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Referência do projecto
Project reference
PTDC/SAU-MII/099118/2008

1. Identificação do projecto
1. Project description -

Área científica principal
Main Area
Ciências da Saúde - Microbiologia, Infecção, Imunologia e Inflamação

Área científica Secundária
Secondary area
Ciências Biológicas - Biologia Microbiana

Título do projecto (em português)
Project title (in portuguese)
A influência dos ácidos biliares na infecção do fígado por malária

Título do projecto (em inglês)
Project title (in english)
The influence of bile acids on malaria liver infection

Financiamento solicitado
Requested funding
179.182,00€

Palavra-chave 1
Plasmodium

Palavra-chave 2
Hepatócitos

Palavra-chave 3
Sinalização celular

Palavra-chave 4
Ácidos biliares

Keyword 1
Plasmodium

Keyword 2
Hepatocytes

Keyword 3
Cellular signalling

Keyword 4
Bile acids

Data de início do projecto
Starting date
01-07-2010

Duração do projecto em meses
Duration in months
36

2. Instituições envolvidas
2. Institutions and their roles

Instituição Proponente
Principal Contractor

Instituto de Medicina Molecular (IMM/FM/UL)
Avenida Professor Egas Moniz
1649-028Lisboa

Instituição Participante
Participating Institution

Faculdade de Farmácia da Universidade de Lisboa (FF/UL)
Av. Professor Gama Pinto
1649-003Lisboa

Unidade de Investigação
Research Unit

Instituto de Medicina Molecular (IMM/FM/UL)
Avenida Professor Egas Moniz
1649-028Lisboa

Unidade de Investigação Adicional
Additional Research Unit

(Vazio)
(Void)

Instituição de Acolhimento
Host Institution

Instituto de Medicina Molecular (IMM/FM/UL)
Avenida Professor Egas Moniz
1649-028Lisboa

3. Componente Científica
3. Scientific Component

3.1. Sumário
3.1 Summary

3.1.a Sumário Executivo (em português)
3.1.a Executive Summary (in Portuguese)

A malária é uma das doenças mais prevalentes no mundo. É causada por um protozoário do género Plasmodium e é transmitida através da picada de mosquitos Anopheles fêmea. Para chegar à corrente sanguínea numa forma passível de causar os sintomas da doença, o parasita atravessa primeiro uma fase de desenvolvimento obrigatória e assintomática, no fígado. Apesar de subsistirem ainda consideráveis lacunas no conhecimento de alguns dos processos fundamentais que ocorrem durante a fase hepática duma infecção por Plasmodium, é cada vez mais evidente que, durante o seu processo de replicação no fígado, os parasitas de Plasmodium utilizam os recursos da célula hospedeira para satisfazerem as suas necessidades de desenvolvimento (1-3). Os factores do hospedeiro são pois determinantes cruciais do desfecho da infecção por Plasmodium, constituindo assim importantes alvos contra a malária.
Resultados preliminares obtidos no nosso laboratório demonstraram que os ácidos biliares (ABs) influenciam fortemente a infecção hepática por Plasmodium in vitro. Os dados mostraram inequivocamente que diversos dos ABs testados impediam, de forma marcada e reprodutível, o desenvolvimento do parasita dentro de células de hepatoma humano. Recentemente, os ABs foram também implicados nas infecções pelo protozoário Cryptosporidium spp. (4) e pelo vírus da hepatite C (5). Assim, a presente proposta visa COMPREENDER DE QUE FORMA OS ÁCIDOS BILIARES INFLUENCIAM A INFECÇÃO DO FÍGADO PELO PLASMODIUM E INVESTIGAR O SEU POTENCIAL ENQUANTO ALVOS PARA INTERVENÇÃO CONTRA A MALÁRIA.
Os ABs são sintetizados nos hepatócitos e foram recentemente reconhecidos como moléculas de sinalização versáteis com funções endócrinas sistémicas, emergindo como importantes alvos terapêuticos contra doenças metabólicas (6,7). Os ABs sinalizam através de vias específicas para regular não apenas a sua própria síntese mas também a homeostase dos triglicéridos, colesterol, energia e glucose. Foram descritos dois mecanismos principais de sinalização mediada por ABs, resultantes do facto destes serem ligandos quer do receptor acoplado à proteina-G (TGR-5), quer de receptores hormonais nucleares, como o FXR (8-12). Assim, investigaremos de que forma as vias de sinalização mediadas por ABs influenciam a infecção do fígado por Plasmodium e revelaremos o seu potencial enquanto alvos contra a malária.
A síntese de ABs constitui a principal fonte de degradação de colesterol no organismo. Para além disso, os ABs participam no metabolismo do colesterol funcionando como hormonas que controlam a transcrição do enzima limitante na sua biosíntese. O colesterol desempenha um papel crucial durante o desenvolvimento hepático do Plasmodium, presumivelmente em resultado da enorme taxa de replicação do parasita durante esta fase. Para além disso, demonstrámos recentemente que o receptor hepático SR-BI, que medeia a entrada de colesterol nos hepatócitos, desempenha um papel fundamental durante o desenvolvimento intracelular do parasita de Plasmodium (13). Importa referir que os ABs também foram implicados na modulação da expressão do gene SRBI (14). Assim, elucidaremos se a regulação da homeostase do cholesterol pelos ABs contribui para o efeito destes no desenvolvimento do Plasmodium no fígado e clarificaremos se o papel do SR-BI nesse desenvolvimento está relacionado com o metabolismo dos ABs. Para além do seu papel crucial na homeostase do colesterol, os ABs foram implicados noutros processos celulares que ocorrem nas

células do fígado, nomeadamente a apoptose (15). A apoptose pode ser usada pela célula hospedeira como mecanismo de defesa contra patagéneos intracelulares. Inversamente, a inibição da apoptose da célula hospedeira é benéfica para um patogéneo como o Plasmodium, permitindo-lhe completar o seu desenvolvimento dentro dos hepatócitos (16). Investigaremos, pois, se as acções (anti-)apoptóticas dos ABs contribuem para os efeitos marcados que estes exercem na infecção por Plasmodium.
Para o cumprimento ds objectivos descritos serão utilizadas em paralelo estratégias in vitro, ex vivo e in vivo. Serão usados agonistas, antagonistas, microscopia de imunofluorescência e modulação da expressão genética por RNA de interferência (RNAi) por forma a dissecar os mecanismos e vias metabólicas através dos quais os ABs influenciam a infecção quer in vitro (linhas celulares de hepatomas) quer ex vivo (hepatócitos primários de roedores). Levar-se-ão a cabo estudos in vivo em modelos roedores de malária, que incluirão as mais recentes tecnologias de RNAi in vivo e, quando apropriado, o uso de linhas de ratinhos transgénicos, knock-out (KO) para genes específicos. Dada a crescente relevância dos ABs enquanto alvos clínicos, o conhecimento dos seus mecanismos de acção durante a infecção por malária encerra um enorme potencial quer no que respeita à compreensão de aspectos fundamentais das interacções parasita-hospedeiro, quer no tocante à identificação de novos alvos de intervenção contra a malária.

3.1.b Sumário Executivo (em inglês)

3.1.b Executive Summary (in English)

Malaria is one of the most prevalent infectious diseases worldwide. It is caused by a protozoan parasite from the genus Plasmodium and is transmitted through the bite of the female Anopheles mosquito. In order to reach the bloodstream in a form capable of causing disease symptoms, the parasite first undergoes an obligate and clinically silent developmental phase in the liver. Whereas important gaps subsist in our understanding of some fundamental processes that occur during the hepatic stage of infection, it is becoming increasingly apparent that, during their replication in the liver, Plasmodium parasites engage or subvert host cell resources in order to fulfil their developmental needs (1-3). Consequently, host factors are crucial determinants of the outcome of Plasmodium infection and, therefore constitute appealing targets for anti-malarial strategies.
Preliminary findings from our laboratory demonstrated that bile acids (BAs), strongly influence Plasmodium hepatic infection in vitro. The data unequivocally showed that several of the BAs tested markedly and reproducibly impaired the parasite's development inside human hepatoma cells. Recently, BAs have also been implicated in infection by the protozoa Cryptosporidium spp. (4) and by hepatitis C virus (5). Thus, the present proposal is aimed at UNDERSTANDING HOW BILE ACIDS INFLUENCE PLASMODIUM INFECTION IN THE LIVER AND INVESTIGATING THEIR POTENTIAL AS TARGETS FOR ANTI-MALARIAL INTERVENTION.
BAs are synthesized in hepatocytes and have recently been recognised as versatile signalling molecules endowed with systemic endocrine functions, emerging as important therapeutic targets for metabolic diseases (6,7). BAs signal through specific pathways to regulate not only their own synthesis, but also triglyceride, cholesterol, energy and glucose homeostasis. Two major BA-mediated signalling mechanisms have been described, arising from the fact that BAs are ligands for both the TGR-5 G-protein coupled receptor (GPCR), and nuclear hormone receptors, such as FXR, whose activation has been implicated in a number of important biological processes (8-12). Thus, we will investigate whether and how BA-mediated signalling pathways influence Plasmodium infection of the liver and unveil their potential as anti-malarial targets.
The synthesis of BAs accounts for the majority of cholesterol breakdown in the body. Moreover, BAs participate in cholesterol metabolism by functioning as hormones to repress the transcription of the rate-limiting enzyme in cholesterol biosynthesis. Importantly, cholesterol plays a crucial role during Plasmodium hepatic development presumably as a result of the outstanding parasite replication rate that occurs during this phase. Furthermore, we recently showed that the host scavenger receptor class B type I (SR-BI), which mediates cholesterol uptake by hepatocytes, plays a crucial role in the intracellular development of the Plasmodium parasite (13). Importantly, BAs have also been implicated in the modulation of SRBI expression (14). Thus, we will elucidate whether the regulation of cholesterol homeostasis by BAs contributes to their observed effect on Plasmodium development in the liver and clarify whether the demonstrated implication of SR-BI in Plasmodium development is related to BA metabolism.
In addition to their crucial role in cholesterol homeostasis, BAs have been implicated in other cellular processes that take place in liver cells, most notably, apoptosis (15). Apoptosis can be used by the host cell as a defence mechanism against intracellular pathogens. Conversely, as we previously demonstrated, inhibition of host cell apoptosis is advantageous for a pathogen such as Plasmodium to complete its development inside hepatocytes (16). We will therefore investigate whether BA-mediated (anti-) apoptotic effects contribute towards the marked effects that BAs exert on Plasmodium infection.
The proposed investigation of the mechanism(s) by which BAs interfere with Plasmodium development will be achieved by undertaking parallel state-of-the-art in vitro, ex vivo and in vivo approaches. Agonists, antagonists, immunofluorescence microscopy and RNA-interference (RNAi-) mediated knock-down of specific genes will be employed to dissect the mechanisms and pathways through which BAs influence infection both in vitro (hepatoma cell lines) and ex vivo (rodent primary hepatocytes). In vivo studies will be carried out in rodent models of malaria and will include state-of-the-art in vivo RNAi delivery techniques and, when appropriate, the use of transgenic lines of relevant knock-out mice.
Given the increasing relevance of BAs as clinical targets, unravelling their mechanisms of action during malaria infection holds immense importance from the points of view of both understanding fundamental aspects of host-pathogen interactions and unveiling novel anti-malarial targets.

3.2. Descrição Técnica

3.2 Technical Description

3.2.1. Revisão da Literatura

3.2.1. Literature Review

Malaria liver stage
In order to reach the bloodstream in a form capable of causing the symptoms of malaria, the Plasmodium parasite first undergoes an obligate developmental phase in the liver. Although important gaps subsist in our understanding of some fundamental processes that occur during this stage of infection (1), it is increasingly apparent that, during their replication in the liver, Plasmodium parasites engage or subvert host cell resources in order to fulfil their developmental needs (2,3). Recent work carried out in the host lab has identified such novel host factors, including 5 kinases (17), a hepatic cholesterol receptor (13) and a set of genes whose expression is modulated throughout infection (submitted). These observations strengthened the notion that host factors are crucial determinants of the outcome of Plasmodium infection, making them appealing anti-malarial targets. Importantly, though, hepatocytes are the only cell type of the vertebrate host that can support complete growth and development of Plasmodium before the blood stage of disease

(3). In considering the physiological properties of the liver, several unique features such as bile acids (BAs) and cholesterol metabolic processes become apparent and thus constitute the focus of this proposal.

BA-mediated signalling
BAs are derivatives of cholesterol synthesized in hepatocytes. They have recently been recognised as versatile signalling molecules endowed with systemic endocrine functions and are emerging as important therapeutic targets for metabolic and hepatic diseases (6,7). BAs signal through specific pathways to regulate not only their own synthesis, but also triglyceride, cholesterol, energy and glucose homeostasis. Two major BA-mediated signalling mechanisms arise from the fact that BAs are ligands for both the G-protein coupled receptor (GPCR), TGR-5, and nuclear hormone receptors, such as FXR. TGR-5 is a ubiquitously expressed membrane receptor. It is activated by multiple BAs, with an impact in metabolic disorders and a suggested role in cell proliferation and apoptosis (8,9). FXR is a nuclear receptor that is activated by several BAs and has an established role in feed-back regulation of BA biosynthesis. Its activation leads to increased expression of genes encoding BA-binding proteins (I-BABP or FABP6), basolateral BA transporters (OSTα and OSTβ) and fibroblast growth factor 19 (FGF19). It also induces expression of SHP, an atypical nuclear receptor that inhibits the activity of several other nuclear receptors, including liver receptor homologue 1 (LRH1) and liver X receptor (LXR) (10-12).

Cholesterol
One of Plasmodium's essential needs during development in the liver concerns the replicating parasites' requirements for cholesterol, presumably to allow the synthesis of the significant amounts of membranes that will surround the newly formed parasites. Cholesterol is the first substrate for BA biosynthesis, which begins with the conversion of the former into oxysterols (e.g. 25-hydroxycholesterol, 25-HC) by CYP7A1. Abnormal accumulation of these oxysterol intermediates, as well as of BAs, blocks the activation of sterol regulatory element binding proteins (SREBPs), transcription factors that regulate the expression of genes in the cholesterol and fatty acid synthesis pathways (18). Moreover, BAs participate in cholesterol metabolism by functioning as hormones that alter the transcription of CYP7A1. Interestingly, we showed that treatment with 25-HC affects infection of hepatoma cells by Plasmodium in vitro (13). A recent RNA interference (RNAi) screen of human genes with roles in cholesterol uptake and lipoprotein assembly identified the host receptor SR-BI, as playing a crucial role in the infection of host liver cells by Plasmodium (13). SR-BI mediates cholesterol uptake by hepatocytes and subsequent studies further revealed that it modulates not only cell invasion by the parasites but also the extent of their intracellular development (13). Importantly, BAs have recently been implicated in the modulation of SRBI expression, through signalling via FXR (14).

Apoptosis
Apoptosis can be used by the host cell as a defence mechanism against intracellular pathogens. Conversely, inhibition of host cell apoptosis is advantageous for the pathogen to prolong life in the infected cell and complete its development (16). Interestingly, numerous studies have shown that elevated BA concentrations in the liver induce hepatocyte apoptosis. Highly hydrophobic BAs, such as DCA and CDCA are able to pass through the membrane and activate pro-apoptotic signalling cascades (19). In contrast to the effect of cytotoxic BAs in the liver, there is growing evidence that UDCA may modulate gene expression to prevent cell death, acting as a potent inhibitor of apoptosis (15).

Preliminary results
In view of the established links between BAs and various biological processes that have been shown to influence malaria liver stage infection, we sought to test whether BAs influence Plasmodium hepatic infection in vitro. A total of 11 primary, secondary, conjugated and unconjugated BAs were used in these experiments. Infection of the human hepatoma cell line Huh7, stably expressing NTCP, a specific receptor for conjugated BAs, incubated with various amounts of BAs was compared to that of untreated controls. The parasite's intracellular development was assessed by a flow cytometry-based method developed in the host lab (20). The results showed a marked effect of 8 of the BAs tested on the parasite's ability to develop inside the cells, without toxic effects on the latter. Importantly, BAs have recently been implicated in infection by the Apicomplexan protozoan Cryptosporidium spp. (4) and by hepatitis C virus (5). This, in conjunction with our own observations of the striking effects of BAs on Plasmodium's ability to develop, constitutes a solid basis for a more detailed investigation of their mechanisms of action during malaria infection.

3.2.2. Plano e Métodos

3.2.2. Plan and Methods

The hepatic stage of a Plasmodium infection constitutes an appealing target for the development of an anti-malarial vaccine or prophylactic drug since it would act before the onset of pathology. Consequently, we sought to focus on the specific features of the hepatocyte that could provide a unique niche for the parasites to differentiate and replicate. Preliminary results from our laboratory showed that bile acids (BAs) markedly and reproducibly influence the ability of Plasmodium parasites to develop inside Huh7 cells, a well-established model for in vitro malaria hepatic infection (20). BAs are synthesised in hepatocytes from cholesterol and interestingly are involved in cellular process that have been shown to affect Plasmodium liver stage infection and/or development, namely, intracellular signalling, cholesterol homeostasis and apoptosis.

In this context, the main OBJECTIVE of this project is TO UNDERSTAND HOW BILE ACIDS INFLUENCE PLASMODIUM INFECTION IN THE LIVER. In particular, we will:
- Establish which bile acids influence a Plasmodium liver infection that occurs in physiologically relevant conditions;
- Understand the interplay that occurs between liver infection by Plasmodium and bile acid production and accumulation;
- Dissect the signalling pathways through which bile acids modulate Plasmodium development in hepatic cells;
- Elucidate how bile acid-mediated cellular processes, notably cholesterol homeostasis and apoptosis, influence Plasmodium liver infection;
- Identify novel targets for anti-malarial intervention.

These objectives will be accomplished through the completion of 5 tasks (please see attached file General_scheme.PDF), designed to provide a complete picture of the role of BAs and BA signalling mechanisms during Plasmodium liver infection and their potential use as targets for clinical intervention:
- The influence of bile acids on ex vivo and in vivo infection by Plasmodium.

- The interplay between in vivo Plasmodium infection and bile acid pool.
- The role of bile acid-mediated signalling pathways during infection by Plasmodium.
- The role of bile acid and cholesterol metabolism during liver infection by Plasmodium.
- The role of bile acid-mediated (anti-)apoptotic effects during liver infection by Plasmodium.

These tasks aim at covering all aspects of BA-related biology that may be relevant during the parasite's development in the liver. Furthermore, they have been constructed in such a way as to maximise their complementarity while avoiding that initiation of one task be dependent upon completion of another. In this way, it is expected that multiple tasks can be carried out in parallel, with a close interplay between the knowledge generated throughout the progress made in each one.

The work will be articulated between three research groups whose individual areas of expertise will be combined to ensure success of the tasks at hand (please see attached file General_scheme.PDF). The Malaria Unit of the IMM, led by Prof. Maria Mota, a consultant in this project, has world-renowned expertise in malaria and, in particular, in the study of host factors that influence liver infection by Plasmodium. The PI, Dr. Miguel Prudêncio, is a Staff Scientist at the IMM-UMA, holding a CIÊNCIA 2007 position towards becoming a fully independent researcher, in which is highly supported by the unit leader. He is a liver-stage malaria expert who has written various reviews on this subject and has participated in numerous studies, published in international peer-reviewed journals, aimed at developing techniques for the study of hepatic malaria and identifying host molecules that modulate infection. The FF-UL group is led by Prof. Cecília Rodrigues, also a consultant in this project, is a world leader in the study of BAs and, in particular, of their role during apoptosis. The Autonomic Nervous System Unit of the IMM, led by Prof. Isabel Rocha, has a vast and proven experience in the use of animal models for the study of disease and possesses all the relevant resources for the isolation of the primary hepatocytes required throughout this project.

State-of-the-art methodologies will be employed throughout this project to tackle the proposed tasks, as outlined below:
o Green fluorescent protein (GFP)-expressing P. berghei (parasite line 259cl2) sporozoites (21) will be obtained from dissection of salivary glands of infected female Anopheles stephensi mosquitoes reared in the insect facilities of the IMM.
o In vitro studies will be carried out in human hepatoma cell lines, cultured in supplemented RPMI medium and maintained at 37°C with 5% CO2. Infection will be assessed by flow cytometry.
o Ex vivo infection studies will be carried out in rat primary hepatocytes freshly isolated by a modified collagenase perfusion method (22). Briefly, the liver will be perfused and digested via the portal vein to detach cells. Isolated cells will be washed and centrifuged through a Percoll gradient to remove damaged cells. Cell viability will be determined by trypan blue exclusion. Hepatocytes will be cultured in supplemented William´s E medium prior to treatment and infection with P. berghei sporozoites. Infection will be assessed either by flow cytometry or by quantitative RT-PCR (qRT-PCR).
o In vivo infection studies will employ C57BL/6 mice, bred in the pathogen-free facilities of the Instituto de Gulbenkian de Ciência (IGC) and housed in the pathogen-free facilities of the Instituto de Medicina Molecular (IMM). Transgenic mice will be purchased from Jackson Laboratories or obtained through an MTA with the research groups that have produced them. Drugs will be administered by intravenous (i.v.) or intraperitoenal (i.p.) injection. All protocols to be used are approved by the Animal Care Committees of both the IGC and the IMM. Parasite liver loads will be determined by qRT-PCR40 h after sporozoite injection. Blood stage infections will be followed by daily monitoring of parasitemia (% infected red blood cells) and disease symptoms.
o In vitro RNAi will be performed as previously described (13,17). Briefly, cells will be seeded in RPMI medium and incubated at 37ºC in 5% CO2. Twenty-four h later, cells will be transfected with individual siRNAs in a final concentration of 100nM per lipofection, in triplicate. Cells will be infected with P. berghei sporozoites two days after siRNA transfection.
o In vivo RNAi will be performed as previously described (13,17). Briefly, mice will be treated with a single i.v. administration of 5 mg/kg of siRNA formulated in liposomal nanoparticles (Alnylam). Thirty-six h later mice will be infected by i.v. injection of P. berghei sporozoites. Remaining specific gene-mRNA levels will be determined by qRT-PCR.
o Measurements of infection by flow cytometry will be performed as previously described (20). Cells infected with GFP-expressing P. berghei, will be sorted by Fluorescent Activated Cell Sorting (FACS) using a Becton Dickinson FACSAria cell sorting system.
o For infection or gene-specific expression determination by qRT-PCR total RNA will be extracted and reverse transcribed into cDNA, which will be used as template for SYBR Green-based qRT-PCR reactions. Amplification reactions will employ PbA 18 S- or specific gene-specific primers. Infection and expression data will be normalized by carrying out amplification reactions with primers for the housekeeping gene HPRT.
o BA profile determination will be carried out as previously described (23). Briefly, BAs will be extracted from bile by liquid-solid extraction. BA conjugates will then be solvolyzed, hydrolyzed, isolated, and analyzed as methyl-trimethylsilyl (Me-TMS) ethers by gas-chromatography.
o For immunofluorescence microscopy, cells seeded on glass coverslip will be fixed and permeabilized as described before (13,16). Observations will be carried-on with a ZEISS LSM510 META scanning confocal microscope.

It can realistically be expected that the close articulation between the proposed tasks and the research groups involved in their completion, as well as the use of state-of-the art experimental approaches, will enable the detailed elucidation of the role of BAs during Plasmodium infection of the host's liver cells. In addition to revealing fundamental aspects of the biology of the host-parasite interactions that take place during this stage of the parasite's life cycle, this will undoubtedly identify novel targets for potential intervention against malaria. Given the imperative need for novel anti-malarial strategies, the relevance of these findings can hardly be overestimated.

3.2.3. Tarefas
3.2.3. Tasks

Lista de tarefas (5)
Task list (5)

Designação da tarefa Task denomination	**Data de início** Start date	**Data de fim** End date	**Duração** Duration	**Pessoas * mês** Person * months

The influence of bile acids on ex viv...	01-07-2010	31-03-2011	9	7

Descrição da tarefa e Resultados Esperados
Task description and Expected results

Preliminary results obtained in our laboratory clearly demonstrated that several bile acids (BAs) have a marked effect on the intra-cellular development of malaria parasites in vitro. These studies employed Huh7 cells, a human hepatoma cell line that constitutes an established model for Plasmodium infection (13,17,20). Huh7 cells stably transfected with the sodium-dependent BA transporter (Huh-BAT) (24) were further employed in these experiments. The results obtained with both cell lines showed that BAs do exert a marked influence on Plasmodium's developmental process in vitro. During this task, these results will be further validated both in an ex vivo and in an in vivo models of infection.

Ex vivo assays
Freshly isolated primary hepatocytes will be seeded in 24-well plates with Primaria surface treatment and allowed to adhere for 4h at 37°C and 5% CO2. Hepatocytes will then be incubated with each BA and subsequently infected with freshly extracted P. berghei sporozoites. Infection will be analysed 40 h later by flow cytometry or qRT-PCR and compared to that of appropriate control cells.

In vivo assays
A rodent model of malaria will be employed to test the effect of selected BAs on liver infection by Plasmodium. C57/Bl6 mice will be treated by intraperitoneal or intravenous injection of bile acids at selected time points relative to infection with sporozoites. Parasite liver load will be determined by qRT-PCR of livers collected 40 h after infection and compared with that of appropriate controls. In parallel, infection of similarly treated mice will be allowed to proceed onto the blood-stage. These mice will be monitored regularly for the appearance of parasites in the blood (parasitemia) and for symptoms of cerebral malaria, in order to evaluate the influence of the BA treatment on pathology.

It can realistically be expected that both the ex vivo and the in vivo systems will confirm our previous observations, at least for some of the BAs that have proven to influence the parasite's intracellular development in the in vitro system. Moreover, results from the ex vivo and in vivo experiments outlined above are expected to provide important additional information about which BAs exert the strongest effect on Plasmodium development in a more physiologically relevant context of infection. This information will constitute a valuable asset not only for an understanding of the biology of BAs' influence on infection but also for their potential usefulness as clinical targets. Furthermore, it will provide important guidance in terms of directing the choice of bile acids to be employed in subsequent experiments.

This task will be carried out in close collaboration between the three teams that are involved in the project. BAs will be provided by FF-UL and primary hepatocytes will be isolated at IMM-ANS. Cell maintenance, infections and infection measurements will be performed at IMM-UMA, with the participation of IR from IMM-ANS. This Task will be initiated at the start of the project, is expected to be accomplished within 9 months, and will involve a total of 7 person-months. The requested budget will cover parasite production, animal model acquisition and flow cytometry and qRT-PCR analyses.

Membros da equipa de investigação nesta tarefa
Members of the research team in this task

(BPD) Bolseiro de Pós-Doutoramento 1; Maria Isabel de Sousa Rocha; Miguel Prudêncio;

Designação da tarefa Task denomination	**Data de início** Start date	**Data de fim** End date	**Duração** Duration	**Pessoas * mês** Person * months
The interplay between in vivo Plasmod...	01-10-2010	30-09-2011	12	10

Descrição da tarefa e Resultados Esperados
Task description and Expected results

Having established that the addition of exogenous bile acids (BAs) markedly influences Plasmodium infection of liver cells, the present task is aimed at determining whether (i) an ongoing liver infection has an effect on the BA pool of a rodent model of malaria and (ii) an overall decrease in the availability of BAs in vivo influences the extent of Plasmodium liver infection. These two goals complement each other in establishing whether interfering with bile acid availability in vivo may influence the extent of malaria liver infection.

(i) Plasmodium infection's influence on bile acid pool
In order to determine whether and to what extent a Plasmodium liver infection influences the BA profile in vivo, C57/Bl6 mice will be infected with defined numbers of P. berghei sporozoites. At specific times, typically 40 h, post-infection, both the bile and the livers of infected mice will be collected. Non-infected, control mice will be used as controls in this experiment. Bile samples will be analysed by gas chromatography in order to determine their composition in terms of BA profile. In parallel, the parasite loads in the collected livers will be assessed by quantitative Real-Time PCR (qRT-PCR). Comparison of both sets of data will establish whether the level of Plasmodium infection in the liver influences either the profiles of individual BAs or the total amount of BAs in the gallbladder.

(ii) Bile acid pool's influence on Plasmodium infection
The effect of in vivo BA availability on Plasmodium liver infection will be assessed by treating C57/Bl6 mice with bile acid binding resins, which are known to reduce BA availability in vivo, prior to infection with defined numbers of P. berghei sporozoites. Parasite loads will then be assessed by qRT-PCR of livers collected 40h post-infection. If the latter are shown to be strongly affected by the BA removal treatment, infection of similarly treated mice will be allowed to proceed onto the blood stage. The time of appearance of parasites in the blood, the extent of parasitemia and disease symptoms will be monitored in order to determine whether they are consequently affected. These results will complement those obtained in the in vivo section of Task 1, where the effect of the reverse treatment, i.e., addition of exogenous BAs prior to infection, will be monitored. In conjunction, these data will inform whether the in vivo (un)availability of BAs bares an effect on malaria infection.

Overall, the results obtained in this task will be complemented by those from Task 1 to provide a complete picture of the effect of Plasmodium infection on the BA pool and vice-versa. Completion of this task will provide detailed information not only about the profile of BAs that exert an influence on infection, but also about the anti-malarial potential of the modulation of that profile. It should be emphasized that, despite their complementary nature, Tasks 1 and 2 can be carried out independently of each other and any difficulties encountered during either of them will not preclude conclusion of the other.

This task will be carried out in close cooperation between the IMM-UMA and FF-UL partners. BAs and BA binding resins will be provided by the FFUL. All the in vivo treatments and infections, bile/liver collections, liver infection quantifications, and blood infection monitoring will be carried out at IMM-UMA. Analysis of gallbladder contents and bile composition will be performed at FF-UL on the samples provided by the IMM-UMA team. This Task will be initiated be during the first trimester of the project and should be completed up to 12 months from then. It will involve a total of 10 person-months. The requested budget will cover parasite production, animal model and reagents acquisition and BA analyses.

Membros da equipa de investigação nesta tarefa
Members of the research team in this task
(BPD) Bolseiro de Pós-Doutoramento 1; Márcia Maria de Almeida Aranha; Miguel Prudêncio;

Designação da tarefa Task denomination	**Data de início** Start date	**Data de fim** End date	**Duração** Duration	**Pessoas * mês** Person * months
The role of bile acid-mediated signal...	01-01-2011	31-03-2013	27	22

Descrição da tarefa e Resultados Esperados
Task description and Expected results
Bile acids (BAs) have recently been recognized as versatile signalling molecules endowed with systemic endocrine functions. The objective of the present task is to address in vitro, ex vivo and in vivo whether and how BAs influence Plasmodium liver infection through the activation of TGR-5- and FXR-mediated signalling mechanisms.

TGR-5
TGR-5 is a dedicated membrane receptor for BAs and the founder of the BA receptor subclass of GPCRs. It is conserved among mammals and is activated by multiple BAs (8,9). Preliminary results have shown that incubation of Huh-BAT cells with LCA or DCA, the two most potent natural agonists of TGR-5, leads to a strong decrease in Plasmodium development. The influence of TGR-5 signalling on Plasmodium hepatic infection will be further assessed in vitro and ex vivo by determining the effect of the non-natural agonists oleanolic acid and 6α-ethyl,23(S)-methyl-CDCA on the parasite's ability to develop inside Huh-BAT cells and rodent primary hepatocytes, respectively. Expression of the gene encoding TGR-5 will be knocked-down (KD) by RNA interference (RNAi) and infection will be assessed both in the presence and in the absence of TGR-5 agonists, in order to establish whether the latter modulate infection via this receptor. In vivo, Tgr5 expression in C57/Bl6 mice will be KD by in vivo RNAi and the resulting effect in terms of Plasmodium infection will be assessed. In parallel, transgenic, TGR-5-KO mice will be infected with P. berghei sporozoites and parasite liver load will be compared to that of their wild-type counterparts.

FXR
FXR is a dedicated nuclear receptor for BAs that is present in rodents and humans. It is activated by various BAs, mostly CDCA and its conjugated form, and plays an established role in the feed-back regulation of BA biosynthesis. FXR induces expression of the short heterodimer partner (SHP), an atypical nuclear receptor that inhibits the activity of several other nuclear receptors, including liver receptor homologue 1 (LRH1) and liver X receptor (LXR) (10-12). Preliminary results have shown that incubation of Huh-BAT cells with CDCA leads to a strong decrease in Plasmodium development.
The relevance of FXR-mediated signalling pathways during liver cell infection by Plasmodium will be evaluated in vitro and ex vivo by assessing the effect of the FXR agonist and antagonist (GW4064 and guggulsterone, respectively) upon Huh-BAT and rodent primary hepatocyte infection, respectively. In order to dissect the mechanisms through which FXR-α influences infection, we will use RNAi to KD the expression of FXR, SHP, LRH1, LXR, SREBP1c and Fgfr4 in vitro and monitor the resulting effects on infection. In vivo RNAi will be used to target these same genes and assess the resulting effect in terms of Plasmodium infection. Additionally, infection of FXR-KO mice will be compared with that of wild-type controls.

This is a pivotal task in the context of the whole project, as signalling is expected to play a crucial role in Plasmodium infection. Upon completion of this task, we will have established whether TGR-5-, FXR-mediated or both signalling mechanisms interfere with Plasmodium infection of liver cells. Results obtained from the experiments proposed here will be closely and continually knitted with those obtained in all other tasks. It is expected that such knowledge will be instrumental in the identification of novel targets for anti-malarial intervention.

This task will be carried out at the IMM-UMA. BAs will be provided by the FF-UL and primary hepatocytes will be produced at the IMM-ANS. All the in vivo treatments and infections and infection quantifications and monitoring will be carried out at IMM-UMA. This Task will initiated be during the second semester of the project, will proceed until its last trimester and will involve a total of 22 person-months. The requested budget will cover parasite production, animal model and reagents acquisition and infection analyses.

Membros da equipa de investigação nesta tarefa
Members of the research team in this task
(BPD) Bolseiro de Pós-Doutoramento 1; Ghislain Cabal; Maria Isabel de Sousa Rocha; Miguel Prudêncio;

Designação da tarefa Task denomination	**Data de início** Start date	**Data de fim** End date	**Duração** Duration	**Pessoas * mês** Person * months
The role of bile acid and cholesterol...	01-07-2011	31-03-2013	21	14

Descrição da tarefa e Resultados Esperados
Task description and Expected results

Bile acids (BAs) are derivatives of cholesterol and important regulators of cholesterol homeostasis. In this task, we will elucidate whether this regulation contributes to the observed effect of BAs on Plasmodium development in the liver. In parallel, we expect to clarify whether the previously demonstrated implication of SR-BI in Plasmodium development inside hepatocytes is related to BA metabolism.

BA and cholesterol metabolism during liver stage infection
BAs and their precursors have been shown to block SREBPs and consequently the expression of genes in the cholesterol and fatty acid synthesis pathways (18). SREBPs are transcription factors that translocate to the nucleus upon activation (25). This activation step can be inhibited either by treatment with 25-HC, an oxysterol derivative of cholesterol, or overexpression of INSIG1, a membrane protein involved in the retention of SREBPs in the ER (25). To confirm the previously observed effect of 25-HC on Plasmodium development (13), we will overexpress INSIG1 by transfecting a construct already available in the host laboratory, prior to in vitro infection assays. Once the implication of the SREBP pathway in liver stage infection is confirmed, the potential role of BAs in such regulation will be evaluated. First, SREBP localization (i.e., nuclear or cytoplasmic) upon BA treatment in infected cells in vitro or ex vivo and in infected livers in situ will be assessed by immunofluorescence microscopy. The expression levels of SREBPs target genes (e.g. HMGCoA reductase and fatty acid synthase) upon BA treatment will then be measured by qRT-PCR on both infected and non-infected cells/hepatocytes, previously sorted by fluorescence activated cell sorting (FACS). Finally, we will check in vitro whether overexpression of the rate-limiting enzymes in cholesterol and fatty acid biosynthesis (i.e. HMGCoA reductase and fatty acid synthase) can bypass the BAs' effect on parasite development. These experiments will elucidate whether the effect of BAs on Plasmodium liver stages is a consequence of their negative feedback regulation of SREBP-related pathways.

SR-BI regulation by BA during infection
SR-BI mediates uptake of HDL-cholesterol by hepatocyte. Interestingly, HDL-cholesterol is the preferred substrate for BA synthesis and SR-BI is regulated in parallel with BA synthesis by LXR (14,26). Besides, several links between the BA-induced FXR pathway and SRBI expression have been described, although their exact nature remains controversial (14,27,28). Considering the effect of SR-BI depletion on Plasmodium development inside hepatocytes (13), we therefore want to establish whether the SR-BI and BA effects on parasite development are linked. Initially, the effect of BA on SRBI expression will be evaluated by qRT-PCR and Western blot on infected and non-infected Huh7 cells in vitro and on hepatocytes ex vivo and in vivo, previously treated with specific BAs and FACS-sorted. The signalling pathways know to be induced by BAs and to regulate SR-BI will then be challenged by performing the same experiments in cells/mice depleted by RNAi for either LXR or FXR. Results will be confirmed by using agonists and antagonists of LXR and FXR. Finally, the causal relationship will be clarified by complementing the BA effect on parasite development in vitro by overexpressing SR-BI. These experiments will elucidate whether and how BAs are involved in the regulation of SR-BI, and whether this regulation contributes to the BA inhibition of parasite liver stage development.

This task will be coordinated by GC, a researcher with experience on cholesterol metabolism, at the IMM-UMA. BAs will be provided by the FFUL and primary hepatocytes will be produced at the IMM-ANS. This Task will be initiated in the second of the year project, will proceed until its last trimester and will involve a total of 14 person-months. The requested budget will cover parasite production, reagents acquisition and infection analyses.

Membros da equipa de investigação nesta tarefa
Members of the research team in this task

(BPD) Bolseiro de Pós-Doutoramento 1; Ghislain Cabal; Maria Isabel de Sousa Rocha; Miguel Prudêncio;

Designação da tarefa Task denomination	**Data de início** Start date	**Data de fim** End date	**Duração** Duration	**Pessoas * mês** Person * months
The role of bile acid-mediated (anti-...	01-05-2012	30-06-2013	14	10

Descrição da tarefa e Resultados Esperados
Task description and Expected results

Accumulation of hydrophobic bile acids (BAs) within the hepatocyte have been show to induce apoptosis of liver cells while hydrophilic bile acids may be cytoprotective (15). Besides, Plasmodium liver stages have been show to inhibit apoptosis of their host cells in order to be able to survive and develop (16). Hence, the present task is intended to assess whether the (anti-)apoptotic effects of BAs contribute to their modulation of Plasmodium development in the liver.

Apoptosis quantification upon BA treatment
To determine to which extend infected hepatoma cells in vitro or hepatocytes in vivo are affected by apoptosis upon BA treatment, apoptosis will be quantified by three fluorescence methods: (i) TUNEL (Roche) and DAPI staining, which detects DNA breaks and access nuclear morphology; (ii) active caspase-3 detection (Promega); (iii) immunofluorescence location of cytochrome c and members of the Bcl-2 family, in conjunction with mitotracker to confirm the mitochondrial co-localization of proteins. Furthermore, the level of expression of anti-apoptotic members of Bcl2 family proteins (e.g. Bcl2 itself) and pro-apoptotic members (e.g. Bax) will be quantified by qRT-PCR and Western blot on previously FACS-sorted infected cells. These experiments will allow to correlate the level of impair of parasite development with the extent of apoptosis induction, for each BA tested.

Inhibition of BA-induced apoptosis
For the BAs showing a pro-apoptotic effect on infected cells, various anti-apoptotic treatments will be applied before BA addition, and parasite development will be subsequently quantified by flow cytometry. cAMP (through the use of the cell-permeable cAMP analog CPT-cAMP), antioxidants (i.e. α-tocopherol, ebselen and idebenone) and blockers of mitochondrial permeability transition (i.e. cyclosporin A and bongkrekic acid) have been shown to specifically inhibit BA induced apoptosis (15). Therefore, those compounds will be used to revert the effect of BAs on parasite development. On the other hand, work from our lab showed that HGF-induced MET

activation protects Plasmodium-infected cells from apoptosis (16). In order to observe the cytoprotective effect of HGF on BA induced apoptosis, HGF itself or another MET agonist (e.g. DO-24 mAb) will be added to cells before the addition of cytotoxic BAs, infection by P. berghei sporozoites and quantification of parasite development. These experimental approaches are expected to elucidate whether the apoptotic induction by BA treatment is the cause of their effect on malaria liver stage development, and hopefully discriminate the major apoptotic pathways through which they act.

Anti-apoptotic effects of UDCA
For the BAs showing an anti-apoptotic effect on infected cells, as one could reasonably expect from UDCA, a hydrophilic BA that protects against the toxicity of hydrophobic BAs, we will determine whether they act through the phosphatidylinositol 3-kinase (PI3K)- and MAPK-dependent survival signalling pathways as has been already shown for Plasmodium-dependent inhibition of apoptosis (16). Hence, PI3K activity upon UDCA treatment in FACS-sorted infected cells will be assessed by measuring phosphorylation of Akt by Western blot. Finally, the UDCA anti-apoptotic effect on infected cells will be challenged by inhibition of PI3K and MAPK, with LY294002 and PD98059 respectively.

This task will be coordinated by the FF-UL, in close cooperation with the IMM-UMA. The expertise of CR (FF-UL), an expert on BA-mediated apoptosis and a consultant in this project, will be crucial throughout completion of this task. This Task will be initiated during the last trimester of the second year of the project, will proceed until the project's completion and will involve a total of 10 person-months. The requested budget will cover parasite production, animal model and reagents acquisition immunofluorescence microscopy, Western blot and cell sorting analyses.

Membros da equipa de investigação nesta tarefa
Members of the research team in this task
(BPD) Bolseiro de Pós-Doutoramento 1; Ghislain Cabal; Maria Isabel de Sousa Rocha; Miguel Prudêncio; Rita Cruz Coelho de Mira Ramalho;

3.2.4. Calendarização e Gestão do Projecto
3.2.4. Project Timeline and Management

3.2.4.a Descrição da Estrutura de Gestão
3.2.4.a Description of the Management Structure
The project will be undertaken through the coordinated efforts of three research units (IMM-UMA, FF-UL and IMM-ANS), six researchers (MP, GC, RR, MA, IR and one post-doc to be hired) and two consultants (MMM and CR). The articulation of the tasks and the relative contributions of each participant are detailed in the attached files General_scheme.pdf, Timeline.pdf and PersonMonths.pdf, to which the reader is kindly referred. Essentially, the project will be coordinated by the PI (MP, IMM-UMA) and the proposed Tasks will be carried out in the different participating units, according to their areas of expertise. Given the close geographical proximity between the three participating laboratories, regular meetings will be held to evaluate progress, discuss results and delineate strategies. The two consultants are world leading researchers in their respective areas of expertise and will also participate in these meetings. As a general rule, the team will meet at least every six months, but further meetings will be held whenever the PI or any of the other participants will deem them useful and necessary. It should be emphasised that all the participants in this project have collaborated in the past and that a solid history of cooperation and fruitful discussions exists between them. Thus, informal meetings may also take place between specific members of the team, whenever these may be relevant for the discussion of issues of relevance to the project. As detailed in General_scheme.pdf, throughout the project, researchers from a given unit will carry out specific work at a different unit. All the three laboratories involved in this project are fully equipped to meet the demands of the proposed tasks and equipment will be shared, if and when needed. Biological material and reagents will also be shared between units, as deemed necessary. Overall, the project has been structured in such a way that all tasks and the participants involved in each of them have been clearly defined so as to capitalise on each one's expertise and maximise resources and productivity.

3.2.4.b Lista de Milestones
3.2.4.b Milestone List

Data Date	**Designação da milestone** Milestone denomination
01-03-2011	Final list of bile acids that influence Plasmodium infection

Descrição
Description
This milestone coincides with the expected conclusion of Task 1, at which point we expect to have established definitively which bile acids influence infection in vitro, ex vivo and in vivo. This list constitutes an important finding in itself and will be instrumental throughout the whole project.

Data Date	**Designação da milestone** Milestone denomination
01-09-2011	Bile acid pool in an in vivo infection

Descrição
Description
This milestone coincides with the expected conclusion of Task 2, at which point we expect to have determined how infection by Plasmodium influences the bile acid profile of infected rodents.

Data Date	**Designação da milestone** Milestone denomination

01-07-2012	Bile acid-mediated signalling pathways

Descrição
Description

This milestone coincides with the end of the second year of the project and is set 18 months after initiation of Task 3, at which point we expect to have clarified whether one or both bile acid (BA)-mediated signalling pathways under study are involved in BA modulation of Plasmodium infection.

Data	**Designação da milestone**
Date	Milestone denomination
01-01-2013	Cholesterol and apoptosis

Descrição
Description

This milestone is set 6 months before the end of the project, 18 and 8 months after initiation of Tasks 4 and 5, respectively. We then expect to have unequivocally established whether bile acid (BA)-mediated cholesterol homeostasis and apoptosis are involved in BA modulation of Plasmodium infection.

Data	**Designação da milestone**
Date	Milestone denomination
30-06-2013	Target identification and validation

Descrição
Description

This milestone coincides with the end of the project, at which point we expect to have identified and validated novel bile acid-related targets for anti-malarial intervention.

3.2.4.c Cronograma
3.2.4.c Timeline

Ficheiro com a designação "timeline.pdf", no 9. Ficheiros Anexos, desta Visão Global (caso exista).
File with the name "timeline.pdf" at 9. Attachments (if exists).

3.3. Referências Bibliográficas
3.3. Bibliographic References

Referência Reference	**Ano** Year	**Publicação** Publication
1	2006	**Prudêncio M, Rodriguez A, Mota MM (2006) "The silent path to thousands of merozoites: the Plasmodium liver stage", Nat. Rev. Microbiol., 4, 849-856**
2	2006	**Prudêncio M, Rodrigues CD, Mota MM (2006) "The relevance of host genes in Malaria", In: Parrington,J. and Coward, K. (Eds) Comparative Genomics and Proteomics in the Identification of New Drug Targets, Taylor & Francis, Oxford, UK, SEB Exp. Biol. Ser., 58, 47-91.**
3	2008	**Silvie O, Mota MM, Matuschewski K, Prudêncio M (2008) "Interactions of the malaria parasite and its mammalian host", Curr. Opin. Microbiol., 11, 352-359**
4	2006	**Feng H, Nie W, Sheoran A, Zhang Q, Tzipori S (2006) "Bile acids enhance invasiveness of Cryptosporidium spp. into cultured cells", Infect. Immun., 74, 3342-3346.**
5	2007	**Chang KO, George DW (2007) "Bile acids promote the expression of hepatitis C virus in replicon-harboring cells", J. Virol., 81, 9633-9640**
6	2007	**Houten SM, Watanabe M, Auwerx J (2006) "Endocrine functions of bile acids", EMBO J., 25, 1419-1425**
7	2008	**Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K (2008) "Targeting bile-acid signalling for metabolic diseases", Nat. Rev. Drug Discov., 7, 678-693**
8	2002	**Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Itadani H, Tanaka K (2002) "Identification of membrane- type receptor for bile acids (M-BAR)", Biochem. Biophys. Res. Commun., 298, 714–719**
9	2003	**Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M (2003) "A G protein-coupled receptor responsive to bile acids", J. Biol. Chem., 278, 9435–9440**
10	1999	**Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B (1999) "Identification of a nuclear receptor for bile acids", Science, 284, 1362–1365**
11	1999	**Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM (1999) "Bile acids: natural ligands for an orphan nuclear receptor", Science, 284, 1365–1368**
12	1999	**Wang H, Chen J, Hollister K, Sowers LC, Forman BM (1999) "Endogenous bile acids are ligands for the nuclear receptor FXR-BAR", Mol. Cell, 3, 543–553**
13	2008	**Rodrigues CD, Hannus M, Prudêncio M, Martin C, Gonçalves LA, Portugal S, Epiphanio S, Akinc A, Hadwiger P, Jahn-Hofmann K, Röhl I, van Gemert GJ, Franetich JF, Luty AJ, Sauerwein R, Mazier D, Koteliansky V, Vornlocher HP, Echeverri CJ, Mota MM. (2008) "Host scavenger receptor SR-BI plays a dual role in**

		the establishment of malaria parasite liver infection", Cell Host Microbe, 11, 271-282
14	2005	Malerød L, Sporstøl M, Juvet LK, Mousavi SA, Gjøen T, Berg T, Roos N, Eskild W (2005) "Bile acids reduce SR-BI expression in hepatocytes by a pathway involving FXR/RXR, SHP, and LRH-1", Biochem. Biophys. Res. Commun., 336, 1096-105
15	2006	Solá S, Amaral JD, Aranha MM, Steer CJ, Rodrigues CMP (2006) "Modulation of Hepatocyte Apoptosis: Cross-talk Between Bile Acids and Nuclear Steroid Receptors", Curr. Med. Chem., 13, 3039-3051
16	2005	Leirião P, Albuquerque SS, Corso S, van Gemert GJ, Sauerwein RW, Rodriguez A, Giordano S, Mota MM (2005) "HGF/MET signalling protects Plasmodium-infected host cells from apoptosis", Cell. Microbiol., 7, 603-609
17	2008	Prudêncio M, Rodrigues CD, Hannus M, Martin C, Real E, Gonçalves LA, Carret C, Dorkin R, Röhl I, Jahn-Hoffmann K, Luty AJ, Sauerwein R, Echeverri CJ, Mota MM (2008), "Kinome-wide RNAi screen implicates at least 5 host hepatocyte kinases in Plasmodium sporozoite infection", PLoS Pathog., 4, e1000201
18	2004	Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J (2004) "Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c.", J. Clin. Invest. 113, 1408-1418.
19	2002	Kim, ND, Im, EO, Choi,YH, Yoo, YH (2002) "Synthetic bile acids: novel mediators of apoptosis", J. Biochem. Mol. Biol., 35, 134-141
20	2008	Prudêncio M, Rodrigues CD, Ataíde R, Mota MM (2008), "Dissecting in vitro host cell infection by Plasmodium sporozoites using flow cytometry", Cell. Microbiol., 10, 218-224
21	2004	Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M. van der Linden, R, Sinden RE, Waters AP, Janse CJ (2004) "A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle", Mol. Biochem. Parasitol., 137, 23-33
22	2007	Goncalves LA, VigarioAM, Penha-Goncalves C (2007) "Improved isolation of murine hepatocytes for in vitro malaria liver stage studies", Malar. J., 6, 169
23	1997	Setchell KDR, Rodrigues CMP, Clerici C, Solinas A, Morelli A, Gartung C, Boyer J (1997) "Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei", Gastroenterology, 112, 226–235
24	2001	Higuchi H, Bronk S, Takikawa Y, Werneburg N, Takimoto R, El-Deiry W, Gores G (2001) "The bile acid glycochenodeoxycholate induces TRAIL-receptor 2/DR5 expression and apoptosis", J. Biol. Chem., 276, 38610-38618
25	2008	Ikonen E (2008) "Cellular cholesterol trafficking and compartmentalization", Nat. Rev. Mol. Cell Biol., 9, 125-138
26	1978	Schwartz CC, Halloran LG, Vlahcevic ZR, Gregory DH, Swell L (1978) "Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man", Science, 200, 62-64
27	2003	Lambert G, Amar MJ, Guo G, Brewer HB, Gonzalez FJ, Sinal CJ (2003) "The farnesoid X-receptor is an essential regulator of cholesterol homeostasis", J. Biol. Chem., 278, 2563-2570.
28	2006	Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, Edwards PA (2006) "Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice", Proc. Natl. Acad. Sci. USA, 103, 1006-1011

3.4. Publicações Anteriores
3.4. Past Publications

Referência Reference	Ano Year	Publicação Publication
1	2006	Prudêncio M, Rodriguez A, Mota MM (2006) "The silent path to thousands of merozoites: the Plasmodium liver stage", Nat. Rev. Microbiol., 4, 849-856
17	2008	Prudêncio M*, Rodrigues CD*, Hannus M, Martin C, Real E, Gonçalves LA, Carret C, Dorkin R, Röhl I, Jahn-Hoffmann K, Luty AJ, Sauerwein R, Echeverri CJ, Mota MM (2008a), "Kinome-wide RNAi screen implicates at least 5 host hepatocyte kinases in Plasmodium sporozoite infection", PLoS Pathog., 4, e1000201 *-Equally contributing authors
20	2008	Prudêncio M, Rodrigues CD, Ataíde R, Mota MM (2008b), "Dissecting in vitro host cell infection by Plasmodium sporozoites using flow cytometry", Cell. Microbiol., 10, 218-224
13	2008	Rodrigues CD*, Hannus M*, Prudêncio M*, Martin C, Gonçalves LA, Portugal S, Epiphanio S, Akinc A, Hadwiger P, Jahn-Hofmann K, Röhl I, van Gemert GJ, Franetich JF, Luty AJ, Sauerwein R, Mazier D, Koteliansky V, Vornlocher HP, Echeverri CJ, Mota MM. (2008) "Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection", Cell Host Microbe, 11, 271-

3	2008	**282 *-Equally contributing authors** **Silvie O, Mota MM, Matuschewski K, Prudêncio M (2008) "Interactions of the malaria parasite and its mammalian host", Curr. Opin. Microbiol., 11, 352-359**

4. Equipa de investigação
4. Research team

4.1 Lista de membros
4.1. Members list

Nome Name	Função Role	Grau académico Academic degree	%tempo %time	CV nuclear Core CV
Miguel Prudêncio	Inv. Responsável	DOUTORAMENTO	25	
Ghislain Cabal	Investigador	DOUTORAMENTO	25	
Maria Isabel de Sousa Rocha	Investigador	DOUTORAMENTO	5	
Rita Cruz Coelho de Mira Ramalho	Investigador	DOUTORAMENTO	5	
Márcia Maria de Almeida Aranha	Bolseiro	LICENCIATURA	15	

(O curriculum vitae de cada membro da equipa está disponível clicando no nome correspondente)
(Curriculum vitae for each research team member is available by clicking on the corresponding name)
Total: 5

4.2. Lista de membros a contratar durante a execução do projecto
4.2. Members list to hire during project"s execution

Membro da equipa Team member	Função Role	Duração Duration	%tempo %time
(BPD) Bolseiro de Pós-Doutoramento 1	Bolseiro	36	100

Total: 1

5. Projectos financiados
5. Funded projects

Lista de projectos financiados
Funded projects list

Referência Reference	Título Title	Estado Status
PTDC/BIA-BCM/71920/2006	O papel do SR-BI na infecção d...	Em curso

(Os detalhes de cada projectos estão disponíveis clicando na referência correspondente)
(Details for each project are available by clicking on the corresponding reference)
Total: 1

6. Indicadores previstos
6. Expected indicators

Indicadores de realização previstos para o projecto
Expected output indicators

Descrição Description	2009	2010	2011	2012	2013	Total
A - Publicações Publications						
Livros Books	0	0	0	0	0	**0**
Artigos em revistas internacionais Papers in international journals	0	0	0	1	2	**3**
Artigos em revistas nacionais Papers in national journals	0	0	0	0	0	**0**
B - Comunicações Communications						
Comunicações em encontros científicos internacionais Communications in international meetings	0	0	2	4	4	**10**
Comunicações em encontros científicos nacionais Communications in national meetings	0	0	0	0	0	**0**
C - Relatórios Reports	0	0	1	1	1	**3**
D - Organização de seminários e conferências Organization of seminars and conferences	0	0	0	1	1	**2**
E - Formação avançada Advanced training						

Teses de Doutoramento PhD theses	0	0	0	0	0	**0**
Teses de Mestrado Master theses	0	0	0	0	0	**0**
Outras Others	0	0	0	0	0	**0**
F - Modelos Models	0	0	0	0	1	**1**
G - Aplicações computacionais Software	0	0	0	0	0	**0**
H - Instalações piloto Pilot plants	0	0	0	0	0	**0**
I - Protótipos laboratoriais Prototypes	0	0	0	0	0	**0**
J - Patentes Patents	0	0	0	0	1	**1**
L - Outros Other						
Review/Book chapter	0	1	0	0	1	**2**
	0	0	0	0	0	**0**
	0	0	0	0	0	**0**

Acções de divulgação da actividade científica
Scientific activity spreading actions

The Communication and Training Unit is the IMM´s first line of interaction with society. The Unit has dedicated members to address Science and Society activities, in collaboration with national and international partners. A science communication programme has been developed which builds on researcher's initiatives as well as projects aiming at promoting a two-way interaction between researchers and a wide range of audiences. Activities include workshops and hands-on activities for younger generations, meetings involving scientists and different publics, the development of educational resources and media training activities for scientists. Schools and the media are among privileged partners/audiences.
The research team will inform the IMM Communication and Training Unit about project results in order to allow the latter to mediate the diffusion of results to the media. In addition, the project team is willing to participate in science communication activities organized by the IMM Communication and Training Unit for the general public (primary and secondary students, teachers, children, adults, etc), contributing effectively with the team members' expertise and that acquired during the project.

7. Orçamento
7. Budget

-

Instituição Proponente
Principal Contractor

Instituto de Medicina Molecular

Descrição Description	**2009**	**2010**	**2011**	**2012**	**2013**	**Total**
Recursos Humanos Human resources	0,00	9.870,00	19.740,00	19.740,00	9.870,00	**59.220,00**
Missões Missions	0,00	2.000,00	2.000,00	2.000,00	2.000,00	**8.000,00**
Consultores Consultants	0,00	0,00	0,00	0,00	0,00	**0,00**
Aquisição de bens e serviços Service procurement and acquisitions	0,00	14.000,00	28.000,00	28.000,00	14.000,00	**84.000,00**
Registo de patentes Patent registration	0,00	0,00	0,00	0,00	5.000,00	**5.000,00**
Adaptação de edifícios e instalações Adaptation of buildings and facilities	0,00	0,00	0,00	0,00	0,00	**0,00**
Gastos gerais Overheads	0,00	2.587,00	4.794,00	4.794,00	3.087,00	**15.262,00**
TOTAL DESPESAS CORRENTES TOTAL CURRENT EXPENSES	**0,00**	**28.457,00**	**54.534,00**	**54.534,00**	**33.957,00**	**171.482,00**
Equipamento Equipment	0,00	0,00	0,00	0,00	0,00	**0,00**
Total	**0,00**	**28.457,00**	**54.534,00**	**54.534,00**	**33.957,00**	**171.482,00**

Instituições Participantes
Participating Institutions

Faculdade de Farmácia da Universidade de Lisboa

Descrição Description	**2009**	**2010**	**2011**	**2012**	**2013**	**Total**
Recursos Humanos Human resources	0,00	0,00	0,00	0,00	0,00	**0,00**
Missões Missions	0,00	0,00	0,00	0,00	0,00	**0,00**
Consultores Consultants	0,00	0,00	0,00	0,00	0,00	**0,00**
Aquisição de bens e serviços Service procurement and acquisitions	0,00	3.000,00	3.000,00	0,00	1.000,00	**7.000,00**
Registo de patentes Patent registration	0,00	0,00	0,00	0,00	0,00	**0,00**
Adaptação de edifícios e instalações Adaptation of buildings and facilities	0,00	0,00	0,00	0,00	0,00	**0,00**
Gastos gerais Overheads	0,00	300,00	300,00	0,00	100,00	**700,00**
TOTAL DESPESAS CORRENTES TOTAL CURRENT EXPENSES	**0,00**	**3.300,00**	**3.300,00**	**0,00**	**1.100,00**	**7.700,00**
Equipamento Equipment	0,00	0,00	0,00	0,00	0,00	**0,00**
Total	**0,00**	**3.300,00**	**3.300,00**	**0,00**	**1.100,00**	**7.700,00**

Orçamento Global
Global budget

Descrição Description	**2009**	**2010**	**2011**	**2012**	**2013**	**Total**
Recursos Humanos Human resources	0,00	9.870,00	19.740,00	19.740,00	9.870,00	**59.220,00**
Missões Missions	0,00	2.000,00	2.000,00	2.000,00	2.000,00	**8.000,00**
Consultores Consultants	0,00	0,00	0,00	0,00	0,00	**0,00**
Aquisição de bens e serviços Service procurement and acquisitions	0,00	17.000,00	31.000,00	28.000,00	15.000,00	**91.000,00**
Registo de patentes Patent registration	0,00	0,00	0,00	0,00	5.000,00	**5.000,00**
Adaptação de edifícios e instalações Adaptation of buildings and facilities	0,00	0,00	0,00	0,00	0,00	**0,00**
Gastos gerais Overheads	0,00	2.887,00	5.094,00	4.794,00	3.187,00	**15.962,00**
TOTAL DESPESAS CORRENTES TOTAL CURRENT EXPENSES	**0,00**	**31.757,00**	**57.834,00**	**54.534,00**	**35.057,00**	**179.182,00**
Equipamento Equipment	0,00	0,00	0,00	0,00	0,00	**0,00**
Total	**0,00**	**31.757,00**	**57.834,00**	**54.534,00**	**35.057,00**	**179.182,00**

Plano de financiamento
Finance plan

Descrição Description	**2009**	**2010**	**2011**	**2012**	**2013**	**Total**
Financiamento solicitado à FCT Requested funding	0,00	31.757,00	57.834,00	54.534,00	35.057,00	**179.182,00**
Financiamento próprio Own funding	0,00	0,00	0,00	0,00	0,00	**0,00**
Outro financiamento público Other public-sector funding	0,00	0,00	0,00	0,00	0,00	**0,00**
Outro financiamento privado Other private funding	0,00	0,00	0,00	0,00	0,00	**0,00**
Total do Projecto Total of the project	**0,00**	**31.757,00**	**57.834,00**	**54.534,00**	**35.057,00**	**179.182,00**

8. Justificação do orçamento
8. Budget rationale

-

8.1. Justificação dos recursos humanos

8.1. Human resources rationale

Tipo
Type
(BPD) Bolsa de Pós-Doutoramento

Nº de pessoas
No. of persons
1

Duração (em meses)
Duration (in months)
36

Custo envolvido (€) *(calculado)*
Total cost (€) *(estimated)*
53.820,00

Outros custos (€)
Other costs (€)
5.400,00

Justificação do financiamento solicitado
Rationale for requested funding

In addition to the current members of the research team, one post-doctoral researcher will be hired. He/she will be working directly under the PI's supervision, and will be fully dedicated to this project. Careful selection of a candidate with the appropriate background and expertise will ensure the completion of the proposed tasks to be carried out at the IMM.
In addition to his/her monthly stipend, funding is requested in order to cover yearly expenses with mandatory work insurance (50€/year), conference attendance (750€/year) and social security costs (approximately 1000€/year).

8.2. Justificação de missões

8.2. Missions rationale

Tipo
Type
Cursos associados à temática do projecto

Nº de deslocações
No. of participations
1

Local
Venue
International

Custo envolvido (€)
Cost (€)
1.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Funding for a training workshop to be attended by the hired post-doc early after joining the team is contemplated (1000 Euros). This is intended provide the new member of the team with the theoretical and experimental tools that will be required throughout the duration of the project.

Tipo
Type
Participação em congressos

Nº de deslocações
No. of participations
7

Local
Venue
International

Custo envolvido (€)
Cost (€)
7.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Funding is requested for the participation of team members in international conferences (besides the hired post-doc, whose funding request already includes this item). Requested funding assumes an average of one participation per semester in the years 2010-2012 and two participations in the first semester of 2013, when the project is approaching completion and a full data set has been gathered. The assumed cost of each participation is 1000 Euros.

8.3. Justificação de consultores

8.3. Consultants rationale

Nome completo
Full name
Cecília Maria Pereira Rodrigues

Instituição
Institution
Faculdade de Farmácia da Universidade de Lisboa (FF/UL)

Fase do projecto
Project phase
Throughout the project, particularly during Tasks 2, 3 and 5

Custo (€)
Cost (€)
0,00

Justificação do financiamento solicitado
Rationale for requested funding

No funding is requested for this consultant

Página na Internet onde pode ser consultado o CV do consultor
Web page where the consultant's CV can be accessed
(Vazio)
(Void)

Nome completo
Full name
Maria Manuel Dias da Mota

Instituição
Institution

Unidade de Malaria - Instituto de Medicina Molecular, Lisboa, Portugal

Fase do projecto Project phase	**Custo (€)** Cost (€)
Throughout the project	0,00

Justificação do financiamento solicitado
Rationale for requested funding

No funding is requested for this consultant

Página na Internet onde pode ser consultado o CV do consultor
Web page where the consultant's CV can be accessed

(Vazio)
(Void)

8.4. Justificação de aquisição de bens e serviços
8.4. Service procurement and acquisitions

Tipo Type	**Custo (€)** Cost (€)
Biological material	24.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover acquisition, breeding and maintenance of parasites and rodent models. This funding will be allocated to the IMM for completion of Tasks 1-5. An estimated 8000€/year, on average, in a total of 24000€ is requested to cover the costs of parasite production, rodent model maintenance and acquisition of transgenic lines of rodent models.

Tipo Type	**Custo (€)** Cost (€)
Bile acid profile analyses	6.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover the analysis of bile acid profiles in the bile of rodent models. This funding will be allocated to the FF-UL, for completion of Task 2. The cost of analysis is 125€/sample, yielding an estimated total of 6000€, to be spent in the second semester of 2010 and the first semester of 2011, during execution of Task 2

Tipo Type	**Custo (€)** Cost (€)
Molecular biology reagents and kits	18.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover the necessary material for analysis of gene expression levels and infection assessments. This funding will be allocated to the IMM for completion of Tasks 1-5. An estimated 6000€/year, on average, yielding a total of 18000€ is requested to cover the costs of RNA extraction, cDNA and qRT-PCR kits

Tipo Type	**Custo (€)** Cost (€)
Antibodies	6.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover expenses with antibodies and will be allocated to the IMM for completion of Tasks 3-5 and to the FF-UL for completion of Task 5. An estimated 2000€/year, on average, yielding a total of 6000€ is requested for this purpose.

Tipo Type	**Custo (€)** Cost (€)
Flow cytometry	13.500,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover in vitro infection analyses and cell sorting procedures by flow cytometry. This funding will be allocated to the IMM for completion of Tasks 1 and 3-5. The cost of utilisation of the flow cytometry analysers is 10€/hour and that of the fluorescence activated cell sorter is 16€/hour. An estimated 4500€/year, on average, in a total of 13500€ is requested for these purposes.

Tipo Type	**Custo (€)** Cost (€)
Confocal microscopy	4.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover confocal microscopy of immunostained samples. This funding will be allocated to the IMM for completion of Tasks 3-5. The cost of utilisation of the confocal microscope is 11€/hour; An estimated total of 4000€ is requested for this purpose.

Tipo Type	Custo (€) Cost (€)
RNA interference	10.500,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover reagents for RNA interference experiments, both in vitro and in vivo. This funding will be allocated to the IMM for completion of Tasks 3-5. An estimated 3500€/year, on average, in a total of 10500€ is requested for the acquisition of small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and transfection reagents.

Tipo Type	Custo (€) Cost (€)
Cell culture	6.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover expenses with cell culture reagents and plastic consumables. This funding will be allocated to the IMM for completion of Tasks 1 and 3-5. An estimated 2000€/year, on average, in a total of 6000€ is requested for the acquisition of consumables such as cell growth media and cell culture plates.

Tipo Type	Custo (€) Cost (€)
Other reagents	3.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Requested funding will cover expenses with reagents other than those previously listed. This funding will be allocated to the IMM for completion of Tasks 1-5. An estimated 1000€/year, on average, in a total of 3000€ is requested for the acquisition of consumables such as agarose, buffers, antibiotics, etc..

8.6. Justificação do Equipamento
8.6. Equipment rationale

8.6.1. Equipamento já disponível para a execução do projecto
8.6.1 Available equipment

Tipo de equipamento Equipment type	Fabricante Manufacturer	Modelo Model	Ano Year
Quantitative Real-Time PCR	Applied Biosystems	ABI PRISM 7000	2003
Quantitative Real-Time PCR	Corbett Research	Rotor Gene 6000	2006
NanoDrop - Spectrophotometer	Thermo Scientific	ND-1000	2003
Bioanalyzer	Agilent Technologies	2100	2006
Fluorescence microscope	Leica	Axiovert 200M	2005
Confocal microscope	Zeiss	LSM 510 Meta	2006
Biosafety cabinet	Tecniplast	BS48	2005
Flow cytometer	BD Biosciences	FACSAria	2005
Flow cytometer	BD Biosciences	FACSCalibur	2005
Flow cytometer	BD Biosciences	FACSCanto	2005

8.6.2. Discriminação do equipamento a adquirir
8.6.2. New equipment requested

(Vazio)
(Void)

8.7. Justificação de registo de patentes
8.7. Patent registration

Tipo Type	Custo (€) Cost (€)
Patent registration	5.000,00

Justificação do financiamento solicitado
Rationale for requested funding

Funding is requested towards the possible registration of one patent at the end of the project. One of the proposed goals of this project is the identification of novel target(s) for anti-malarial intervention. The identification of such target(s) and its/their validation constitutes the final proposed milestone (M5), as detailed in the Timeline.PDF file. If this endeavour meets with the expected success, the host lab will consider filling in a patent application for the identified anti-malarial target(s).

8.8. Justificação de adaptação de edifícios e instalações
8.8. Adaptation of buildings and facilities

(Vazio)
(Void)

9. Ficheiros Anexos
9. Attachments

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Nome Name	Tamanho Size
General_scheme.pdf	197Kb
PersonMonths.pdf	53Kb
Timeline.pdf	63Kb

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