_2O_2 is degraded by catalase (6) (Adapted from Nelson & Cox 2004). **(B)** Core peroxisomal β-oxidation enzymes in the degradation of different fatty acid substrates. VLCFA, very-long-chain-fatty acids; C24:6 tetracosahexaenoic acid; PRIS, pristanic acid; D/THCA, di- and trihydroxycholestanoic acid; DCA, long-chain dicarboxylic acid; CoASH, free unesterified coenzyme A (Adapted from Wanders & Waterham 2006).

Detoxification of reactive oxygen species in peroxisomes

Reactive oxygen species (ROS) are a group of highly-reactive oxygen-containing molecules generated by normal cellular metabolism, as well as by exposure to environmental oxidants and stresses like heat shock and UV radiation. ROS is a broad term that includes free radical species (i.e. with unpaired electrons), such as superoxide anion ($O^{\bullet}_2^-$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, but also non-radical species like hydrogen peroxide (H_2O_2) (Circu & Aw 2010). Reactive nitrogen species (RNS) have many functions in common with ROS. RNS include the free radical nitric oxide ($NO^{\bullet}$) and the highly cytotoxic peroxynitrite ($ONOO^-$), which results from a reaction between NO and $O^{\bullet}_2^-$ (Nordberg & Arnér 2001). Intracellular oxidative stress arises from a significant increase in ROS or from

impairment of their detoxification mechanisms (Schrader & Fahimi 2006). The effects of ROS are dose-dependent. At low and moderate concentrations they have a physiological role in responses to noxia, including defense against infectious agents and mediation of cellular signaling pathways (Valko *et al*. 2007). High levels of ROS exert damage on biomolecules, including DNA, proteins and lipids, leading to an accumulation of oxidative damage in diverse cellular locations and to the deregulation of ROS-mediated metabolic and signaling pathways (Finkel 2011). Along with ER monooxygenases and plasma membrane NADPH oxidases, peroxisomal oxidases (including acyl-CoA oxidases of the FA β-oxidation pathway) are a source of cytosolic ROS under physiological conditions (Circu & Aw 2010). To sustain equilibrium between production and scavenging of ROS, as well as to respond to the diffusion of ROS generated in other intra- and extracellular locations, peroxisomes harbor several antioxidant enzymes (**Fig.5**) (reviewed in Schrader & Fahimi 2006). The peroxisomes are only a small part of a larger interacting network of ROS/RNS-detoxifying enzymes and low molecular weight antioxidant molecules that preserve the several levels of intracellular redox homeostasis (Circu & Aw 2010).

Although not a free radical, H_2O_2 is highly important due to its ability to penetrate biological membranes, its role in the production of more reactive ROS molecules, and its functions as an intracellular signaling molecule (Nordberg & Arnér 2001). H_2O_2 is removed by three types of enzymes: catalases, glutathione peroxidases and peroxiredoxins. Catalase is a heme-containing enzyme and the classical marker enzyme of peroxisomes. Its major function is the dismutation of H_2O_2 to H_2O and O_2, but it also detoxifies other substrates such as phenols and alcohols through coupled reduction of H_2O_2, and lowers the risk of $^{\bullet}OH$ formation from H_2O_2 via the Fenton reaction catalyzed by metal ions (Nordberg & Arnér 2001). Glutathione peroxidases (GPx) are present in virtually all cellular compartments, but are primarily cytosolic. These enzymes catalyze the degradation of H_2O_2 with concomitant conversion of reduced glutathione (GSH), a key intracellular antioxidant molecule, to glutathione disulfide (GSSG). There are four mammalian GPxs (GPx1-4), all of them with a selenocysteine-containing active site (Ursini *et al*. 1995). Peroxiredoxins (PRDXs) are a recently characterized family of thioredoxin-dependent peroxidases capable of degrading H_2O_2 and different alkyl-hydroperoxides, and in which conserved cysteine residues (Cys) are the primary site of oxidation (Rhee *et al*. 2001; Rhee *et al*. 2005). Mammalian cells express six distinct peroxiredoxin isoforms: PRDX1-6. These can be divided into three groups: typical 2-Cys PRDXs (PRDX1-4), atypical 2-Cys PRDXs (PRDX5) and 1-Cys PRDXs (PRDX6). This classification is based on the Cys residues required for catalytic function (reviewed in Rhee *et al*. 2005). PRDXs make up a dynamic network spread out over different sub-cellular localizations and, among them, PRDX1 and PRDX5 are peroxisomal (Rhee et al 2005; Immenschuh *et al*. 2003), but also found in the cytoplasm, nucleus and mitochondria of

mammalian cells. PRDX1 is highly homologous to the cytoplasmic and mitochondrial PRDX2, but their subtle structural differences give PRDX2 a more efficient peroxidase activity, while PRDX1 is more sensitive to inactivation by H_2O_2 (overoxidation) and a better molecular chaperone (Lee *et al*. 2007). These two PRDXs are the most abundant in most types of mammalian tissues and cultured mammalian cells (Rhee *et al*. 2001).

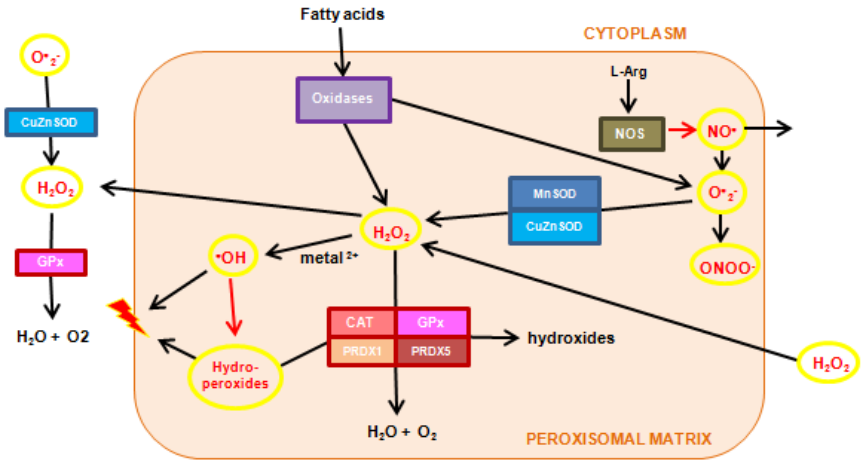

Figure 5. ROS detoxification in peroxisomes. H_2O_2 and hydroperoxides are degraded by catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxins (PRDX1, PRDX5) or converted to $^{\bullet}OH$ by Fenton reactions catalyzed by metal ions. $^{\bullet}OH$ reacts strongly with biomolecules and damages membranes by lipid peroxidation. $O^{\bullet}_2^-$ is scavenged by manganese (MnSOD) and copper/zink (Cu/ZnSOD) superoxide-dismutases. Nitric oxide synthase (NOS) oxidizes L-arginine (L-Arg) to nitric oxide ($NO^{\bullet}$), which can react with $O^{\bullet}_2^-$ to form the highly toxic $ONOO^-$ (Adapted from Schrader *et al*. 2006 & PeroxisomeDB 2.0).

Host peroxisomes in malaria liver stage: aims

The rapidly developing *Plasmodium* EEF has heavy lipid requirements, specifically for FAs, in order to support membrane biogenesis during liver stage (Prudêncio *et al*. 2006a; Vaughan *et al*. 2009). Although *Plasmodium* possesses a FA synthesis pathway (Waller *et al.* 1998) which is crucial for late liver stage development (Vaughan et al. 2009), host lipid metabolism also seems to be very important to the EEF (Mikolajczak *et al*. 2007; Rodrigues et al. 2008). Peroxisomes, abundant liver organelles where β-oxidation of specific FA substrates occurs (Wanders & Waterham 2006), are conspicuously absent in *Plasmodium* (Ding *et al*. 2000; Gardner *et al*. 2002; McIntosh *et al*. 2005). Thus, the hypothesis of a possible host-dependency at the level of peroxisomal lipid metabolism was formed. Microarray and proteomics data from Dr. M. Mota's lab (IMM, Lisbon) revealing changes in peroxisome-related pathways during infection supported this hypothesis, and further suggested an anti-oxidative stress-related role. The major aim of the present project was to uncover interactions between *Plasmodium* and host peroxisomes, which could potentially provide targets for new chemotherapeutic strategies against liver stage malaria. The effects of infection on the general properties of the host peroxisomal population and the contribution of the two major peroxisomal pathways, β-oxidation and ROS detoxification, were studied.

MATERIALS AND METHODS

Cell culture

Adherent human hepatoma cells (Huh7) were cultured at 37ºC 5% CO_2 in Roswell Park Memorial Institute medium (RPMI-1640) with phenol red and supplemented with 10% fetal bovine serum (FBS), 1% HEPES pH 7, 1% Minimum Essential Medium-Eagle with Non-Essential Amino Acids (MEM-NEAA), 1% L-Glutamine, 1% Penicillin/Streptomycin (Pen/Strep). Only Huh7 passaged at least 3 times and no more than 12 times after thawing from -80ºC storage were used for infection assays. Cells passaged on 10cm Petri dishes were washed with phosphate buffer saline (PBS), detached with the trypsin-like TrypLE Express for 5min at 37ºC 5% CO_2 and collected in supplemented RPMI. After 5min of centrifugation at 290 x *g*, cells were resuspended, counted by microscopy in a Neubauer-Improved chamber, appropriately diluted in RPMI and plated for assays. In general, $8x10^3$–10^4 cells were plated on 96-well plates, $4x10^4$–$5x10^4$ on 24-well plates and glass coverslips, and $3x10^5$–$4x10^5$ on 35mm glass bottom dishes. Medium, supplements and trypsinization reagent were purchased from Gibco and Neubauer-Improved chamber from LO – Laboroptik.

Parasite lines

Rodent malaria parasite *Plasmodium berghei* ANKA (*Pb*A) wild-type clone 2.34 (*Pb*WT) (Sinden *et al*. 2002) and three transgenic parasite lines created by genetic modification of *Pb*A clone cl15cy1 were used in this study. Each modified line constitutively expresses a transgene under the control of the elongation factor 1-alpha (*eef1α*) promoter during the entire life cycle, without compromising parasite viability and infectivity. Line 259cl2 expressing Green Fluorescent Protein (GFP) (*Pb*-GFP_{con}, RMgm-5, Franke-Fayard *et al*. 2004) and line 733cl1 expressing the Red Fluorescent Protein derivative RedStar (Pb-RFP_{con}, RMgm-86, Sturm *et al*. 2009) were used for flow cytometry analysis, while line 676m1cl1 expressing a GFP-firefly luciferase fusion protein (*Pb*GFP-LUC_{con}, RMgm-29, Janse *et al*. 2006) was used for luciferase assays. All four lines were used for immunofluorescence stainings followed by confocal fluorescence microscopy.

In vitro infection and culture of liver stages

Cells were infected *in vitro* by exposure to *P. berghei* sporozoites freshly extracted from the salivary glands of female *Anopheles stephensi* mosquitoes (Mota & Rodriguez 2000). Mosquitoes were infected by feeding on the blood of infected mice and dissected after 21-35 days. The salivary glands of dissected mosquitoes were collected into Dulbecco's Modified

Eagle's Medium (DMEM, Gibco), mechanically homogenized to release the sporozoites within and filtered through a 70µm strainer. Sporozoites were counted in a Neubauer-Improved chamber, diluted in supplemented RPMI medium containing the antimycotic solution Fungizone (Gibco), and added to cells. After 5min centrifugation at 1810 x *g*, to allow invasion by the sporozoites, cells were cultured at 37ºC 5% CO_2 for the duration of the infection. The number of sporozoites used for a single infection varied between $8x10^3$ and 10^4 in 96-well plates for luciferase assay, between $2x10^4$ and $4x10^4$ in 24-well plates for fluorescence microscopy or flow cytometry, and between 10^5 and $1{,}5x10^5$ in 35mm glass bottom dishes for live imaging.

Luciferase assay

Infection with *Pb*GFP-LUC_{con} parasites in 96-well plates was quantified by measuring the bioluminescence resulting from luciferase activity (Ploemen *et al.* 2009) with Firefly Luciferase Assay Kit (Biotium). A fluorescence-based cell viability assay was routinely performed before each infection, as well as before each luciferase assay. Plated cells were incubated with supplemented RPMI medium containing alamarBlue (Invitrogen) for 1h30m, and fluorescence emission at 590±20nm after excitation at 520±9nm was measured by an Infinite M200 microplate reader. Between 44 to 48 hours after cell invasion, after the second cell viability assay, cells were washed with PBS and vortexed in lysis buffer for 20min. The 96-well plate was centrifuged to pellet cell debris, and a portion of the lysate of each well was pipetted to an opaque white 96-well plate. Firefly Luciferase Assay Buffer (FLAB) containing the luciferase substrate D-luciferin at 20µg/mL was added to each well, and luminescence was immediately quantified by microplate reader.

Flow cytometry

Taking advantage of the green and red fluorescent proteins constitutively expressed by *Pb*-GFP_{con} and *Pb*-RFP_{con} parasites, respectively, the effects of different experimental conditions on infection were analyzed by flow cytometry (FCM) (Prudêncio *et al.* 2008). Cells seeded and infected in 24-well plates were washed with PBS and detached with TrypLE Express at certain time-points after invasion. Detached cells were collected in PBS 10% FBS, centrifuged 5min at 200 *x g*, and resuspended in PBS 10% FBS. When quantification of total cell number was necessary, a fixed number of fluorescent beads (Flow-Count Fluorospheres from Beckman Coulter) was added to each sample. Cells were analyzed in BD LSRFortessa (*Pb*-GFP_{con} infections) or FACSAria III (*Pb*-RFP_{con} infections and *Pb*-GFP_{con} infections of DsRed-PTS1-transfected cells). Signal bleed-through between channels was appropriately

compensated and in DsRed-PTS1 quantification experiments fluorescence intensity was normalized by cell size (geometric mean of forward scatter). Flow cytometry data was processed with FlowJo software.

Drug tests

The effects of the peroxisomal fatty acid β-oxidation inhibitor thioridazine hydrochloride (thioridazine, Sigma) and of the catalase inhibitor 3-amino-1,2,4-triazole (3-AT, Sigma) on infection were tested by luciferase assay and flow cytometry. Thioridazine was resuspended in dimethyl sulfoxide (DMSO Hybri-Max, Sigma), while 3-AT was resuspended directly in culture medium. Plated cells were incubated with different concentrations of drug and with different incubation schedules. Concentrations in the range of 0–15μM for thioridazine and 0–10mM for 3-AT were tested for cytotoxicity by alamarBlue assay. Thioridazine at 5μM and 3-AT at 1mM were used for luciferase assay and flow cytometry analysis.

Expression knockdown by siRNA

Single-sequence (Ambion, kindly provided by Dr. Michael Hannus) and SMARTpool four-sequence pools (Dharmacon) of exogenous small interfering RNA (siRNA) duplexes against several human genes – CAT, PRDX1, PRDX2, ACOX1, HSD17B4, SCP2 – were used to evaluate the effects of host gene expression knockdown on infection (Prudêncio *et al*. 2008). Briefly, siRNAs were incubated in an Opti-MEM I (Gibco)-Lipofectamine RNAiMAX (Invitrogen) solution for 20min at room temperature. Cells were detached and collected as described in a previous section, but using Pen/Strep-free RPMI. Cells were then reverse transfected by being plated on wells containing 30nM final concentration of siRNA, or a multiple of 30nM in the case of simultaneous knockdown of different targets. Cells transfected with siRNA not targeting any annotated genes in the human genome were used as negative control, while cells transfected with siRNA targeting the SR-BI-coding gene SCARB1 were used as positive control (Rodrigues *et al*. 2008; Yalaoui *et al*. 2008). Before infection, the transfected cells were incubated at 37ºC, 5% CO_2 for 36 to 48 hours, with the medium being changed to fully supplemented RPMI with antibiotics 16 to 24 hours after transfection. Cell viability assay, infection and posterior analysis by luciferase assay and flow cytometry were performed as previously described.

Quantitative real-time PCR

Knockdown efficiency by RNAi was assessed through quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). DNase I-treated RNA extracted from

siRNA-transfected cells was reverse transcribed with random primers into cDNA through the following RT-PCR program: 25ºC for 10min, 55ºC for 30min, 85ºC for 5min, cooling to 4-10ºC. This cDNA was amplified by quantitative real-time PCR with incorporation of SYBR Green reagent. The qPCR program consisted of a holding stage of 20sec at 50ºC and 10min at 95ºC, a cycling stage with 50 cycles of 15sec at 95º and 1min at 60ºC, and a melting curve stage of 1min at 60ºC, 30sec at 95ºC and 15sec at 60ºC. To avoid the amplification of genomic DNA remnants, qPCR primer pairs were designed to span exon-exon junctions or to flank an intron with a minimum of 900 nucleotides. Data was normalized by the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT1) housekeeping gene and analyzed by the comparative C_T method ($\Delta\Delta C_T$) to produce relative gene expression levels. High Pure RNA Isolation Kit and Transcriptor First Strand cDNA Synthesis Kit were purchased from Roche, and DyNAmo HS SYBR Green qPCR Kit from Finnzymes.

Cloning

Invitrogen's ViraPower Adenoviral Expression System coupled to Gateway Technology was used to clone and overexpress mouse cDNA sequences coding GFP-tagged SCP-x and SR-BI. RNA extracted from livers of BALB/c or C57BL/6 mice was reverse transcribed to total liver cDNA, which was used as template for amplification with restriction-site containing primers. When possible, proof-reading *Pfu* DNA polymerase (Fermentas) was used to guarantee a low chance of amplification errors, but in some instances the use of *Taq* DNA polymerase (Fermentas) with higher amplification rate was necessary to assure the production of enough insert for cloning. Each cDNA insert was first cloned in-frame with EGFP in a pEGFP-C1 or pEGFP-N2 vector (Clontech), depending on the desired GFP-tag position, by ligating digested vector and insert with T4 DNA Ligase (Roche) and transforming *E. coli* DH5α competent bacteria (Invitrogen) by heat-shock at 42ºC. GFP-tagged clones were selected with kanamycin, purified at a small scale and sequenced. Each GFP-tagged insert was sub-cloned in the entry vector pENTR1A by another round of PCR, digestion, ligation, DH5α transformation, purification, and sequencing. The selected pENTR1A-GFP-tagged constructs were recombined with the adenoviral destination vector pAd/CMV/V5-DEST by Gateway Clonase II enzyme mix, and the final pAd-GFP-tagged clones were purified at a large scale from transformed DH5α. PCR products and digested inserts and vectors extracted from 0.8% agarose gels were purified with High Pure PCR Product Purification Kit from Roche, minipreps of plasmid DNA were performed with Wizard Plus SV Minipreps DNA Purification System from Promega, and maxiprep of the final adenoviral constructs were performed with JETSTAR 2.0 Plasmid Purification Kit from GenoMed. Schematics of the adenoviral constructs generated can be found in **Annex I, Fig.S1**.

Adenovirus production and cell transduction

To remove bacterial sequences and to expose the viral Inverted Terminal Repeat (ITR) sequences for proper viral replication and packaging, prior to cell transfection the pAd-GFP-tagged clones were digested with *Pac* I and purified with High Pure PCR Product Purification Kit (Roche). For each construct a 6-well plate well with $5x10^5$ 293A cells was plated and transfected the next day with Lipofectamine 2000 (Invitrogen). Transfected cells were incubated in the 6-well plate for 48 hours at 37ºC, 5% CO_2, and each well was trypsinized and transferred to a 10cm Petri dish. Culture medium was replaced with fresh supplemented medium every 2 or 3 days for a period of 7-10 days, until small regions of cytopathic effect were observed. Medium was replenished and 2-3 days later, when approximately 80% cytopathic effect was observed, adenovirus-containing cells were harvested in the spent medium into a 15mL Falcon tube. A crude lysate was prepared by 3 freeze/thaw cycles consisting of 30min incubation in dry ice followed by 15min in a 37ºC water bath. To pellet cell debris the lysate was centrifuged at 1810 x *g* for 15min, at room temperature, and the resulting supernatant of viral particles in spent medium was aliquoted into cryovials and stored at -80ºC. Each aliquot of viral particles was never thawed and refrozen more than 5 times. For overexpression assays by flow cytometry or live fluorescence microscopy, plated cells were transduced by simply adding adenoviral particles diluted in supplemented RPMI medium and incubating for 36-48 hours at 37ºC 5% CO_2 before infection with *P. berghei*.

DsRed-PTS1 transient transfections

To study the dynamics of the peroxisomal compartment, infected cells transiently transfected with a plasmid coding for DsRed-Serine-Lysine-Leucine (DsRed-PTS1) were imaged (Wiemer *et al*. 1997; Schrader *et al*. 2000). Briefly, cells in suspension or plated on the previous day were transfected with plasmid DNA using FuGENE 6 Transfection Reagent (Roche), and incubated for 24-48 hours at 37ºC 5% CO_2 before infection.

Live imaging and immunofluorescence

For live imaging of adenoviral transductions and transient transfections, cells were cultured in 35mm glass bottom dishes with 10mm microwell (MatTek Corp). Cells were either imaged in culture medium or, when using Hoechst 3342 (Invitrogen) to stain nuclei, culture medium was replaced with RPMI without phenol red but containing Hoechst (1:1000) 30 min prior to imaging. For immunostainings, infected and non-infected cells on glass coverslips were fixed for 10min with 4% paraformaldehyde (PFA) at 24 or 48 hours post-infection, permeabilized with 0.5% Triton X-100 in PBS for 20-30 min and blocked with 0.1% Triton X-100 1% BSA in

PBS for 1 hour. Fixed cells were incubated with primary antibodies for 1-2 hours and washed with blocking solution, followed by 30min incubation with secondary antibodies coupled to Alexa Fluor (AF) 488, 555, 568, 594, 633 or 647 fluorophores. Finally, cells were washed with PBS, incubated 10-15min with 4',6-diamidino-2-phenylindole (DAPI) in PBS, and mounted on glass slides with Fluoromount-G (SounthernBiotech). Vibratome sections of infected mouse liver, 50µm thick, were fixed with 4% PFA, washed with PBS and permeabilized/blocked overnight at 4ºC with 0.5% Triton 1% BSA in PBS. They were incubated with primary antibodies overday at 4ºC and washed with blocking solution, followed by incubation with secondary antibodies, DAPI, and Phalloidin-AF660 overnight at 4ºC. Stained sections were washed with PBS and mounted between 2 glass slides with Fluoromount-G. Rabbit anti-bovine catalase (1:2500) was obtained from Rockland, mouse anti-human catalase (1:100) from Santa Cruz, rabbit anti-human PRDX2 (1:500) from Sigma, rabbit anti-GFP-488 (1:50) from Santa Cruz, DAPI (5µg/mL) from Sigma, and phalloidin-AF660 (1:100) from Molecular Probes. Mouse anti-*Pb*Hsp70/2E6 (1:500) was produced in-house (M. Mota's lab, IMM, Lisbon, Portugal), while chicken anti-*Pb*EXP1 (1:500) and rabbit anti-UIS4 (1:500) were kindly provided by Dr. Volker Heussler and Dr. Stephan Kappe, respectively. Live and fixed samples were examined under a Zeiss LSM 710 laser point-scanning confocal microscope (live cells at 37ºC). Image processing was performed with ImageJ software.

ROS detection

The general oxidative stress indicator 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (C400 from Molecular Probes) is a nonfluorescent molecule that becomes fluorescent when its acetate groups are removed by intracellular esterases and oxidation occurs within the cell. This reagent was used to detect ROS by flow cytometry in FACSAriaIII (Eruslanov & Kusmartsev 2010). Immediately prior to use, C400 was resuspended in DMSO to a final concentration of 100mM and diluted to a working concentration of 10µM in warm supplemented RPMI without phenol red or FBS. Cells plated with FBS-containing medium were washed with warm HBSS and incubated for 45min with the freshly-prepared 10µM C400 solution. Cells were washed again and incubated with H_2O_2 (Sigma), sodium azide (Sigma), or sodium pyruvate (Sigma and Gibco) in RPMI without phenol red or FBS. The treatments lasted 45min, after which the cells were washed, tripsinized and resuspended in PBS 10% FBS for analysis. C400 was excited by the 488nm laser and emitted fluorescence was detected by the FITC detector, while RFP-expressing parasites were excited by the 561nm laser and detected by PE. Due to C400's high sensitivity to light and oxygen, these assays were, as much as possible, sheltered from excessive exposure to light and air.

RESULTS AND DISCUSSION

PART I: Dynamic properties of host peroxisomes in malaria liver stage

The conditions established within a hepatocyte during infection by intracellular trypanosomatid parasites of the genus *Leishmania* have been observed to significantly alter the morphology and dynamics of the host peroxisomal population (Raychaudhury *et al*. 2003; Gupta *et al*. 2009). To assess if the presence of the developing liver stage *Plasmodium* parasite can also affect host peroxisomal population properties, hepatoma cells transiently transfected with a DsRed-PTS1 plasmid were infected with *Pb*-GFP_{con} and analyzed by live confocal fluorescence microscopy and flow cytometry. Exclusive labeling of peroxisomes by DsRed-PTS1, comparable to the GFP-PTS1 construct already established for mammalian cells *in vitro* (Wiemer *et al*. 1997; Schrader *et al*. 2000), was confirmed by co-localization of DsRed-PTS1 with known peroxisomal matrix proteins. DsRed-PTS1-transfected cells were fixed and immunolabeled for catalase, the most common peroxisomal marker enzyme (**Fig.6A**), or imaged live after transduction with AdGFP-SCP-x, an adenoviral construct that overexpresses the peroxisomal fatty acid thiolase SCP-x (**Fig.6B**).

The general spatial distribution of peroxisomes observed by live fluorescence microscopy of DsRed-PTS1 appeared unaltered in infected cells at different time-points post-infection (**Fig.6C**) when compared to non-infected cells (**Fig.6D**). Although peroxisomes were observed in relatively close proximity to the exoerythrocytic form of the parasite (EEF) (**Fig.6C**), this appears to be a consequence of the random distribution of dynamic structures within the smaller space that is available in the infected cell cytosol, and not a particular accumulation around the parasite. No Dsred-PTS1 signal was seen to co-localize with the EEFs, indicating that internalization of intact host peroxisomes by the parasite does not occur. The possibility of interactions between the peroxisomal membrane and the parasite's parasitophorus vacuole membrane (PVM), however, cannot be excluded.

The geometric mean of DsRed-PTS1 fluorescence intensity in cells infected with *Pb*-GFP_{con} was analyzed by flow cytometry at two time-points post-infection (**Fig.6E**). While at 2 hours post-infection there is no difference between DsRed-positive infected and non-infected cells, at 16 hours post-infection a 30% reduction in the geometric mean of the DsRed-PTS1 signal is observed in infected cells when compared to non-infected cells. Although the evolution of DsRed-PTS1 signal at later time-points post-infection needs to be assessed, this result already suggests that in infected cells the number and/or size of peroxisomes decreases as infection progresses.

It thus seems that *Plasmodium* EEFs may affect some properties of the host cell peroxisomal dynamics. Compilation of more fluorescence microscopy data is underway in

order to quantify the changes in number or size that are suggested by the flow cytometry results. Further detailed characterization of these changes will be carried out through an immunoelectron microscopy study of peroxisome morphology and distribution in infected cells (Funato *et al*. 2006).

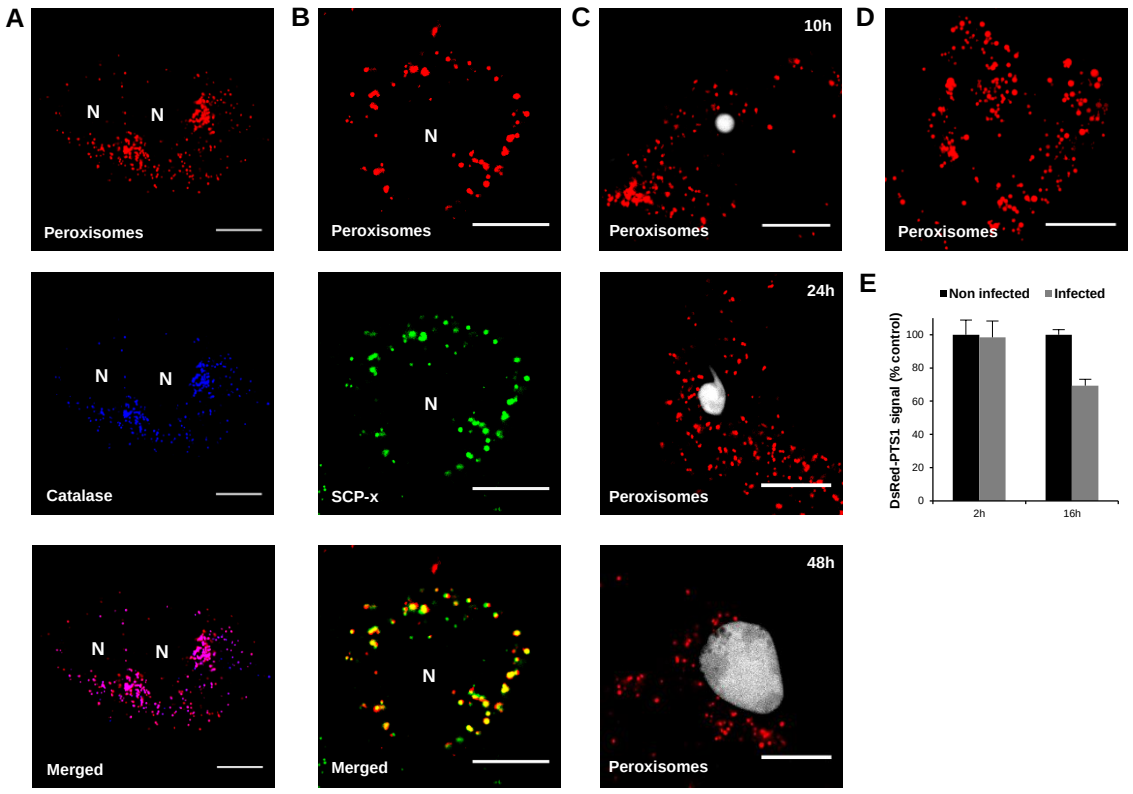

Figure 6. Host peroxisomal population may be altered in *Plasmodium* infected cells. (A) DsRed-PTS1-transfected cells were fixed and immunolabeled for catalase. DsRed-PTS1-labeled peroxisomes (red) co-localize with the most common peroxisomal marker enzyme, catalase (blue). **(B)** DsRed-PTS1-transfected AdGFP-SCP-x-transduced cells were imaged live. DsRed-PTS1 (red) also co-localizes with the FA β-oxidation thiolase SCP-x (green). **(C-D)** Live confocal microscopy of DsRed-PTS1-transfected cells. The DsRed-PTS1-labeled peroxisomal population (red) appears unaltered in **(C)** infected cells (EEFs in grey) at different time-points when compared to **(D)** non-infected cells. **(E)** Quantification of DsRed-PTS1 in infected (grey) and non-infected (black) cells by flow cytometry. A 30% decrease is observed at 16 hours post-infection (t-test, $p<0.05$). N denotes nuclei. Scale bar, 10µm.

RESULTS AND DISCUSSION

PART II: Role of host peroxisomal fatty acid β-oxidation in malaria liver stage

Impairment of host peroxisomal FA β-oxidation affects Plasmodium liver stage

Following the observation that *Plasmodium* infection may interfere with peroxisome population dynamics of hepatocytes, host-parasite interactions at the functional level of peroxisomes were sought out. Firstly, peroxisomal fatty acid β-oxidation, one of the two major pathways of mammalian peroxisomes, was studied. Thioridazine, a drug of the phenothiazine group, has been shown to selectively inhibit hepatic peroxisomal β-oxidation in isolated hepatocytes (Leighton *et al*. 1984), as well as *in vivo* (Van den Branden, C. & Roels 1985). In order to assess the effects of host peroxisomal β-oxidation inhibition on infection, cells were exposed to thioridazine for different periods of time, with the drug being added before or after *in vitro* host cell invasion by *Plasmodium* (**Fig.7A**). The cytotoxicity of increasing thioridazine concentrations was measured, and 5μM was chosen as the highest concentration that is not excessively cytotoxic (**Annex II, Fig.S2A**). Infection levels after exposure to 5μM of drug were quantified by luciferase assay (**Fig.7B**) and flow cytometry (**Fig.7C**).

Exposure to 5μM of thioridazine during the 2-hour period of invasion (**Fig.7B 'Inv'**) exerted no effect on the subsequent progress of infection, which suggests that this time-window is too limited and that thioridazine does not target the parasite itself directly. All the remaining schedules of exposure, from the pre-invasion 4-hour schedule (**Fig.7B 'Preinv+Inv'**) to the post-invasion 46-hour schedule **(Fig.7B 'Postinv+46h'**) resulted, without significant difference, in a 30-45% decrease of infection. When analyzed by flow cytometry, the pre-invasion 48-hour exposure schedule lead to a 35% decrease in parasite development (**Fig.7C**), supporting the previous luciferase assay results. Thus, it seems that thioridazine's effect is not dependent on the duration of exposure or the moment during infection when drug exposure starts. Although significant, the negative effect on infection is not cumulative and does not cross the 30-45% plateau. This suggests that host peroxisomal β-oxidation is somehow important, but not crucial for parasite survival.

The effects of FA β-oxidation impairment on infection were also studied through siRNA-mediated knockdown of peroxisomal enzymes active at different levels of this pathway (**Fig.7D-E**). The knockdown target genes were ACOX1, HSD17B4 and SCP2, which respectively code for the acyl-CoA oxidase ACOX1; the hydratase/ dehydrogenase known as D-bifunctional protein (D-BP); and two proteins which function as a thiolase and a lipid transfer protein, SCP-x and SCP-2. Additionally, PEX14, a gene that codes for a

peroxin involved in peroxisomal biogenesis, was also targeted. Scrambled siRNA sequences that do not have targets in human cells were used as negative controls in these experiments, and the targeting of *scavenger receptor* class B member 1 (SR-BI), which is known to decrease *Plasmodium* liver stage infection (Rodrigues *et al*. 2008; Yalaoui *et al*. 2008), was used as positive control. Both single-sequence siRNAs (**Fig.7D**) and siRNA pools of 4 sequences (**Fig.7E**) were tested. Of the three single-sequences tested for each target, only the less cytotoxic with an appropriate target knockdown is shown. Knockdown efficiencies were confirmed at the mRNA level by qRT-PCR (**Annex III, Fig.S3A-B**), and will also be assessed at the protein level by Western Blot in the near future.

Knockdown of SR-BI resulted in 65% reduction of infection when compared to the negative control, whereas the targeting of PEX14 and D-BP lead to 40% and 60% reduction, respectively (**Fig.7D**). The siRNA pools targeting ACOX1 and SCP-x/2 resulted in 40% and 15% reduction, respectively, in contrast to the 60% reduction obtained with SR-BI knockdown (**Fig.7E)**. It seems that peroxisomal β-oxidation enzymes may play a significant role in the mediation between host cell peroxisomal β-oxidation and the parasite. The fact that SCP-2 knockdown had a very small effect on infection can be explained by the existence of several other peroxisomal enzymes that possess SCP-2 domains as part of their multifunctional structure (including D-BP), and therefore non-specific lipid transport in the absence of SCP-2 could be carried out by one of the other functionally redundant proteins. The analysis of additional peroxisomal β-oxidation enzymes, as well as double and triple simultaneous knockdowns, will be performed to further complement this data and assess the relevance of different branches of the pathway.

To overcome the functional redundancy of SCP-2, overexpression of GFP-tagged SCP-x through adenoviral transduction (Ad-GFP-SCP-x) was conducted in order to evaluate if increased levels of this key peroxisomal thiolase have any effect on *Plasmodium* infection (**Fig.7C-D**), since expression knockdown did not. Overexpression of GFP-tagged SR-BI was the positive control and overexpression of GFP alone in the same adenoviral vector as the other constructs was the negative control. At 2 hours post-infection (**Fig.7F**), it is clear that overexpression of SR-BI and SCP-x increases the percentage of infected cells by 55% and 30%, respectively. At 48 hours post-infection, the percentage of infection is still higher by 50% in the case of AdSR-BI-GFP, when compared to control, and by 45% in the case of Ad-GFP-SCP-x (**Fig.7G)**. No change in parasite development, quantified as the geometric mean of RFP signal emitted by the *Pb*-RFP_{con} transgenic parasite was observed (**Fig.7G**).

The evidence gathered from inhibition by thioridazine, siRNA knockdown and enzyme overexpression points to a relevant, although not crucial, role for peroxisomal β-oxidation during *Plasmodium* host cell invasion and development. It can be speculated that this role does not consist of a direct interaction between host proteins and the parasite, but

probably arises from the substantial part that peroxisomes play in the overall intracellular lipid metabolism of the host.

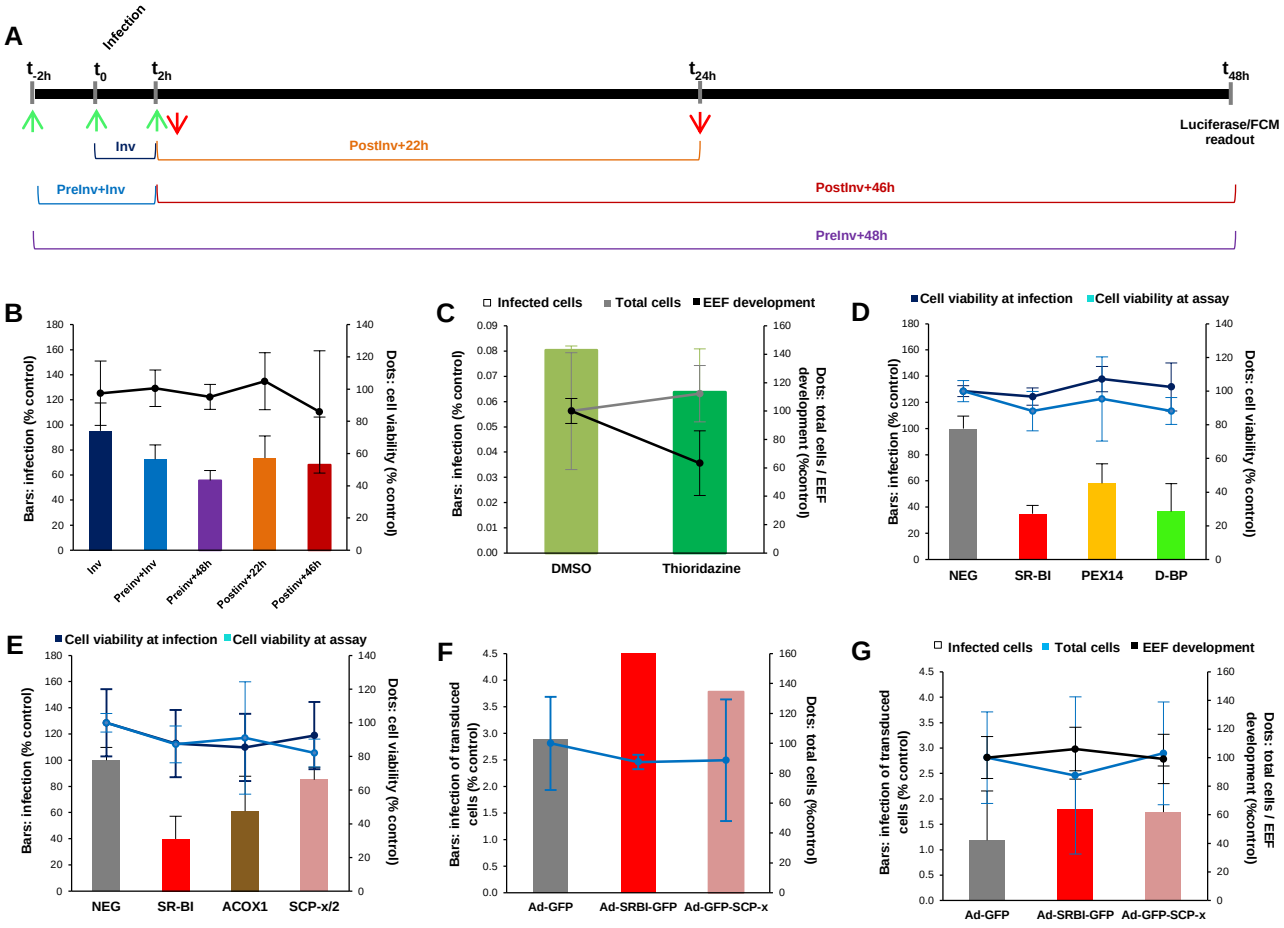

Figure 7. Impairment of host peroxisomal fatty acid β-oxidation affects *Plasmodium* liver stage development. (A) Overview of thioridazine exposure schedules. Green arrows indicate the addition of drug to the medium and red arrows indicate drug removal. **(B)** Quantification of infection by luciferase assay in cells subjected to treatments of 5µM of thioridazine. Drug exposure during invasion only has no effect, whereas all other treatment schedules decrease infection by 30-45%, without significant difference between them (t-test, $p>0.05$). **(C)** Flow cytometry analysis of 5µM thioridazine treatment on a pre-incubation 48-hour exposure schedule. Percentage of infected cells is not significantly affected by thioridazine (t-test, $p>0.05$), but EEF development decreases by 35% (t-test, $p<0.05$). **(D)** Quantification by luciferase assay of the effects on infection of knockdown of β-oxidation enzymes with single-sequence siRNA and **(E)** siRNA pools. All knockdowns significantly decreased infection by 40-60% (t-test, $p<0.05$), with the exception of SCP-x/2. **(F)** Quantification of the effects on infection of Ad-GFP-SCP-x overexpression by flow cytometry at 2 hours and **(G)** 48 hours post-infection. Both SR-BI and SCP-x overexpression increase percentage of infection by 30-50% (t-test, $p<0.05$) without affecting EEF development.

SCP-x localization does not correlate with Plasmodium EEFs

Once SCP-x overexpression increases infection, the localization of this enzyme in relation to the EEF was analyzed by fluorescence microscopy (**Fig.8**). No co-localization between Ad-GFP-SCP-x and the EEF was observed, not even between Ad-GFP-SCP-x and the PVM, at the level of which direct host-parasite interactions could occur. This observation supports the previously mentioned speculation that the role of host peroxisomal β-oxidation enzymes during *Plasmodium* infection does not lie in direct interaction, but is probably related to the setting-up of favorable intracellular metabolic conditions for the parasite at the level of general lipid and fatty acid availability or even the production of specific classes of lipid metabolites.

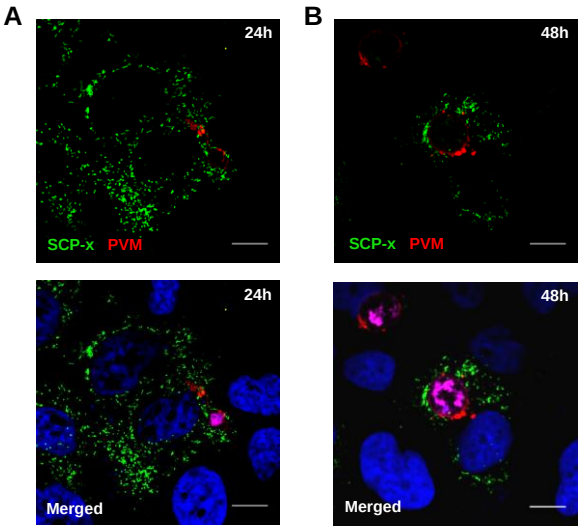

Figure 8. **Ad-GFP-SCP-x does not co-localize with *Plasmodium* EEFs *in vitro***. Cells transduced with the adenoviral construct Ad-GFP-SCP-x were fixed at **(A)** 24 hours and **(B)** 48 hours post-infection. Samples were stained for the EEF (magenta), the parasitophorus vacuole membrane as revealed by *Pb*UIS4 staining (PVM, red), and nuclei (blue). GFP-SCP-x (green) was not seen to co-localize with either the EEF or the PVM. Scale bar 10µm.

RESULTS AND DISCUSSION

PART III: Role of host peroxisomal antioxidant system in malaria liver stage

Impairment of host peroxisomal peroxidases does not affect Plasmodium liver stage

The second major pathway of mammalian peroxisomes is the detoxification of reactive oxygen species (ROS). For this purpose, peroxisomes harbour many antioxidant enzymes, particularly those that degrade hydrogen peroxide (H_2O_2). In order to assess if this peroxisomal function also plays a role in *Plasmodium* infection, the approaches previously described for FA β-oxidation were applied again.

Firstly, 3-amino-1,2,4-triazole (3-AT), a heterocyclic organic compound that specifically and covalently binds to the active centre of catalase (Margoliash & Novogrodsky 1957; Margoliash et al. 1959; Chang & Schroeder 1972), was used to study the effects of H_2O_2-detoxification inhibition. Cells were submitted to different schedules of exposure to 1mM 3-AT, before and after *Plasmodium* invasion (**Fig.9A**). This concentration was chosen due to being the highest 3-AT concentration that is not excessively cytotoxic (**Annex II, Fig.S2B**). None of the 3-AT exposure schedules, from the shortest 4-hour exposure (**Fig.9B 'Preinv+Inv'**) to the longest 50-hour exposure **(Fig.9B 'Preinv+48h'),** significantly affected infection. The longest schedule was also tested by flow cytometry, yielding the same result (**Fig.9C**).

Two key peroxisomal peroxidases, catalase and the peroxiredoxin PRDX1, where knockdowned along with PRDX2, a cytosolic peroxiredoxin of the same antioxidant network and that is known to be uptaken by blood stage forms of *Plasmodium* (Koncarevic *et al*. 2009). Catalase knockdown by siRNA pool resulted only in a 25% decrease in infection, despite the 90% knockdown efficiency. Although both single-sequence (**Fig.9D**) and pooled siRNAs (**Fig.9E**) knockdowned the expression of these enzymes by 85% (**Annex III, Fig.S3C-D**), single-sequences targeting PRDX1 and PRDX2 decreased infection by 50% and 70%, respectively (**Fig.9D**), whereas four-sequence siRNA pools lead only to a 20% decrease (**Fig.9E**). Two possible and mutually exclusive conclusions can be drawn from these results. Either the strong decrease in infection after single-sequence siRNA knockdown of PRDX1 and PRDX2 is real and the siRNA pools yielded false negatives (Brown *et al*. 2005; reviewed in Smith 2006), or the single-sequence siRNAs yielded false positives but the siRNA pools did not because they have less off-target effects (reviewed in Smith 2006; Dharmacon RNAi Technologies 2010). Considering that inhibition by 3-AT (**Fig.9B**) did not affect infection and that only a 25% decrease was observed by expression knockdown of catalase (**Fig.9E**), the most abundant peroxisomal peroxidase, it is perhaps

more reasonable to lean towards the latter possibility. This also seems to be supported by the fact that intracellular ROS levels, as revealed by the sensitive general ROS indicator C400 (**Fig.9F**) and quantified by flow cytometry, are not significantly higher in infected cells at 24 hours post-infection when compared to non-infected cells (**Fig.9G**). Thus, managing oxidative stress may not be a severe problem faced by liver stage *Plasmodium*, in contrast to its blood stage counterpart (reviewed in Postma *et al*. 1996 & Becker *et al*. 2004), and so EEFs may not need to take significant advantage of host peroxisomal peroxidases. However, it is not truly possible to conclude that H_2O_2-detoxification is not one of the functional roles of host peroxisomes during malaria liver stage without first assessing the effects of simultaneous knockdown of the different peroxidases, which is currently underway.

Catalase, but not PRDX2, co-localizes with Plasmodium EEFs

The localization of two host peroxidases regarding the developing EEF was also assessed by immunofluorescence microscopy (**Fig.10**). A substantial amount of catalase was seen to localize inside EEFs at different developmental stages *in vitro* (**Fig.10A**), and also at 48 hours post infection in mouse liver slices (**Fig.10C**). Fluorescence microscopy of infected cells after catalase knockdown by siRNA was carried out to confirm the specificity of catalase staining. Catalase knockdown with siRNA pool was quantified to be 90% by qRT-PCR (**Annex III, Fig.S3D**). However, when compared to samples subjected to scramble siRNA (**Fig.10A**), some staining for catalase was still clearly visible in the catalase knockdown samples (**Fig.10B**), although the overall staining was greatly reduced. Interestingly, after catalase knockdown some of the staining that was previously observed to be inside EEFs remains, apparently only as reduced as the overall signal (**Fig.10B**). This suggests that co-localization between host catalase and the developing EEF indeed occurs. Further confirmation is being obtained by Western Blot for mammalian catalase in infected cells and sporozoite samples. It seems inconsistent that catalase knockdown does not yield stronger negative effects on infection if this host enzyme is recruited by the parasite during its liver development. *Plasmodium* has two complex redox systems of its own, and it may be that, like it was observed for PRDX2 in blood stage (Koncaveric *et al*. 2009), the parasite only takes advantage of host redox enzymes in order to save resources, but is not truly dependent on them for its survival and can use other functionally redundant proteins when necessary. In contrast, immunostaining for PRDX2 shows the cytosolic distribution of this peroxiredoxin and that it does not co-localize with EEFs at 24 or 48 hours post infection (**Fig.10D**).

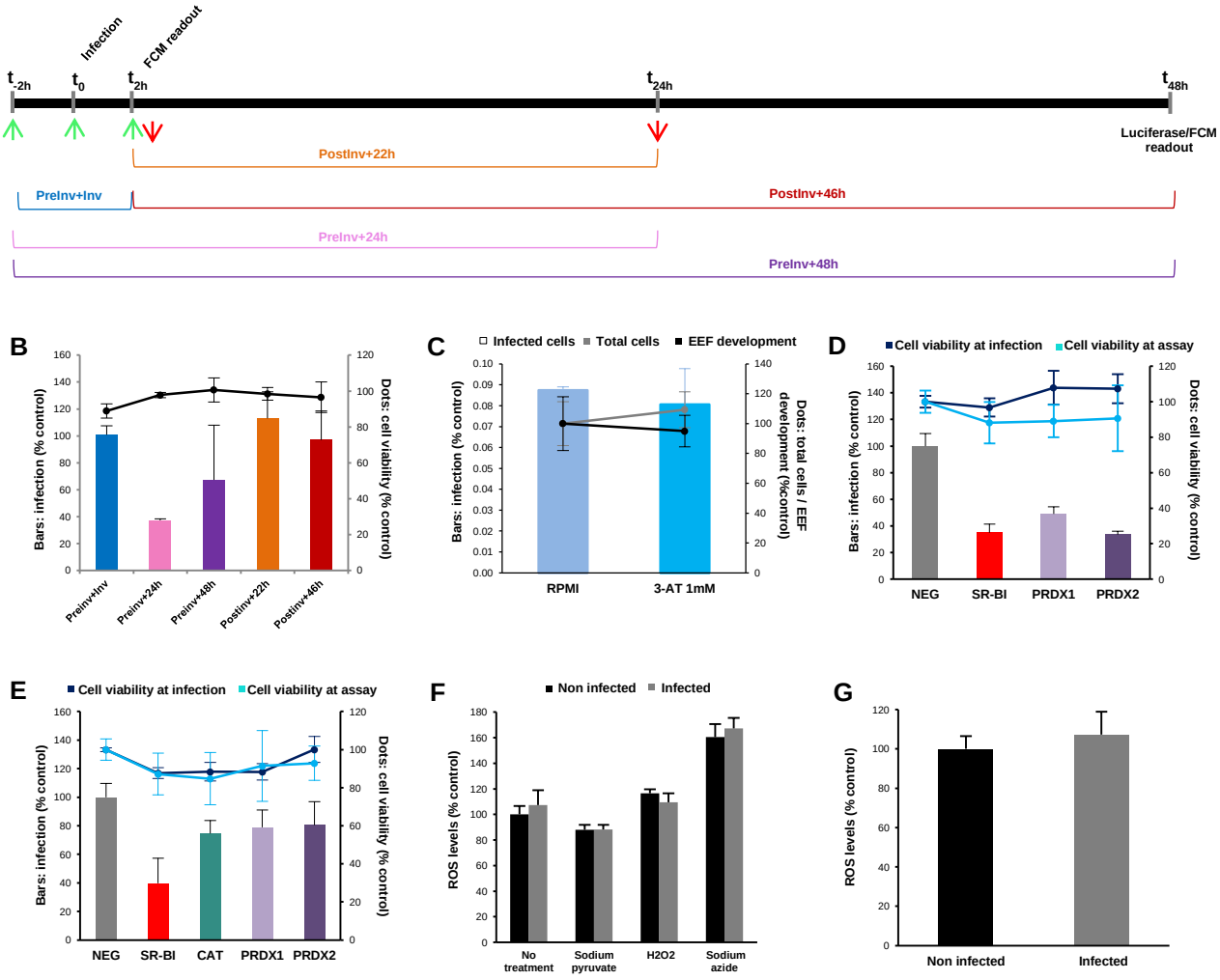

Figure 9. Impairment of host peroxisomal H_2O_2-detoxification does not affect *Plasmodium* liver stage development. (A) Overview of 3-AT exposure schedules. Green arrows indicate the addition of drug to the medium and red arrows indicate drug removal. **(B)** Quantification of infection by luciferase assay in cells subjected to treatments of 1mM of 3-AT. Drug exposure during *Plasmodium* invasion leads to a drastic 80% decrease of infection (t-test, $p<0.05$), whereas all other treatment schedules have none (t-test, $p>0.05$). **(C)** Flow cytometry analysis of 1mM 3-AT treatment on a pre-incubation 48-hour exposure schedule. Neither percentage of infected cells nor EEF development are significantly affected by 3-AT (t-test, $p>0.05$). **(D)** Quantification by luciferase assay of the effects on infection of knockdown of host peroxidases with single-sequence siRNA and **(E)** siRNA pools. Although single-sequence siRNAs targeting PRDX1 and PRDX2 appear to affect infection (t-test, $p<0.05$), siRNA pools offer the opposite result (t-test, $p>0.05$). Catalase knockdown by siRNA pool yielded only 25% reduction of infection (t-test, $p<0.05$). **(F)** Quantification of intracellular ROS levels by flow cytometry. The general ROS indicator used, C400, responds well to ROS scavengers such as sodium pyruvate (20mM) and ROS promoters such as H_2O_2 (200µM) and the highly toxic sodium azide (5mM). **(G)** Intracellular ROS levels, as revealed by C400 and quantified by flow cytometry at 24 hours post-infection, are not significantly increased in infected cells when compared to non-infected cells (t-test, $p>0.05$).

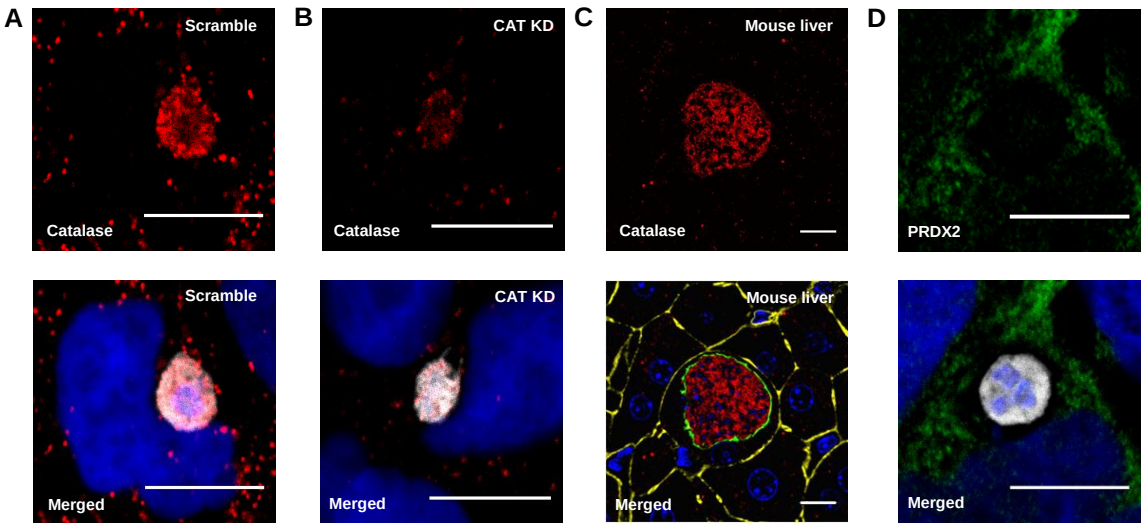

Figure 10. Catalase, but not PRDX2, localizes with Plasmodium EEFs *in vitro* and *in vivo*. (A) Immunostaining for host catalase in cells transfected with scramble siRNA. Substantial catalase signal (red) is observed inside EEFs at 12, 24 and 48 hours post-infection. A representative 24-hour EEF is shown (gray). **(B)** The same staining was performed for cells transfected with a siRNA pool targeting catalase. Overall levels of catalase (red) were greatly reduced, but a proportional degree of staining remained visible within the 24-hour EEF. **(C)** Catalase staining (red) also co-localizes with 48-hour EEFs (contoured in green by *Pb*EXP1 staining) in liver slices of infected mice. Cell contours were revealed by filamentous actin staining by fluorochrome-conjugated phalloidin. **(D)** PRDX2 staining does not co-localize with EEFs at 24 or 48 hours post-infection. A representative 48-hour EEF is shown. Nuclei in blue in all merged panels. Scale bar, 10 µm.

CONCLUSIONS

The study of host-pathogen interactions in liver stage malaria remains a key approach for the uncovering of novel targets for preventive and therapeutic antimalarial strategies with potential to arrest the onset of malaria disease (reviewed in Mota *et al*. 2004; Prudêncio *et al*. 2006). The role played by the host in sustainment of *Plasmodium* infection is revealing itself to be multi-faceted and complex, much as the intracellular parasite itself. *Plasmodium* seems to be completely self-sufficient in certain aspects of its biology and life cycle, while in other cases it is opportunistically or strictly dependent on its host. Taking into account the metabolic landscape of hepatocytes and the parasite's major requirements during liver stage development, the present work proposed to uncover functional interactions between two compartmentalized host pathways – peroxisomal FA β-oxidation and ROS detoxification – and the growing intracellular parasite.

Peroxisome labeling with the DsRed-PTS1 construct revealed that the intracellular presence of *Plasmodium* can influence the properties of the mammalian hepatic peroxisomal population. Peroxisomes are highly plastic and dynamic organelles which can be regulated by many extracellular and intracellular cues (reviewed in Smith & Aitchison 2009 & Yan *et al*. 2005). The effect over peroxisome size and/or number that the results suggest can be mediated by the host cell or by the parasite. In the former case, the cell can be purposefully changing aspects of its metabolism as part of its defense mechanism against the presence of the parasite, in order to save resources or to funnel them to the most crucial cellular functions in a time of need. It the latter case, the parasite-mediated effect can be direct or indirect. Either the parasite provides a signal that directly regulates peroxisomal behavior (proliferation, turnover, matrix protein import and enzymatic activity) or the variation in intracellular resources and metabolic conditions due to host-cell subversion by the parasite gives rise to a signal that regulates peroxisome population and metabolism.

Results also showed that host peroxisomal functions at the level of fatty acid (FA) β-oxidation, and possibly also anti-oxidative stress, can contribute in different degrees to the successful progress of *Plasmodium* liver stage infection. The moderate dependency that the parasite seems to have regarding peroxisomal β-oxidation is probably tied to its metabolic requirements for membrane biosynthesis during growth and replication in the liver. The exact lipid requirements of *Plasmodium* are not known, but it probably needs a plentiful supply of FAs (Vaughan *et al*. 2009). The parasite itself possesses a prokaryotic type II FA synthesis pathway, through which it is able to synthesize FAs from derivatives of acetate and malonate (Waller *et al*. 1998; Vaughan *et al*. 2009). However, considering that *Plasmodium* can achieve one of the fastest growth rates among eukaryotic cells (Prudêncio *et al*. 2007), it

may not be able to produce the quantity of FAs that it needs or its requirements in terms of specific FA classes may go beyond its synthetic ability. Thus, breakdown of very-long-chain fatty acids (VLCFAs), which are peroxisomal-specific substrates (Wanders & Waterham 2006a), into chain-shortened FAs may be a source of particular classes of FAs for *Plasmodium*. The composition of the plasma membrane and the ability of the host cell to grow in order to accommodate the developing parasite are also other aspects that can be considered when it comes to specific lipid requirements. Plasma membrane extension and turnover in infected cells certainly demands more from the intracellular lipid resources and probably biases lipid breakdown and production toward specific substrates. While inhibition by thioridazine and expression knockdown results suggest that FA β-oxidation is important for parasite growth, increased percentage of infection after SCP-x overexpression also suggests that β-oxidation may improve host cell permissiveness to invasion. Perhaps by providing great amounts of particular FAs, increased peroxisomal β-oxidation may lead to changes in the lipid composition of the host plasma membrane, from which the parasite's PVM derives (Vaughan *et al*. 2009). Thus, the regulation of host FA metabolism in order to alter plasma membrane composition and allow for fruitful parasitic interactions and host cell subversion could be another reason why host peroxisomal FA β-oxidation is relevant in *Plasmodium* infection.

Regarding peroxisomal anti-oxidative stress systems, results seem to indicate that this antioxidant network does not play a strong functional role in *Plasmodium* infection, but that specific proteins such as the peroxisomal marker enzyme catalase may be recruited by the parasite to complement its own antioxidant systems in the maintenance of redox homeostasis during extremely rapid growth.

Overall this first set of data concerning host peroxisomes during *Plasmodium* liver stage infection is promising and can give rise to many other lines of questioning to be further studied, perhaps involving other host cell organelles. The dissection of the contribution of peroxisomal and possibly also mitochondrial β-oxidation as sources of specific classes of FAs, the characterization of the mechanisms employed by the parasite in order to take advantage of metabolic pathways that are tightly regulated and confined within host organelles, and profiling of enzymatic activity of lipid metabolic pathways in infected cells are particularly interesting future research perspectives.

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ANNEX I

Adenoviral constructs for protein overexpression

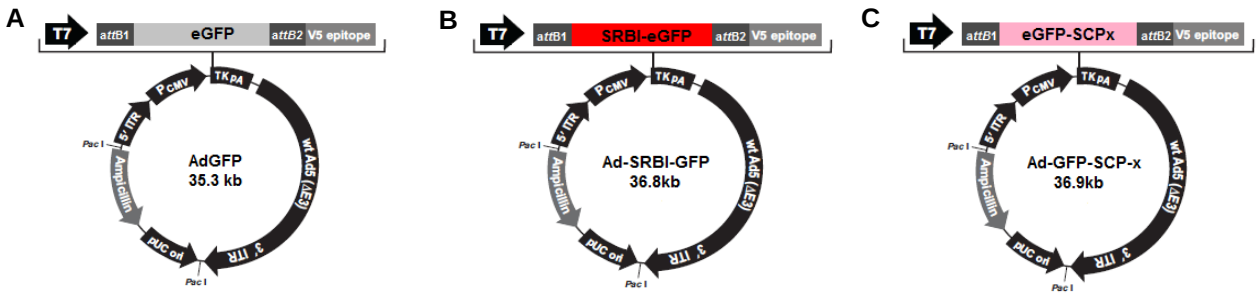

Figure S1. Adenoviral overexpression constructs. (A) GFP, **(B)** SR-BI-GFP and **(C)** GFP-SCP-x DNA inserts were generated in Clontech pEGFP-C1/pEGFP-N2 vectors and sub-cloned into the pAd/CMV/V5-DEST adenoviral vector for *in vitro* protein overexpression in mammalian cells (Adapted from Invitrogen's Gateway-adapted destination vectors User Manual 2010).

ANNEX II

Drug-induced cytotoxicity

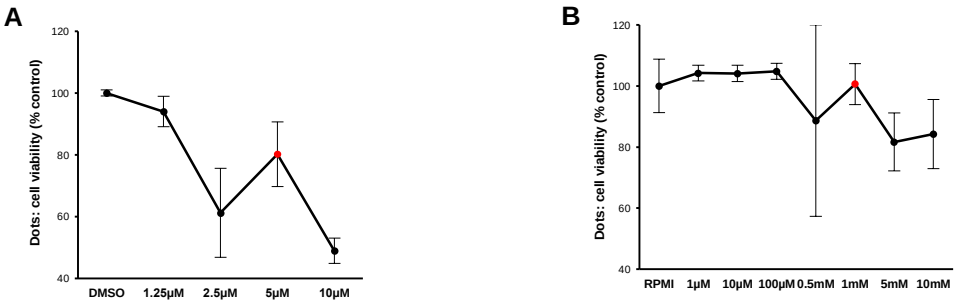

Figure S2. Drug-induced cytotoxicity. The effects of increasing concentrations of **(A)** thioridazine and **(B)** 3-AT inhibitors on cell viability, as quantified by alamarBlue assay, were tested. 5µM of thioridazine and 1mM of 3-AT, the highest concentrations without excessive cytotoxicity, were chosen for further study.

ANNEX III

Efficiency of siRNA-mediated knockdowns

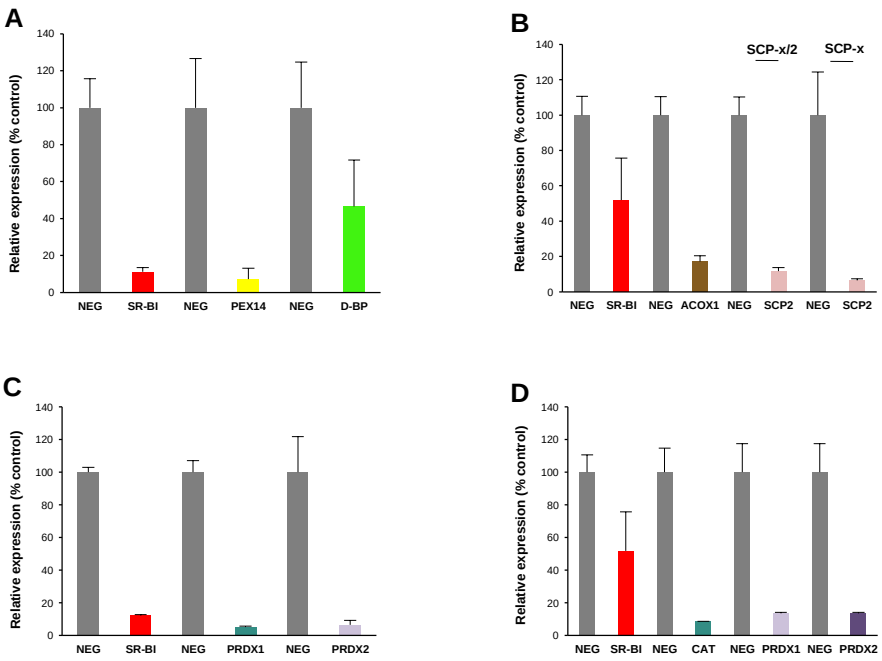

Figure S3. Efficiency of siRNA-mediated knockdowns. The efficiency of expression knockdown of (**A-B**) peroxisomal FA β-oxidation enzymes and (**C-D**) peroxisomal peroxidases was quantified by qRT-PCR. (**A, C**) Single-sequence siRNAs against each target. (**B,D**) Pools of four siRNA sequences against each target.