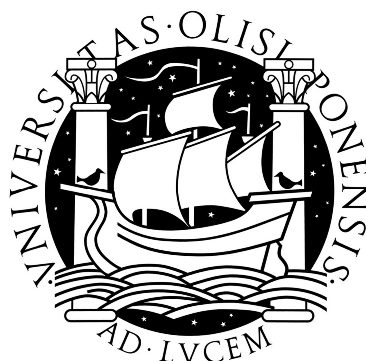
View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Universidade de Lisboa: Repositório.UL

Universidade de Lisboa

Faculdade de Medicina



Plasmodium infection of mammalian host:

Effects of vitamin D

Nuno Baltazar do Carmo

Mestrado em Microbiologia Clínica

2007

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Cientifico da Faculdade de Medicina de Lisboa em reunião de (17/07/2008).

Universidade de Lisboa

Faculdade de Medicina

Plasmodium infection of mammalian host:

Effects of vitamin D

Nuno Baltazar do Carmo

Mestrado em Microbiologia Clínica

Dissertação orientada pela Prof Doutora Maria Manuel

Mota

Todas as afirmações efectuadas no presente documento dão da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados

http://www.youtube.com/watch?v=zL7V_SgP_oA

Ismael Lo – Jammu Africa

Agradecimentos

À Prof. Drª Maria Mota pela opotunidade de desenvolver esta minha ideia, pelo orientação, revisão e comentários a este trabalho.

Ao Prof. Luis Moita por gentilmente fornecer os plasmideos e protocolos para a produção dos lentivírus utilizados neste trabalho e pelos comentários motivadores para este projecto.

A todos os colaboradores da Unidade Malária.

Ao Ricardo Ataíde pela ajuda, amizade, companheirismo e óptimo sentido de humor.

Ao Miguel pela disponibilidade e apoio inesgotáveis, pela revisão e sugestões, pela amizade.

À Cristina, por ser quem é, um modelo para mim como cientista.

Ao meu filho, pelas horas que tem dado à ciência.

À Sandra, por dar tons de Primavera à minha vida mesmo nos dias de Inverno.

À minha familia, que apesar de não compreender porque vivem assim os cientistas, acreditam que sou feliz.

A todos presentes e ausentes, UM GRANDE BEM HAJAM!

Table of Contents

Abstract

Resumo

1. Introduction and Objectives 2

1.1. The Malaria Burden 2

1.2. Plasmodium life cycle 3

1.3. Malaria pathology 6

1.3.1. Cerebral malaria 7

1.3.2. Severe malaria anaemia 8

1.4. Host-targeted antimalaria drugs 9

1.5. Vitamin D 10

1.5.1 Vitamin D metabolism and physiology 11

1.5.2. Molecular actions of Vitamin D 13

1.6. Immunological effects of Vitamin D 15

1.6.1. Anti-inflammatory actions of Vitamin D 15

1.7. Vitamin D and infectious diseases 16

1.8.Aims 18

2. Materials and Methods 20

2.1. Cells 20

2.2. Mice, mosquitos and parasites 21

2.3. Sporozoite isolation and purification 21

2.4. Lentiviral production and cell transduction 22

2.5. In vitro cell infections and treatment with1,5_-dihidroxivitamin D 24

2.6. Immunochemestry 25
2.7. Migration assay.. 25
69
2.8. Flow cytometry.. 26
2.9. Quantitative Real-time PCR (qRT-PCR) 26
2.10. In vivo liver infections.. 27
2.11. In vivo blood infections and disease assessment 28
2.12. Statistical analysis.. 29
3. Results 31
3.1. Liver stage 31
3.1.1. Vitamin D effect in P. berghei in vitro infection 31
3.1.2. Effect of vitamin D in P. berghei liver infection 36
3.2. Blood stage.. 38
3.2.1 Vitamin D effect in a Cerebral Malaria (CM) model 38
4. Discussion and Clonclusions.. 43
5. Conclusions.. 48
6. References.. 50

Abstract

Malaria is one of the most important parasitic infectious diseases in terms of human and economical losses in developing countries. This disease is caused by *Plasmodium* and is transmitted to the vertebrate host by *Anopheles* mosquitoes. During malaria infection, *Plasmodium* parasites infect first hepatocytes and after the erythrocytes, here being responsible for serious pathological complications and some times to death. These two obligatory parasite developmental stages give to scientists two different approaches to fight malaria. The first, in the liver, preventing malarial disease associated symptoms and the second in the blood, to attenuating exacerbated clinical complications.

Vitamin D, and particularly its active metabolite, 1,25α(OH)2D3, have been showed to be a potent immunoregulator. 1,25α(OH)2D3 can tune the immune system towards an anti-inflammatory state. Paradoxically, vitamin D is also associated with the production of antimicrobial peptides by the innate immune system.

In the present work we decided to take advantage of the particular immunoregulatory properties of 1,25α(OH)2D3 to study a potential antimicrobial effect in the *Plasmodium Berghei* liver stage and a potential anti-inflammatory effect in a cerebral malaria rodent model.

In the *P. berghei* liver stage infection our results show a *in vitro* reduction in the of infection of 1,25α(OH)2D3 treated human Huh7 and mouse Hepa1-6 hepatoma cell lines by flow cytometry. *In vivo*, no differences were observed between 1,25α(OH)2D3 treated and untreated infected C57/Bl6 mice livers.

However, lower doses of 1,25α(OH)2D3 tend to reduced infection whether higher doses show the opposite tendency.

1,25α(OH)2D3 had no effect in the outcome of cerebral malaria in a murine cerebral malaria model.

In conclusion, 1,25α(OH)2D3 might have a potential antimicrobial effect in *P. berghei* liver stage. However, further studies should be made.

Keywords:

Malaria, *Plasmodium*, vitamin D, liver stage, cerebral malaria

Resumo

A Malária é uma das mais importantes doenças infecciosas no que respeita a perdas humanas e económicas nos paises em vias de desenvolvimento. Esta doença é causada pelo *Plasmodium* e é transmitida ao hospedeiro vertebrado por mosquitos *Anopheles*. Estes parasitas, no hospedeiro vertebrado, infectam primeiro hepatocitos e seguidamente os eritrocitos, fase responsavel por complicações patologica séria que levam por vezes à morte. Estas duas fases de desenvolvimento necessárias ao parasita dão aos cientistas duas aboradagens deiferentes para combater a malaria. A primeira, no fígado, prevenindo os sintomas associados a esta doença e a seguinda, no sangue, atenuando as complicações clinicas.

A vitamina D, e em particular a sua forma activa, 1,25α(OH)2D3, são potentes reguladores do sistema imune. 1,25α(OH)2D3 pode modular o sistema imune para um estado anti-inflamatório. Paradoxalmente, a vitamina D está também associada com a produção de péptidos antimicrobianos pelo sistema imune.

No presente trabalho decidimos tirar partido das propriedades imuno-regulatorias de 1,25α(OH)2D3 e estudar tanto potenciais efeitos antimicrobianos na fase hepática como potenciais efeitos anti-inflamatórios na fase sanguinea da infecção pelo *Plasmodium Berghei* tendo por modelo roedores.

Durante a fase hepatica da infecção do *P. Berghei* os nossos resultados mostram uma redução *in vitro* da infecção de linhas celulares de hepatoma de ratinho (Hepa1-6) e humano (Huh7), quando tratadas com 1,25α(OH)2D3 por

citometria de fluxo. *In vivo*, não foram observadas diferenças entre a infecção de figados de ratinhos C57/Bl6 tratados ou não com 1,25α(OH)2D3. Contudo, ratinhos sugeitos a doses reduzidas de 1,25α(OH)2D3 mostraram uma tendência para ter uma nenor infecção, enquanto doses mais altas mostraram uma tendência inversa.

1,25α(OH)2D3 não teve qualquer efeito no resultado final no modelo de malaria cerebral.

Em conclusão, 1,25α(OH)2D3 poderá ter um efeito antimicrobiano na fase hepática do *P. Berghei.* Contudo, deverão ser efectuados mais estudos.

Palavras chave:

Malaria, *Plasmodium*, Vitamina D, fase hepática, malária cerebral

1. Introduction and Objectives

1. Introduction and Objectives

1.1. The Malaria Burden

Malaria infection has strained the biological and cultural resources of human populations since ancient times, in an evolutionary associantion between host and parasite [23,68,99]. The advent of agriculture was critical in establishing malaria infection in human population. Animal and plant domestication increased population size and an increased population density was able to support malaria propagation. Moreover, agriculture led to forest clearing and related environmental modifications that favoured breeding of malaria vector [43,45].

Human efforts to control malaria have markedly restricted its distribution during the 20th century [37]. The global program of malaria eradication, coordinated and supported by the World Health Organization (WHO) started in 1957, has been successful in most countries in the temperate climate zones of the globe. However, by the end of the 1960s it became evident that technical problems, such as resistance of mosquito vectors to insecticides and resistance of malaria parasites to drugs, presented serious obstacles to the pursuit of eradication programs in many tropical countries [4,8].

The malaria situation deteriorated during the 90´s [37]. Today malaria is one of the most important infectious diseases to humankind. Every year this parasitic disease affects 300-500 million people and causes 1-3 million deaths worldwide [84]. It is a public health problem that affects around 40% of the world population. Ninety percent of malaria cases occur in the sub-Saharian Africa.

Mortality due to malaria is especially high among children under 5 years of age and pregnant women [33,37]. Malaria represents a huge drawback to the development of the affected countries, both economically and socially, in a vicious cycle of poverty and disease [84].

In 1998, World Health Organization (WHO) launched an international partnership, the Roll Back Malaria (RBM), whose goal is to attain a 50% reduction of the malaria burden based in a transversal approach to fight malaria by sustained country development [71]. Although very naïve in their objectives, the RBM brought to discussion the world malaria burden and mobilized a huge effort to fight this disease.

1.2. *Plasmodium* life cycle

Plasmodium (*P.*), the malaria parasite exhibits a extremely complex life cycle (Fig. 1) involving two hosts, an insect vector, the female *Anopheles* mosquito, and a vertebrate host. This parasitic protozoan infects a wide range of vertebrate hosts including reptiles, birds, rodents, and primates and humans. Four *Plasmodium* species infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is by far the deadliest of the four and is responsible for most of the mortality and morbidity associated with malaria. All four species exhibit a similar life cycle with only minor variations [3,98].

When a female *Anopheles* mosquito probes the human skin to obtain a blood meal, it injects saliva mixed with anticoagulants. If the mosquito is infected with *Plasmodium*, it will also inject an estimate average of around 15

sporozoites into the skin [59,81]. Sporozoites glide through the skin and are carried by the blood stream, rapidly reaching the liver sinusoids [5,26,78]. After being sequestered in the sinusoids, sporozoites reach their initial site of multiplication in the mammalian host, the hepatocytes, by passing through Kupffer cells [77]. *Plasmodium* parasites traverse several hepatocytes before invading a final cell. This traversing behaviour increases the susceptibility of host hepatocytes and activates the parasite, promoting its entry into a final hepatocyte, in which a parasitophorous vacuole (PV) is formed [64,66].

After the final invasion, each *Plasmodium* sporozoite develops and undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocyte [29], generating thousands of new parasites (merozoites). *P. vivax* and *P. ovale* can go through a dorment period in the liver, forming so-called hypnozoites [16]. After full development, parasite-filed vesicles (merosomes) bud from the infected hepatocytes which transport mature merozoites to the blood stream [89]. The *Plasmodium* liver stage is the first obligatory step and is clinically silent.

Symptomatic disease is caused by merozoites that proliferate during the erythrocytic stage of the life cycle. The erythrocytic stage begins when merozoites emerge from the liver and invade erythrocytes. Each merozoite develops sequentially into a ring form, a trophozoite, and a schizont in an enormous numerical expansion through many cycles of mitotic replication. Schizont-infected erythrocytes rupture and release merozoites, which invade other erythrocytes [35].

As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes [3,97]. When

the mosquito takes up an infected blood meal that contains sexual stages, microgametes exflagellate within minutes and fuse with a macrogamete to form a diploid zygote. The zygote transforms into a motile ookinete that traverse epithelial gut cells, encounters the basal lamina where it arrests and transforms into an oocyst. The oocyst undergoes several mitotic divisions and forms sporoblasts. Once formed, sporozoites actively move through the mosquito haemolymph to the salivary glands and start to acumulate in the salivary duct [57]. The cycle is complete when the infected mosquito bites another host.

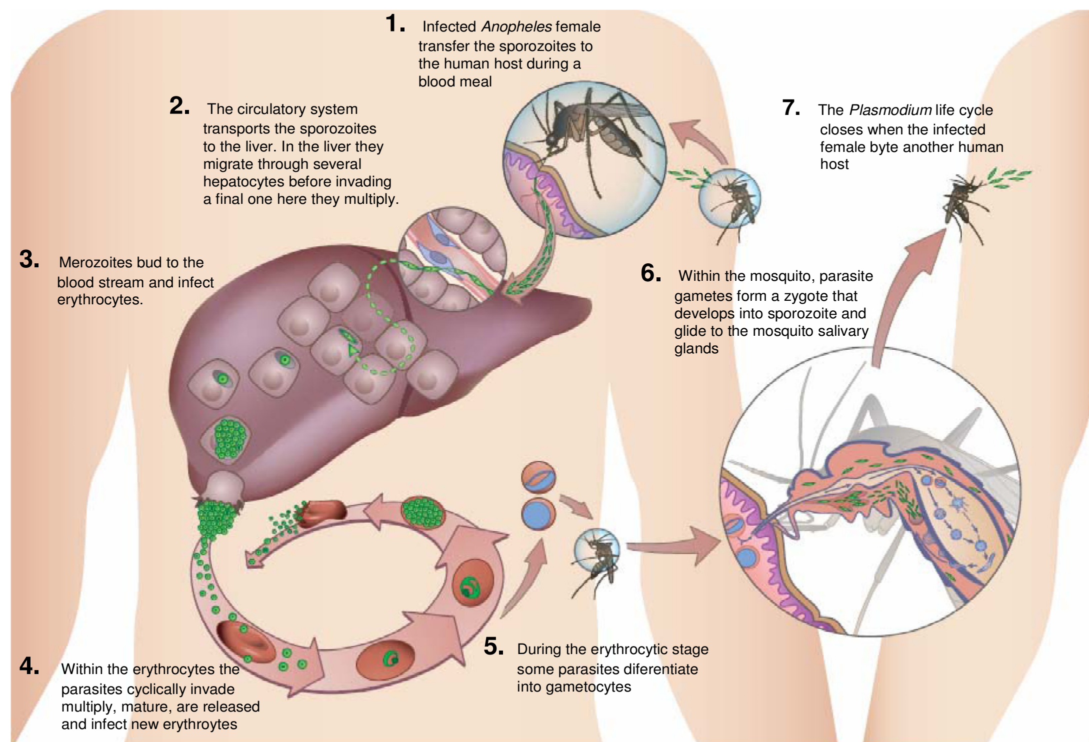


Figure 1.1. The *Plasmodium* life Cycle

1.3. Malaria pathology

Symptoms associated with malaria are restricted to the erythrocytic stage of the *Plasmodium* life cycle in the vertebrate host. In humans, these symptoms display a large spectrum of intensity ranging from slight headache or mild fever to extremely severe life-threatening complications such as metabolic acidosis, cerebral malaria (CM) and severe malarial anaemia [61,85]. The most severe pathologies are observed in non-immune individuals such as children in endemic areas, individuals living in areas of episodic transmission or migrants from non-endemic areas [101]. The development of severe malaria probably results from a combination of parasite-specific factors, such as adhesion and sequestration in the vasculature and the release of bioactive molecules, together with host inflammatory responses [52,76]. Traditionally, approaches to understanding pathogenesis have involved clinical studies in humans, experimental infections in animal models and *in vitro* modelling of pathogenic processes [52].

The observation of large numbers of parasites accumulated in specific organs, such as the brain and the placenta, associated with adverse clinical outcomes, suggests that organ-specific accumulation of parasites is important in the pathogenesis of malarial disease. As circulating parasites mature, antigens expressed on the erythrocyte surface enable adhesion to endothelial cells and non-infected erythrocytes, forming rosettes and sequester in vascular beds. This adhesion phenotype, although detrimental to the host, seems to be

adaptive to the parasite since erythrocytes with mature parasites are sequestered from peripheral circulation and, thus, protected from spleen clearance [6,20].

Toll like receptors (TLR) are responsible for the recognition of parasite molecules by innate immune system. Their activation in endothelial, macrophages and dendritic cells induces widespread expression Th1 pro-inflammatory cytokines such as Tumour necrosis factor (TNF) and Interferon γ (IFN- γ) [31,76]. The Th1 responses are important for the host to kill the parasite, however, in non immune individuals a cytokine storm is initiated with an unbalance between Th1 and Th2 immune responses.

1.3.1. Cerebral malaria

CM is a major life-threatening complication of *Plasmodium* infection which affects mainly children between 2-6 years old. CM pathology seem to include abnormally high production of Th1 cell-derived cytokines such as tumor necrosis factor (TNF)-α and IFN-γ induced by infected erythrocytes [15,31,32]. These cytokines may play an important role in causing certain pathological changes, by up-regulating the expression of cell surface markers like CD36, ICAM-1, VCAM-1 and chondroitin sulfate A, thus leading to the sequestration of infected erythrocytes, leukocytes and monocytes in the cerebral capillaries. This accumulation induces flow perturbations that led to obstruction and hypoxia [76].

Endothelial damage of brain microvasculature induce changes in the blood brain barrier exposing the brain parenchyma to cytokines and parasite antigens, activating the microglia and damaging astrocytes [44,58].

1.3.2. Severe malaria anaemia

Severe malarial anaemia is seen most frequently in areas of very high malaria transmission and most commonly in young children and pregnant women [12].

Anaemia results from the destruction of both infected and non-infected red blood cells and the decrease production of these cells, mainly due to an inadequate erythropoietic response of the bone marrow [60,61,97]. The spleen plays a major role in removing parasitized and non-parasitized erythrocytes from circulation. In fact, this clearance was observed to be increased in patients with splenomegaly [61]. Studies on bone marrow indicated a depression and a defective erythropoiesis during malaria infection [60,97]. The removal of non-infected red blood cells has been proposed as the major mechanism leading to persistence and worsening of anaemia following parasitaemia clearance. This could explain the lack of correlation between the parasitaemia level and anaemia observed in some studies that reported cases of patients with severe anaemia and low parasitaemias [45,61,68].

1.4. Host-targeted antimalaria drugs

The alarming increase in *P. falciparum* resistance to commonly used anti-malarial drugs represents a major public health threat. The current most important strategy used to fight resistance is the use of drug combinations such as arteminisin-based combination therapy [70]. This strategy may delay the future

emergence of resistant strains but has limited application and do not solve the problem of existing antimalarial drug resistance with the addicional drawback of higher cost of the combination.

Most antimalarial drugs target *Plasmodium*. However, *P. falciparum* is a highly variable parasite with immense recombination abilities. Recently, researchers are focusing on the modulation of host pathways as a different approach to a therapeutic strategy to fight malaria . Furthemore, resistance is less likely to occur if the targeted host component remains outside the microorganism [17].

Host molecules are being studied as potential targets to fight *Plasmodium* infection. Liver molecules are potential prophylactic targets [17]. Promising results have been achieved with the use of tyrosine kinase inhibitors to target Hepatocyte Growth Factor (HGF) and its receptor c-MET which impairs *Plasmodium* liver development [11,17]. Harrison and co-workers, blocked invasion and development of *Plasmodium* erythrocytaic stages by inhibiting host erythrocyte C_s signalling [36,69]. Both these host molecules are promising antimalarial drug target candidates.

1.5. Vitamin D

Vitamin D is mainly produced by the skin [42]. This steroid hormone serves as a chemical messenger that triggers biological responses in many target tissues with different physiological actions including maintenance of calcium homeostasis, effects on the immune, cell proliferation and differentiation. The importance of this vitamin in human heath was first described in rickets almost a

hundred years ago [38]. In the last years, vitamin D has emerged as a promising drug against autoimmune diseases, cancer and as an antimicrobial agent [24,39,51]. Vitamin D deficiency is associated with higher susceptibility to infection.

1.6. Vitamin D metabolism and physiology

The metabolism of vitamin D is well known. During sun exposure, UVB radiation is absorbed by the skin of 7-dehydrocholesterol resulting in the formation of Vitamin D_3 [41]. Alternatively vitamin D, in the form of either vitamin D_2 or D_3 can be derived from dietary sources [30]. Vitamin D_3 is then transported in the blood by the vitamin D binding protein (DBP) to the liver where it is hydroxylated at C-25 by 1α-hydroxylase resulting in the formation of 25-dihydroxyvitamin D_3 (25(OH)D_3) [13]. 25(OH)D_3 is transported by DBP to the kidney and hydroxylated at the 1α position by 25-dihydroxyvitamin 1 α-hidroxylase, resulting in the formation of the active form of vitamin D [13,40].

The active form of vitamin D has different target tissues where it can modulate a wide range of physiological actions (Table 1.).

The high potency of 1,25α(OH)2D3 in elevating serum calcium and phosphate levels requires its circulating levels to be tightly regulated. Control of serum 1,25α(OH)2D3 usually involves reciprocal changes in the rates of synthesis and degradation. Hypocalcemia increases serum 1,25α(OH)2D3 levels by stimulation of the kidney 25-dihydroxyvitamin 1α-hydroxylase, whereas hypercalcemia depresses α-hydroxylase activity and increases 24-hydroxylase activity, the 24-hydroxylase is ubiquitously present in vitamin D target tissues, attenuating the response to the vitamin D hormone, and reducing

the levels of 1,25α(OH)2D3. Parathyroidectomy severely blunts the induction of the renal 1-hydroxylase by hypocalcemia. Finally, calcium can, by itself, directly suppress 1α-hydroxylase activity and mRNA levels [7].

Table 1. 1,25α(OH)2D3 actions in classical and nonclassical target tissues [9]

	Tissue	Cell type	Action
Classic			
	Intestine	Epithelial	Enhancement of calcium and phosphate absortion
	Bone	Osteoblast	Enhance of bone matrix protein synthesis, bone mineralization, and synthesis of mediators of osteo-clastogenesis and osteoclastic activity
		Osteoclast	Enhancement of bone resorption
	Kidney	Epithelial (proximal and distal)	Inhibition of 1,25$(OH)_2D_3$ synthesis and induction of 24-hydroxylase
			Enhancement of calcium and phosphate reabsortion
	Parathiroid gland	Chief	Inhibition of cell growth and PHT synthesis
Nonclassic			
	Hematopoetic tissues	Myeloid cell precursors	Antiproliferative, prodiferentiating
		Colony forming units	Prodiferentiating
	Immune System	Monocyte/macrophages	Enhancement of immune function to control viral and bacterial infections and tumour growth
		Lynphocyte	Immunosuppression
	Skin	Keratinocytes, fibroblasts, hair folicule, Langerham cells, melanocytes	Antiproliferative, prodiferentiating
	Muscle	Smoth muscle cell, myoblast	Antiproliferative, prodiferentiating
	Heart	Cardiac muscle cell	Antiproliferative, prodiferentiating
		Atrial myocytes	Inhibition of antinatriuretic factor synthesis
	Pancreas	β-cells	Enhancement of insulin synthesis and secretion
	Cancer cells	Melanoma, breast carcinoma, leukemia, osteosarcoma, fbrosarcoma, pituitary medullary thyroid carcinoma, adenoma, neuroblastoma, pancreatic adenocarcinoma, bladder, cervical, prostate and colon carcinomas	Antiproliferative, prodiferentiating
	Adrenal gland	Medullary cells	Control of catecolamine metabolism
	Brain	Hypocampus/selected neurons	Neuronal regeneration, enhacement of nerve growth factor and neurotrophin synthesis, control of sphyn-gomyelin cycle
	Cartilage	Chondrocyte	Antiproliferative, prodiferentiating
	Female reproductive	Myometrial and endometrial cells	Antiproliferative, prodiferentiating
	Liver	Parenchymal cell (fetal, adult)	Enhancement of liver regeneration, control of glycogen and transferrin synthesis
	Lung	Fetal pneumocytes	Enhancement of maturation, phospholipid synthesis and surfactant release
		Adult pnemocytes	Cell growth
	Male reproductive organs	Sertoli/semminiferus tubule	Enhancement of Sertoli cell fuction and spermatogen-Esis
	Pituitary production	Somatomammotroph	Control of T_3-induced growth hormone, prolactin and Tyrotrophyn
	Thyroid	Follicular cells (C cells)	Inhibition of cell function and calcitonin synthesis

1,25$(OH)_2D_3$. 1,25-dihydroxyvitamin D_3: PHT. Parathyroid hormone: T_3. triiodothyronine

1.7. Molecular actions of Vitamin D

Most of the biological actions of 1,25α$(OH)_2D_3$ are mediated by binding to a high-affinity nuclear receptor, Vitamin D Receptor (VDR) that acts as a ligand-activated transcription factor [19,50,]. Nuclear receptors are ligand-activated regulators of gene transcription with a conserved domain structure [74]. The highly conserved DNA-binding domain (DBD) contains two zinc fingers that form a single structural domain containing a α-helical reading head that controls specific DNA sequence recognition. The VDR ligand-binding domain (LBD) not only binds ligand but also contains a ligand-regulated C-terminal AF-2 domain (activating function-2) that is essential for its capacity to activate transcription [82]. Strong interactions between VDR and RXR LBDs are essential for ligand-dependent dimerization and high-affinity DNA binding. Nuclear receptors regulate target gene transcription by ligand-controlled recruitment of several accessory proteins known collectively as coregulators. Coregulators are essential for the histone modifications, chromatin remodeling and recruitment of RNA polymerase and ancillary factors that are necessary for the initiation of transcription [22].

Vitamin D response elements (VDREs) are composed of tandem motifs with the consensus PuG(G/T)TCA often arranged as direct repeats separated by three base pairs [50].

The active metabolite 1,25(OH)2D enters the cell and binds the vitamin D receptor (VDR). This complex forms a heterodimer with the retinoid receptor

and binds to a vitamin D responsive element on a responsive gene, such as that of osteocalcin, calcium binding protein or 24-hydroxylase. This is followed by transcription and translation, generating proteins such as the calcium binding protein or osteocalcin [39]. The classic effect of 1,25(OH)2D on active calcium transport occurs in the intestinal cell. Like the other members of the steroid receptor family, the VDR acts as a ligand-activated transcription factor. The domains of the VDR involved in the major steps for VDR control of gene transcription are [50]: *1*) ligand binding, *2*) heterodimerization with retinoid X receptor (RXR), *3*) binding of the heterodimer to vitamin D response elements (VDREs) in the promoter of 1,25$(OH)_2$D-responsive genes, and *4*) recruitment of VDR-interacting nuclear proteins (coregulators) into the transcriptional pre-initiation complex, which markedly enhance or suppress the rate of gene transcription by the VDR.

1α,25(OH)2D3 is a conformationally flexible molecule and is known to stimulate a wide array of rapid responses (RR); some examples include the rapid intestinal absorption of calcium, secretion of insulin by pancreatic T3-cells, opening of voltage-gated Ca^{2+} and Cl^- channels in osteoblasts, and the rapid migration of endothelial cells. One conformation of 1α,25(OH)2D3 is used for genomic responses, and an alternative one serves as an agonist of rapid responses. It is clear that VDR, DBP, and the receptor for 1α,25(OH)2D3-mediated RR each have a characteristic ligand confromation. Binding of 1α,25(OH)2D3 to the caveolae-associated VDR may result in the activation of one or more second messenger systems, including phospholipase C (PKC), protein kinase C, G protein-coupled receptors, or phosphatidylinositol-3-kinase (PI3K). There are a number of possible outcomes including opening of the

voltage-gated calcium or chloride channels or generation of the indicated second messengers. Some of these second messengers, particularly RAF/MAPK, may engage in cross-talk with the nucleus to modulate gene expression [73].

1.8. Immunological effects of Vitamin D

Vitamin D receptors have been found in most cells of the immune system and $1,25(OH)_2D_3$ is a potent immune system modulator [56]. There is considerable scientific evidence that 1,25(OH)2D has a variety of effects on immune system function that may enhance innate immunity and inhibit the development of autoimmunity [34,,55,94].

1.8.1. Anti-inflammatory actions of Vitamin D

An anti-inflammatory role for vitamin D has been recognised for some time and it has been specifically identified as exerting protection in conditions that are associated with polarization of T-cell responses to the Th1. $1,25(OH)_2D_3$ has been shown to reduce the inflammatory changes associated with delayed hypersensitivity, inflammatory bowel disease and experimental encephalomyelitis as well as with allograft survival [2,9].

$1,25(OH)_2D_3$ promotes the differentiation of helper T cells from Th1 to Th2 reducing the production of interferon γ (IFN- γ), interleukin-2 (IL-2) and

tumour necrosis factor-α (TNF-α) [48]. Th1 cell activation is essential for strong cell-mediated immune responses, including host responses to tumours and pathogens [1]. By inhibiting IFN-γ transcription, the major positive feedback signal for antigen-presenting cells, $1,25(OH)_2D_3$ prevents further antigen presentation to and recruitment of T lymphocytes [49]. IL-2 is an autocrine growth factor for T lymphocytes and inhibition of its expression by $1,25(OH)_2D_3$ prevents their further activation and proliferation. While inhibiting the synthesis of the Th1 cytokines IL-2 and IFN-γ, and thus indirectly inducing $CD4^+$ T lymphocytes to polarize towards a Th2 phenotype, increases the production of Th2 cytokines such as tumour growth factor (TGF) and interleukin 4 (IL-4) [9,49].

As result of the anti-inflammatory properties of$1,25(OH)_2D_3$ and its analogues, these molecules became ackowledged as immune suppressants [2,21].

1.8.2. Vitamin D and infectious diseases

Although, $1,25(OH)_2D_3$ has anti-inflammatory properties, $1,25(OH)_2D_3$ has been shown to have no effect on the susceptibility of mice to infections with herpes simplex virus or *Candida albicans* with doses that had been shown previously to prolong allograft survival [10]. Surprisingly, little is known about the effect of vitamin D status on the ability of the host to fight infections. One experiment in VDR KO mice showed that they mice exhibited increased

granulomatous inflammation (slightly more severe infection) during *Schistosoma mansoni* infection, compared with WT mice [28].

1,25$(OH)_2D_3$ reduced inflammation and parasite load in experiments with *Trypanosoma cruzi* in mice [86] and inhibited *in vivo* and *in vitro* the intracellular growth of *Toxoplasma gondii* [70]. In the 80's, Vial and co-wprkers showed that *P. falciparum* growth is inhibited in the presence of vitamin D [95].

The only example of an explored immune antimicrobial effect of 1,25$(OH)_2D_3$ is with tuberculosis. In the pre-antibiotic era, vitamin D was used for treatment of tuberculosis [53]. 1,25(OH)2D induces anti-mycobacterial activity *in vitro* in both monocytes and macrophages. Exogenous 1,25(OH)2D induces a superoxide burst and enhances phagolysosome fusion in *M. tuberculosis*-infected macrophages. Both phenomena are mediated by phosphatidylinositol 3-kinase, suggesting that this response is initiated by ligation of membrane vitamin D receptor (VDR) [54]. 1,25(OH)2D3 also modulates immune responses by binding nuclear VDR and TLR activation upregulates protective innate host responses, including induction of nitric oxide synthase and cathelicidin, and down-regulates IFN γ gene expression [51].

1.9. Objectives

The main aim of this project is to investigate the involvement of vitamin D during *Plasmodium* infection of a mammalian host. Specifically, we propose to answer the following questions:

a) Is liver infection by sporozoites impaired by the active form of vitamin D?
b) Will the anti-inflammatory properties of vitamin D interfere in the outcome of CM?

To achieve our aims, both *in vitro* and *in vivo* models of malaria infection were used, a hepatoma cell line culture system and a rodent model of CM, respectively. This enabled exploring different biological approaches for addressing the effects of vitamin D in the modulation of *Plasmodium* infection. In view of the dual effects of vitamin D, both anti-inflammatory and antimicrobial, it can be expected that vitamin D may hold prophylactic/therapeutic potential against malaria infection.

2.Material and Methods

2. Materials and Methods

2.1. Cells

Two hepatoma cell lines were used for *in vitro* experiments: HuH7 a human hepatoma cell line [72] and a mouse hepatoma cell line Hepa 1-6 (ATCC, CRL-1830) [18]. Both cell lines are efficiently infected by rodent malaria parasites which are capable of undergoing complete development [67,87]. This was the *in vitro* model for malaria liver stage infection during the experimental studies. Hepatoma cells were maintained and kept in cell incubators at 37˚C with a 5%. Hepa1-6 cells were maintained in Dubelco's MEM medium (DMEM, Invitrogen) supplemented with 10% heat inactivated Foetal Calf Serum (FCS)(Gibco/Invitrogen) and 1% Penicilin-Streptomycin (PS) (Gibco/Invitrogen). Huh7 were maintained in RPMI medium (RPMI 1640/Invitrogen) supplemented with 10% FCS (Gibco/Invitrogen), 1% PS, 1% Hepes (Gibco/Invitrogen) and 1% Non Essential Anino Acids (NEAA) (Gibco/Invitrogen). For Lentiviral production, HEK293T human embryonic kidney cells were maintained and kept in cell incubators at 37˚C with a 5%. Hepa1-6 cells were maintained in Dubelco's MEM medium (DMEM, Invitrogen) supplemented with 10% heat inactivated Fetal Calf Serum (FCS) (Gibco/Invitrogen) and 1% Penicilin-Streptomycin (PS) (Gibco/Invitrogen).

Cells were periodically tested for mycoplasma infections, as previously described [91].

2.2. Mice, mosquitos and parasites

C57/BL6 mouse strains aged between 6-8 weeks were used as a cerebral malaria model [83] and were supplied by Instituto Gulbenkian de Ciência's (IGC) animal house. Animals were bred, maintained and experimentally used in a pathogen-free animal facility. Mice were feed with RM3 food (SDS-England) with a vitamin D content of 4000UI/Kg. All animal care and procedures were in accordance with European regulations.

Anopheles stephensi infected mosquitos were supplied by the University Medical Center St. Radbound (Nijmegen, The Netherlands) and our own *insectarium* and maintained under adequate controled conditions until being used.

Plasmodium berghei ANKA sporozoites expressing GFP [25] were obtained from infected mosquitoes and were used either *in vitro* or *in vivo* experiments. Parasites were maintained by alternate cyclic passages in mosquitos and mice [92].

2.3. Sporozoite isolation and purification

Female *Anopheles staphensi* mosquitos were fed on infected *Plasmodium berghei* ANKA mice. Sporozoites were obtained by dissection from their salivary glands at days 18-21 post feeding. Dissections of mosquito salivary glands were performed in RPMI medium (Gibco, Invitrogen). The

glands were mechanically disrupted and homogenized to free the parasites. The debris were pelleted after spinning at 20g for 5 min at 4˚C. Sporozoites were then collected, counted and maintained on ice until use. The number of sporozoites per infected mosquito was determined using a hemacytometer (Newbauer chamber).

2.4. Lentiviral production and cell transduction

To knockdown the expression o the vitamin D receptor (VDR) an incompetent lentivirus was produced by transfection of a packaging cell line with the lentiviral genome split in different plasmids [63,75]. Lentiviral particles were produced by cotransfection of pLKO.1 puro plasmid (Fig. 2.1.) with the packaging construct pCMV Delta 8.9, an envelope coding plasmid pCMV-VSV-G. This method allow persistent expression of small RNA molecules that will target specific gene mRNA degradation, named RNA interference (RNAi); and resistance to puromycin to the transduced cells [88].

In a 96 well plate, 2,6X10^4 HEKC 293T cells were seeded in a 96 well plate 24 hours before transfection. Transfection was performed by mixing 100 ng of pCMV-VSV-G, 10ng pCMV Delta 8.9 and 10ng VDR-pLKO.1 or pLKO.1 for controls (table 2.1.) plasmids and 0,6µL of Fugene 6 (Roche) with OptiMEM (Invitrogen) in a total volume of 50µl. After 30 min. of incubation the mixture was added to the 60%-80% confluent cells. Supernatants with lentiviral particles were collected every 8/16 hours for 48 hours and then aliquoted and stored in -80˚C.

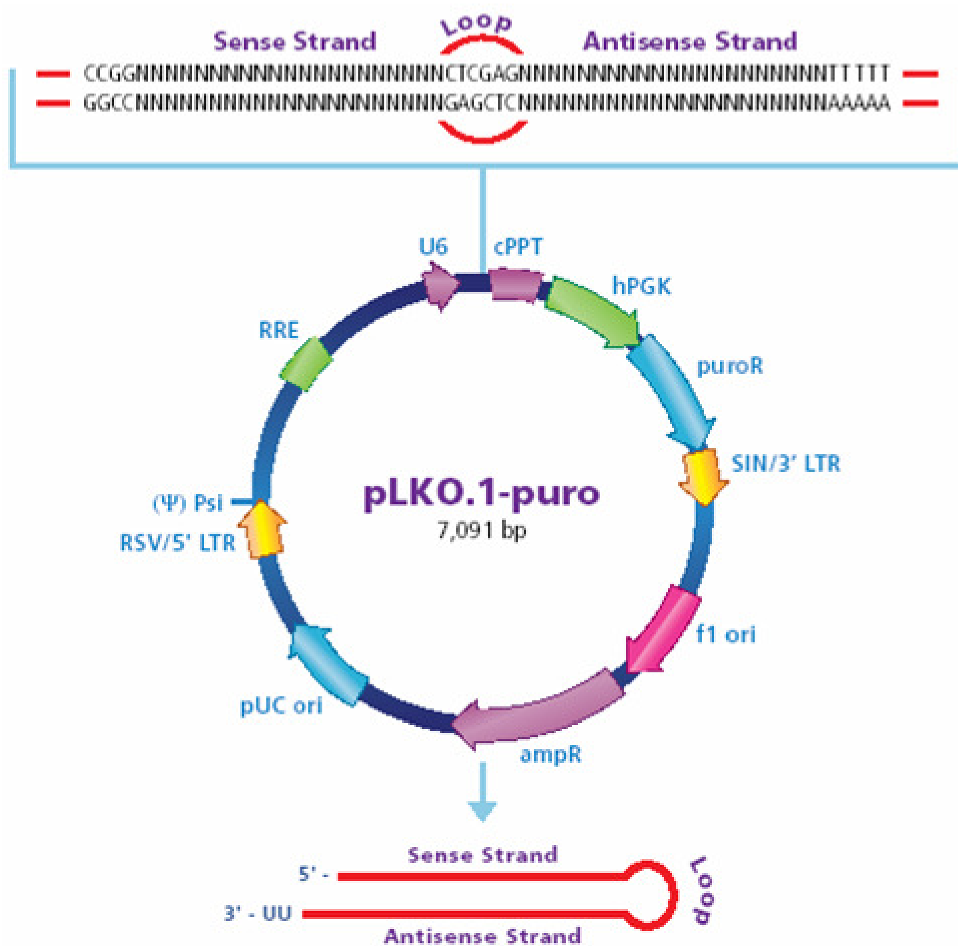


Figure 2.1. pLKO.1-puro vector. Description and features: cppt - Central polypurine tract; hPGK - Human phosphoglycerate kinase eukaryotic promoter; puroR - Puromycin resistance gene for mammalian selection; SIN/LTR - 3' self inactivating long terminal repeat; f1 ori - f1 origin of replication; ampR - Ampicillin resistance gene for bacterial selection; pUC ori - pUC origin of replication; 5' LTR - 5' long terminal repeat; Psi - RNA packaging signal; RRE - Rev response element.

Transduction of Huh7 cells were performed by seeding 24 hours before $3x10^4$ cells in 96 well plates. In the day of transduction, lentivirus aliquots were thawed in ice for 30 min. After collection of the cell media, 10µL of supernatant in 40µL of media and polybrene (Sigma) was added to the cells. Cells were then centrifuged at 2200 rpm, 37˚C for 90 min., after centrifugation, the mixture with the lentivirus was removed and fresh media was added to the cells. Cells were incubated in the cell incubator for 36hours at 37˚C with 5% CO^2. Transduced cells were then selected by adding 12,5 µg/mL of puromycin (Sigma) for 36hours at 37˚C with 5% CO^2. Cells were the expanded and used for *Plasmodium* infection assays.

2.5. In vitro *cell infections and treatment with1,5α-dihidroxivitamin D*

Three to one day before infection, $1,5x10^5$ Huh7 cells or $2x10^5$ Hepa1-6 cells were seeded in 24 well plates with the cell line respective medium and different concentrations of 1,5α-dihidroxivitamin D (Sigma) for control DMSO (Sigma) was added and grow in the respective media. *P. berghey* sporozoites, were added to the Huh7 and Hepa1-6 cell, $3x10^4$ and $5x10^4$ respectively. Cells were centrifuged for 5 min. at 1800g and incubated at 37˚C with 5% CO^2. After 24 hours cells were washed processed either for immunochemistry, flow cytometry analysis or real-time PCR for quantification of *P. berghei* ANKA infection.

2.6. Immunochemestry

For quantification of *in vitro* infection by immunochemestry, after incubation with *P. berghei* ANKA sporozoites cells were fixed with a 2% PFA for 30 min. After fixation, cover slips containing infected cells were incubated with a protein blocking solution (3% Bovine Serum Albumin, 100mM Glycine, 10% FCS) to avoid unspecific reaction, containing 0.1% Saponin (Sigma) for permeabilization, for 30 minutes, rinsed twice shortly in PBS, and incubated 45 minutes RT with a monoclonal antibody against anti HSP70 (also known as 2E6, produced by a hybridoma cell line). After smooth rinsing of the coverslips to remove exceeding mAb, they were incubated with mAb Cy2 Alexa Fluor® 488 for mice IgG (dilution 1:2000) for 45 minutes a room temperature. Following, removal of exceeding mAb. Then, the coverslips are mounted in glass slides, with a mounting medium, Mowiole and then observed in a Fluorescence Microscope. Infection rate was quantified by counting the total number of parasite infected cells per cover slip.

2.7. Migration assay

Plasmodium migration assay was performed as describe elsewhere with some adaptations [65]. Huh7 cells were seeded 24 hours before infection $75x10^3$ cells for each 24 well plate. Before adding $30X10^3$ sporozoites cells were washed and was added 200µL of 50mg of Dextran (Sigma) diluted in media to the cells. During 2 hours the added sporozoites were allow to migrate through

the cells that able Dextran dye to enter in the cells. Cells were then washed with PBS trypsinized, washed again by centrifugation, ressuspended in PBS. The percentage of Dextran positive cells were recorded by flow cytometry .

2.8. Flow cytometry

After incubation with *P. berghei* ANKA sporozoites cells were detached by trypsinization, washed and pelleted in a solution of 20% FCS and ressuspended in 10%FCS in PBS before flow cytometry analysis. Not infected cells were used has control. EEfs sizes was accessed by GFP intensity in GFP positive cells [79].

In vivo peripheral blood was collected by tail bleeding and diluted in PBS with heparin (50U/mL). The percentage of GFP *P. berghei* infected cells was monitored over time from days 3 pos-infection until the end of the experiment.

2.9. Quantitative Real-time PCR (qRT-PCR)

After incubation with *P. berghei* ANKA sporozoites cell RNA was extracted using RNeasy Micro Kit (Qiagen) *in vitro* or RNeasy Mini Kit (Qiagen) for livers, using the supplier instructions. One microgram of RNA from each sample was then used to produce cDNA by reverse transcription using the First Strand cDNA Synthesis Kit (Roche). The synthetized cDNA was used to amplify *P. berghei* 18S rRNA or VDR mRNA and HPRT (Table 2.1.) using SYBRgreen (Power SYBRgreen, Applied Biosystems). Amplification was performed in

Corbertt RG6000 (Corbett Life Sciences). Control plasmids with standard concentrations of 18S rRNA gene, from 10^2 to 10^8 copies were used in the assay and were synthesized as described elsewhere. A similar qRT-PCR was performed for a house-keeping gene, Hypoxanthine Guanine Phosphoribosyltransferase (HPRT), for standardization of values obtained (Witney, 2001). For VDR gene serial dilutions were used to give a relative concentration curve.

Table 2.1. Hairpin and primer sequences used for qRT-PCR and iRNA

Name	Sequence
hVDR hp 1	5' - CCGGCGAAGTGTTTGGCAATGAGATCTCGAGATCTCATTGCCAAACACTTCGTTTTT - 3'
hVDR hp 2	5' - CCGGGTCATCATGTTGCGCTCCAATCTCGAGATTGGAGCGCAACATGATGACTTTTT - 3'
hVDR hp 3	5' - CCGGCCTCCAGTTCGTGTGAATGATCTCGAGATCATTCACACGAACTGGAGGTTTTT - 3'
hVDR hp 4	5' - CCGGCCCTGGAGACTTTGACCGGAACTCGAGTTCCGGTCAAAGTCTCCAGGGTTTTT - 3'
hVDR hp 5	5' - CCGGCGCGTCAGTGACGTGACCAAACTCGAGTTTGGTCACGTCACTGACGCGTTTTT - 3'
hHPRT For	5’ – TGC TCG AGA TGT GAT GAA GG – 3’
hHPRT Rev	5’ – TCC CCT GTT GAC TGG TCA TT – 3’
mHPRT For	5’ – GTA ATG ATC GTC GTC AAC GGG GGA – 3’
mHPRT Rev	5’ – CCA GCA AGC TTG CAA CCT TAA CCA – 3’
Pb 18S For	5’ – GGA GAT TGG TTT TGA CGT TTA TGT G – 3’
Pb 18S Rev	5’ – AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC – 3’
mVDR For	5’ – CTC CTC GAT GCC CAC AAG ACC TAC G – 3’
mVDR Rev	5’ – GTG GGG CAG CAT GGA GAG CGG AGA CAG – 3’
hVDR For	5’ – CCA GGA TAA GAT CCG GCT GG – 3’
hVDR Rev	5’ – AAA GTA GCG AGA GGC ACC AA – 3’

h: human; m:mouse; VDR: Vitamin D Receptor; Pb: *Plasmodium berghei*; Rev: Reverse; For: Forward;

2.10. In vivo *liver infections*

Mice pretreated with 1,5α-dihydroxivitamin D or vegetal oil (Sigma) alone was infected with $2x10^4$ sporozoites by intravenous injection. Livers were dissected 40 hours post infection, and immediately suspended in 4 ml volume of

Denaturing Solution (4M Guanidium Thiocyanate, 25mM Sodium Citrate pH 7, and 0.5% N-Laurosyl-sarcosine) plus 0.1M β-mercaptoethanol, and stored at 4ºC until homogenization. Livers were homogenized with a Tissue Tearer bladder. Liver homogenates were aliquoted (3×300μl per sacrificed animal) and stored at -80ºC (Witney, 2001). Liver homogenates were then used to quantify *P. berghei* liver infection by qRT-PCT (Section 2.8.)

2.11. In vivo *blood infections and disease assessment*

One mouse per experiment was infected with *P. berghei* infected blood from -80˚C vials (passage mouse). When the mouse had a 1-10% parasitemia, it was sacrificed and blood collected by heart puncture. Blood was washed 3 times with PBS (centrifuging at 1699g at 4˚C for 3 min.), and then diluted in PBS to achieve 10^5 infected erythrocytes per 200μL. The mice for blood stage experiments were then infected with the diluted blood by intra peritoneal injection with a dose of 10^5 *P. berghei* infected erythrocytes. Parasitemias were monitored by flow cytometry as described above. Infected mice were monitored twice daily for clinical symptoms of CM including hemi- or paraplegia, head deviation, tendency to roll over on stimulation, ataxia and convulsions.

2.12. Statistical analysis

Analyses were performed using the unpaired Student's *t* parametric test or analysis of variance (ANOVA) parametric tests. Normal distributions were confirmed using the Kolmogorov-Smirnov test. To make multiple comparisions versus control group was used the Holm-Sidak method. The tests were statistically significant when $p \leq 0{,}05$. Correlations were made using the Pearson's correlation coefficient.

3. Results

3. Results

3.1. Liver stage

3.1.1. Vitamin D effect in *P. berghei in vitro* infection

To study the effect of the active form of vitamin D (1,5$(OH)_2D_3$) on hepatoma cell lines (Hepa1-6 and Huh7) we made a preliminary assay by treating Huh7 with 10^{-6} of the tested drug against a solvent treated control for 48 hours. After 24hours $20x10^3$ sporozoites were added to cells. After another 24hours of incubation cells were fixed and stain agains *P. berghey*, cell actin and nucleus. Total EEFs were count by fluorescent microscopy (Fig. 3.1.1.).

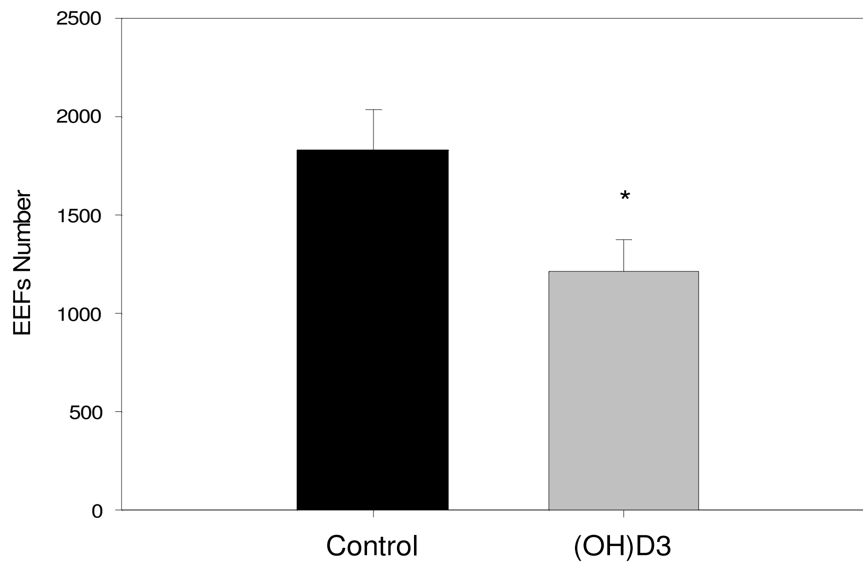


Figure 3.1.1. – Effect of 1,25$(OH)_2D_3$ on *Plasmodium* sporozoite infection of Huh7 cells. 10^{-6}M of 1,25$(OH)_2D_3$ was added or with DMSO (control) to Huh7 cells. After 24 hours of incubation $20x10^3$ sporozoites were added to cells. Infection was quantified 24 hours pos-infection by counting the total number of infected cells (EEFs) per well (average±stdev; t-test, $p \leq 0,05$).

The active form of vitamin D reduced the number of EEFs in comparison to the control cells by around 35%.

To address if the difference observed between treatments was associated with an effect during migration phase of the parasite a migration assay with dextran was performed (Fig. 3.1.2.).

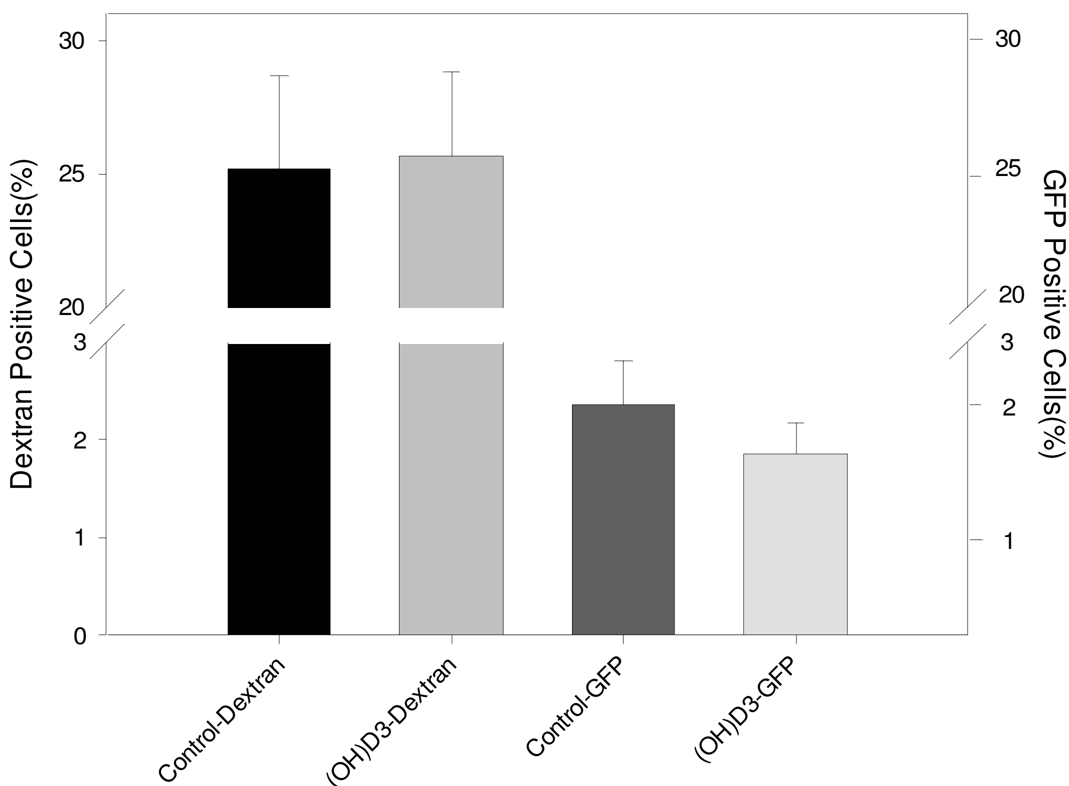


Figure 3.1.2. – Quantification of dextran positive (transversed) cells in both control (DMSO) and 1,5$(OH)_2D_3$ treated cells and GFP positive cells 2 hours after adding $5x10^4$ *P. berghei* sporozoites (average+stdev; t-test; *p*>0,05).

To address if this effect was cell line specific we used mouse Hepa1-6 cell line using the same protocol. Flow cytometry and quatitative real-time PCR (qRT-PCR) was used to quantify *P. berghei* infection.

Both cell lines were susceptible to 1,25-$(OH)_2D_3$ with less infected cells. However Huh7 cells show a less striking effect in the percentage of infected cells. The same tendency was seen when the number of *P. berghei* copies versus HPRT was quantify but with no statistical differences (Fig. 3.1.3.).

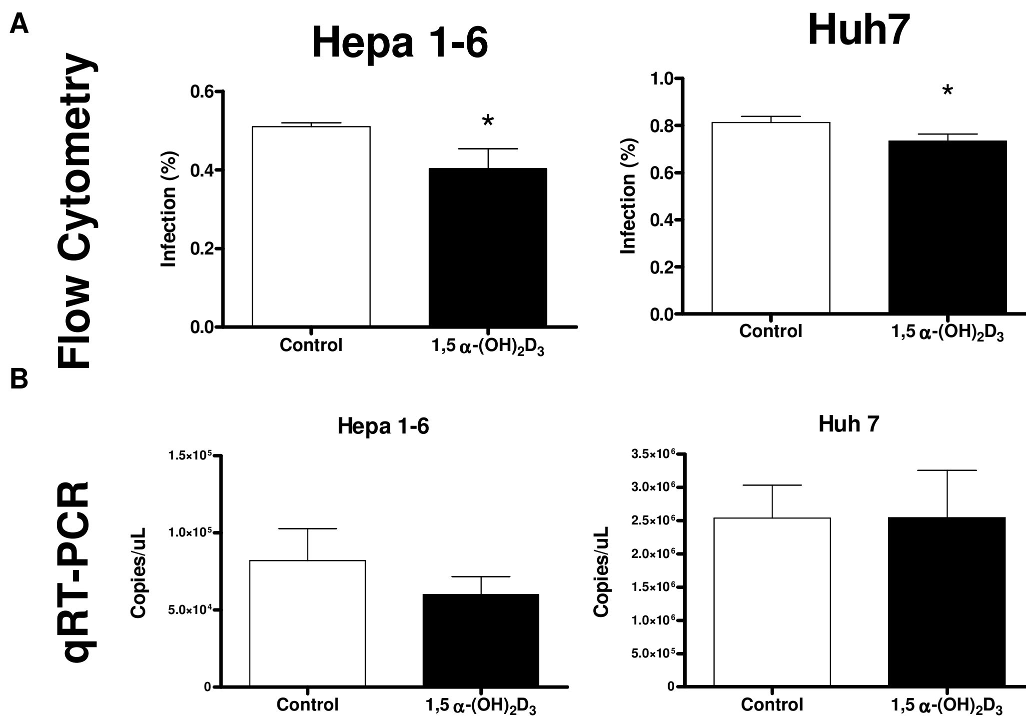


Figure 3.1.3. – Quantification of *P. berghei* infection in mouse (Hepa1-6) and human (Huh7) hepatoma cell lines. Infection of 1,25-$(OH)_2D_3$ treated and untreated (control) cells was quantified 24 hours pos-infection by (A) flow cytometry and (B) qRT-PCR (average+stdev; t-test, * $p \leq 0,05$)

Parallel to the former experiment, Huh7 and Hepa1-6 hepatoma cells were treated with different concentration of 1,25-$(OH)_2D_3$ to see if the effect of the reduction in parasite number was dependent in drug concentration. However, real-time PCR didn't show again any differences between control samples and samples with different concentration of 1,25-$(OH)_2D_3$ (Fig. 3.1.4.).

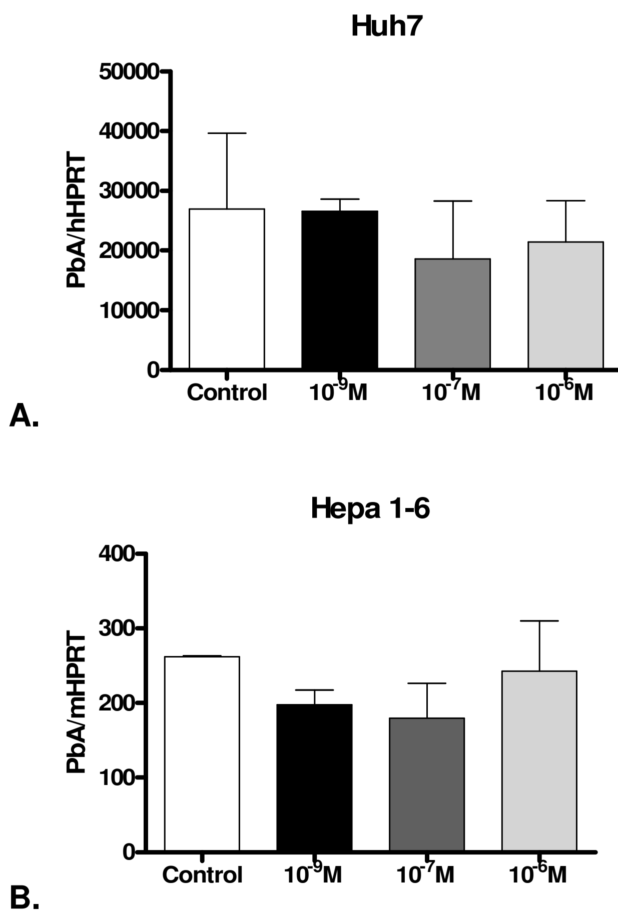


Figure 3.1.4. – Titration of 1,25-$(OH)_2D_3$ in Huh7 and Hepa1-6 hepatoma cells. Cells were treated with different drug concentrations. In both treated and untreated cells, infection was measured 24 hours pos-infection. The Y axis is in arbitrary units (mean±stdev)

This difference between flow cytometry and real-time PCR could be explained if somehow the few number of the EEFs present in the 1,25-$(OH)_2D_3$ treated samples had more parasites (and so, parasite DNA copies). Acessment of EEFs size by GFP intensity by flow cytometry analysis no differences were observed between the EEFs of treated and untreated samples (Fig. 3.1.5.)

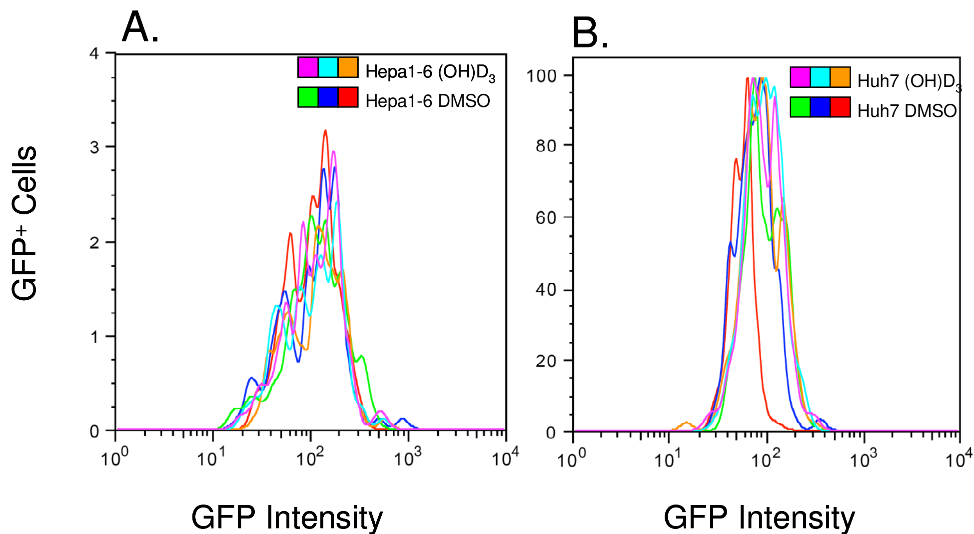


Figure 3.1.5. EEFs size acessment by GFP intensity: A- Hepa1-6 hepatoma cells; B- Huh7 hepatoma cells.

To study whether the knock down of the Vitamin D receptor influences the infection of *P. berghei* Huh7 cells were transduced with lentivirus this method allow persistent expression of interfering RNAs targeting the VDR mRNA leading to a knock down of this gene. Lentiviral transduction with the hairpins 1, 2, 3 and 4 reduced the percentage of infection compared with the control (ANOVA;Holm-SidaK; $p \leq 0,05$) (Fig. 3.1.6). This result suggest an important role of VDR in *P. berghei* infection. However it was not possible to quantify the VDR knock down and this way correlate the differences in infection with the level of knock down.

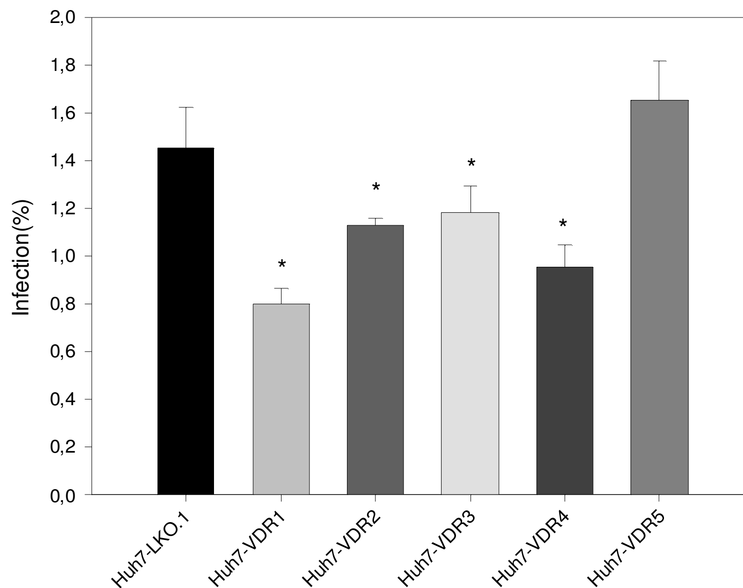


Figure 3.1.6. Comparison between the percentage of *P. berghei* infection in different Huh7 hepatoma cell lines transduced with different VDR targeted hairpins (ANOVA; Holm-Sidak; $p \leq 0,05$)

3.1.2. Effect of vitamin D in *P. berghei* liver infection

In vivo, C57BL/6 were injected intra peritonially (IP) with 0,5µg/Kg on day 3 and 1 before infection with $20x10^3$ sporozoites intravenously (IV) by caudal vein. After 40 hours livers were collected and *P. berghei* infection by qRT-PCR. Mice treated with 1,25-$(OH)_2D_3$ show a tendency to have less DNA copies of *P. berghei*, this tendency is, however, not statistically significant different (t-test; $p>0,05$) between 1,25-$(OH)_2D_3$ treated and untreated mice (Fig. 3.1.6.).

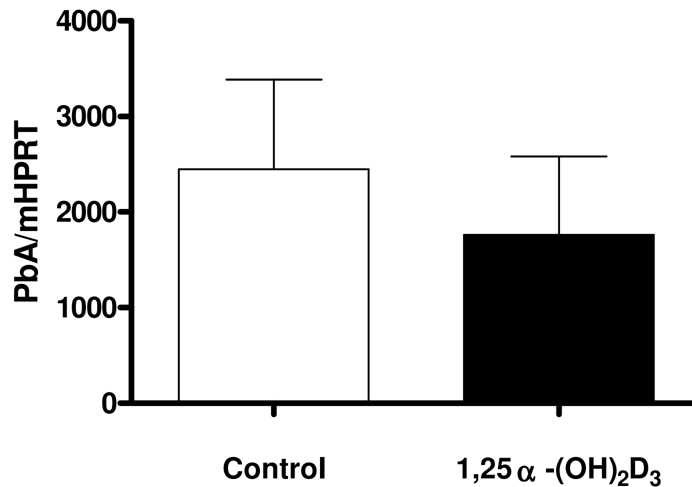


Figure 3.1.6. – Quantification by qRT-PCR of *P. berghei* infection in livers of mice injected IP with 0,5µg/Kg or vehicle (control) of 1,25-$(OH)_2D_3$ on day 3 and 1 before infection. Infection was quantified 40 hours after infection. (average±stdev;n=5; t-test; p>0,05)

A second experiment was made, this time using a higher dose (5µg/kg) delivered by gavage for longer alternate days, 15 days before infection. After infection livers were processed using the same methodology quantifying *P. berghei infection* and Vitamin D Receptor (VDR) both in treated and untreated mice (Fig. 3.1.7.A). Treated mice showed a tendency to have higher *P. berghei* DNA copy numbers, this tendency was not statistically significant (t-tet; *p*>0,05). However, the Vitamin D Receptor (VDR) expression was higher in 1,25-$(OH)_2D_3$ pre treated mice livers (t-test, p≤0,05) (Fig. 3.7.B) showing that this dose of 1,25-$(OH)_2D_3$ had an effect in VDR number in the mice's livers. Mice with higher expression of VDR had a positive correlation with higher number of *P. berghei* copies (Pearson correlation, r^2=0,4337)(Fig. 3.1.7.C).

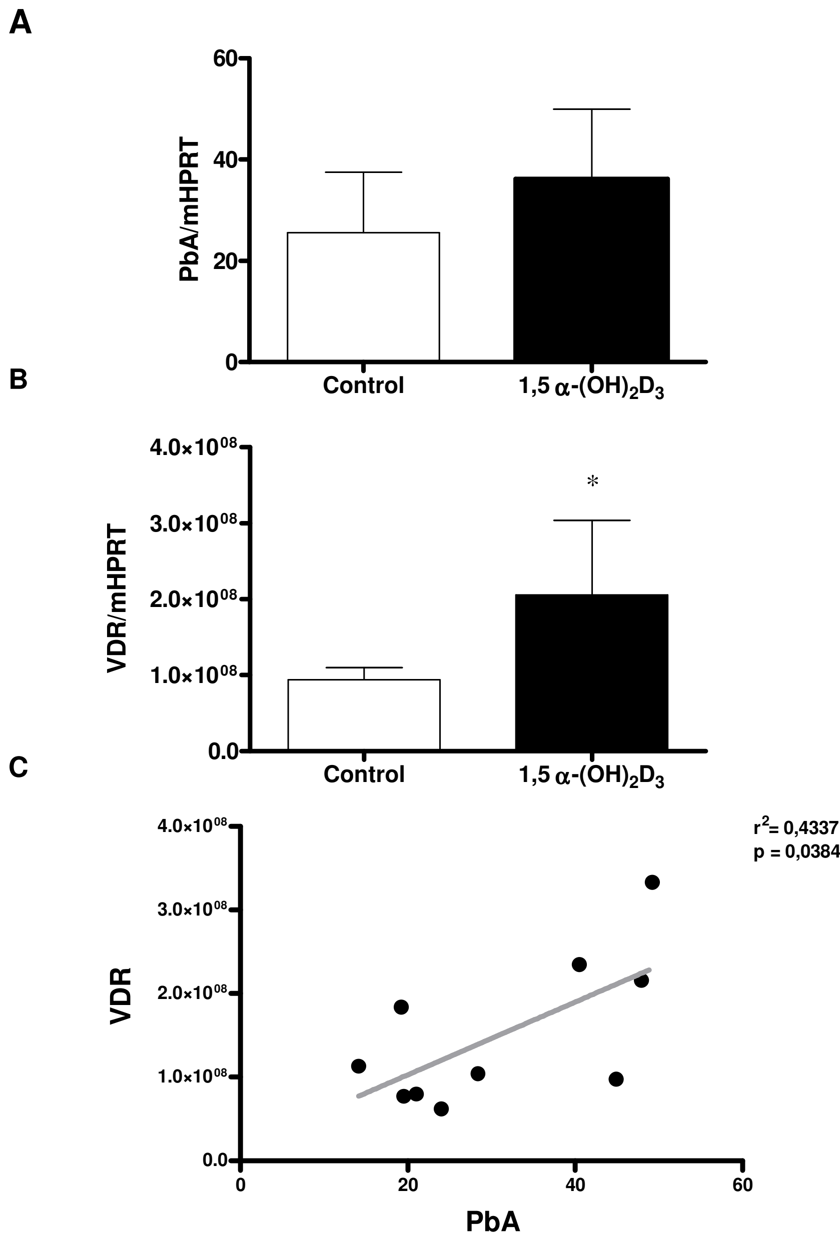


Figure 3.1.7. – Effect of 1,25-$(OH)_2D_3$ in *in vivo* infection of *P. berghei* in livers of mice treated by gavage for 15 alternately with 5µg/kg (average+stdev;n=5) **A**; this dose raised the Vitamin D Receptor (VDR) numbers in treated mice (average+stdev;n=5;t-test; p<0,05) **B**; the increase of VDR copy number was correlated with *P. berghei* copy number (Pearson; r^2=0,4337) **C**.

3.2. Blood stage

3.2.1 Vitamin D effect in a Cerebral Malaria (CM) model

To study the possible effect of Vitamin D (1,25-$(OH)_2D_3$) in a CM model C57/Bl6 mice were pre-treated different doses of 1,25-$(OH)_2D_3$ infected with 10^5 *P. berghei* infected red blood cells by vein injection and parasitemia (Fig. 3.2.1.) and survival rates (Fig. 3.2.2.) were observed.

The peripheral parasitemia of the three different mice groups was not different between the different treatment compared with the controls (t-test; $p>0,05$) and all mice died between day 7 and day 10 pos-infection with neurological symptoms including hemi- or paraplegia, head deviation, tendency to roll over on stimulation, ataxia and convulsions. These findings suggest us no influence of the administration of 1,25-$(OH)_2D_3$ in the peripheral blood parasitemias nor in the CM. Again a more controlled designed experiment with a vitamin D depleted diet and monitoring the circulating titers of the vitamin D forms would give as a more conclusive result.

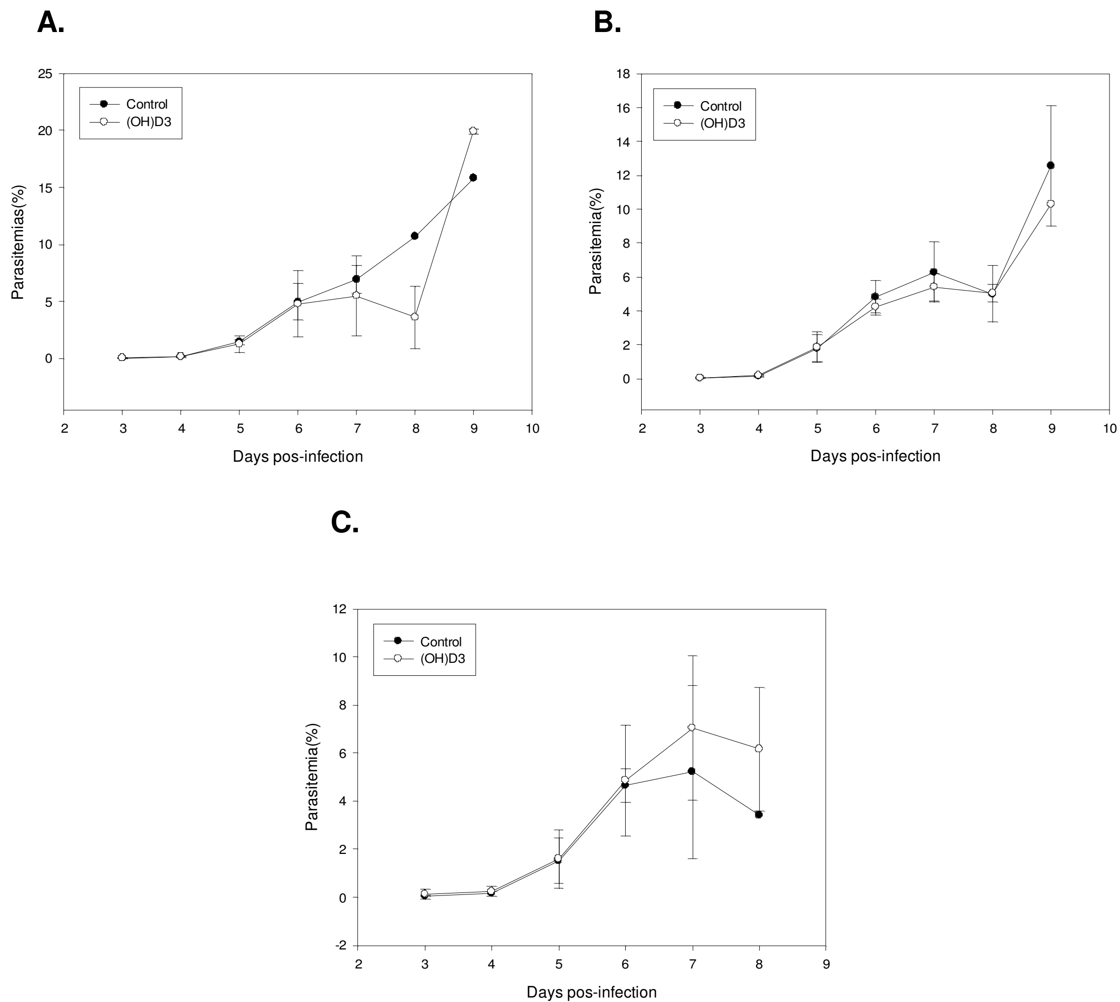


Figure 3.2.1. – Parasitemia of both 1,25-$(OH)_2D_3$ treated and untreated C57/Bl6 with different doses and routes of administration. Mice were injected IP with 10^5 *P. berghei* infected red blood cells, and parasitemia was monitored by flow cytometry from day 3 pos-infection until death. **A.** Mice treated with 2,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by gavage; **B.** Mice treated with 2,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by intra peritoneal injection; **C.** Mice treated with 0,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by intra peritoneal injection.

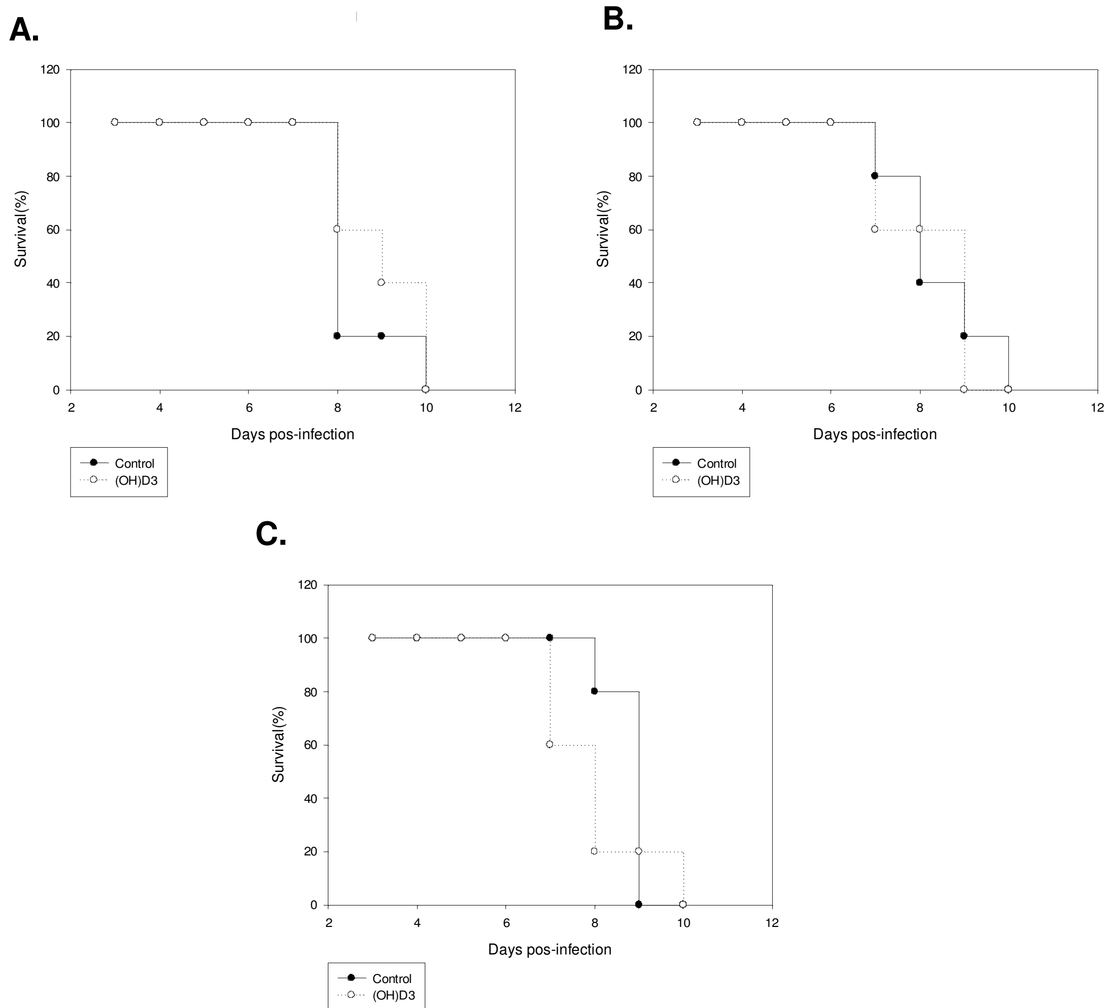


Figure 3.2.2. Comulative survival curves of both 1,25-$(OH)_2D_3$ treated and untreated C57/Bl6 with different doses and routes of administration. Mice were injected IP with 10^5 *P. berghei* infected red blood cells, survival and acute cerebral malaria syndromes was monitored. **A.** Mice treated with 2,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by gavage; **B.** Mice treated with 2,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by intra peritoneal injection; **C.** Mice treated with 0,5µg/Kg of 1,25-$(OH)_2D_3$ or vehicle only (vegetal oil) administered by intra peritoneal injection.

4. Discussion

4. Discussion

Vitamin D has a well known role in the regulation of calcium homeostasis. More recently, vitamin D has become a hot topic in immune regulation. The vitamin D and particularly its active form 1α-25-dihydroxyvitamin D_3, has showed anti-inflammatory and paradoxically antimicrobial properties. The innate immune responses provide a rapid host mechanism for defence against microbial pathogens. Activation of Toll-like receptors induces expression of antimicrobial peptides and this expression is directly link to vitamin D.

In this study, we took advantage of the present knowledge of vitamin D as an immune modulator and proposed to study the effect of vitamin D in the outcome of the *Plamodium berghei* infection in both the liver stage and in the outcome of cerebral malaria in a murine model.

4.1. Liver stage infection

4.1.1. *In vitro P. berghei* infection

A reduced number of infected hepatoma cells were observed in the presence of 1,25α-dihydroxyvitamin D (1,25α(OH)D3) by immunichemestry and flow cytometry. However, when the infection was quantified by qRT-PCR no differences was found. This could be possible if the drug reduce *P. berghei* invasion but promote intracellular development. No differences in EFF size was found to support this hypothesis. These differences might be due to some artefact associated by these techniques. The genomic analysis by witch hepatoma Huh7 cells were transducer with lentivirus encoding a hairpin to

produce iRNA targeting human Vitamin D Receptor (VDR) had less EEfs than the cells transduced with a lentivirus without any hairpin. However, it was impossible to correlate the level of VDR knock down with this decrease of in the number of EEFs.

To successfully infect a liver cell *Plasmodium* migrates trough several hepatocytes and then invades a final hepatocyte were it develops in thousands of merozoites [69]. Our results, that can be considered as preliminary, show that the active form of the vitamin D may have a detrimental effect in the invasion or development of *P. berghei* of hepatocytes. More controlled experiments should be made, particularly the effect of the drug directly in the parasite and in the development of the EEFs inside the hepatocytes.

4.1.2. *In vivo P. berghei* infection

In vivo, both small doses of 1,25α(OH)D_3 given in small periods of time and higher doses given for larger periods had no effect in *P. berghei* liver infection. A tendency in the results could be observed were small doses of 1,25α(OH)D_3 administered in a short period of time reduced the number of *P. berghei* DNA copies, in mice livers whether bigger amounts administered for longer was found a tendency for an increased of *P. berghei* DNA copies compared with controls. Also, mice treated with higher amounts of 1,25α(OH)D_3 had higher expression VDR and this expression was significantly correlated with higher copies of *P. berghei* in the mice livers. This has been already described for *Toxoplasma gondii* in which 1,25α-(OH)D_3 enhanced mice susceptibility to toxoplasmosis, however reducing it's development [80]. A host inflammatory

response seems to be detrimental to *Plasmodium* liver infection [93,27]. Hosts models with less pronounced inflammatory responses are more susceptible to liver infection and induction of liver inflammatory response by *P. berghei* reduces liver greatly reduces *P. yoelii* liver infection. The anti-inflammatory effect that 1,25α(OH)D_3 induces may be promoting *P. berghei* invasion and/or development.

To clearly define *in vivo* relationship between Vitamin D and *P. berghei* liver infection a better experimental design should be used, such as comparing *P.berghei* liver infection in both mice fed in depleted and complete vitamin D and also quantifying circulating titters of vitamin D forms.

4.2. Blood stage infection

Understanding the pathogenesis of malaria infection is crucial for the development of more efficient clinical interventions. Sequestration of infected erythrocytes and leukocytes and the inflammatory response triggered by malaria infection are thought two key events in the pathogenesis of cerebral malaria [14].

From the Vial and co-workers report 1,25α(OH)D_3 was an "effective inhibitor of the *in vitro* intraerythrocytic growth of *Plasmodium falciparum*" they showed that this drug had schizonticidal action [95]. When we proposed this study, we were expecting some effect of 1,25α(OH)D_3 on peripheral blood parasitemias *in vivo*. In this work, was not possible to test the effect of 1,25α(OH)D_3 in *in vitro P. berghei* blood cultures, it would be very interesting to test this hypothesis.

Vitamin D has been suggested to affect the balance between T helper Th1 and Th2 type cytokines by favouring Th2 domination [48].This inhibition of pro-inflammatory and boost of anti-inflammatory make as think that 1,25α(OH)D_3 would have a beneficial effect in the outcome of experimental cerebral malaria. Our results don't show differences between the different doses of 1,25α-(OH)D_3 or between different routes of administration both in parasitemia nor in CM incidence, all mice died in the cerebral malaria window and with similar parasitemias.

5. Conclusions

5. Conclusions

The results presented in this work suggest that vitamin D, particularly its active form 1,25α(OH)D_3, may have some influence in the liver phase infection of *Plasmodium Berghei*. More controlled experiments and bigger sample size, specially *in vivo*, may elucidate the existence or not of a Vitamin D Receptor-vitamin D influence in *P. berghei* hepatocytes infection. 1,25α(OH)D_3 did not affected the parasitemias nor the outcome of cerebral malaria suggesting that vitamin D supplementation has no effect on *P. berghei* blood stage infection.

6. References

6. References

1. Abbas, A.K. and Lichtman, A.H. 2004. *Basic Immunology: Functions and Disorders of the Immune System.* 2nd Edition: Philadelphia: Sauders.

2. Adorini, L. 2005. Intervention in autoimmunity: the potencial of vitamin D receptor agonists. *Cell Immunology*. **233**(2):115-124.

3. Alano, P. 1990. Sexual differentiation in malaria parasites. *Annual Review of Microbiology*. **44**:429-449.

4. Alonso, P.L. 2006 Malaria: deploying a candidate vaccine (RTS.S/AS0"A) for an old scourge of humankind. *International Microbiology*. **9**(2):83-93.

5. Amino, R., Ménard, R. and Frischknecht, F. 2005. *In vivo* imaging of malaria parasites – recent advances and future directions. *Current Opinion in Microbiology*. **8**:407-414.

6. Beeson, J.G. and Brown, G.V. 2002. Pathogenesis of Plasmodium falciparim malaria: The roles of parasite adhesion and antigenic variation. *Celular and Molecular Life Sciences*. **59**(2):258-271.

7. Brown, A.J., Dusso, A. and Slatopolsky, E. 1999. Vitamin D. *The American Journal of Physiology*. **277**(2 Pt 2):F157-F175.

8. Bruce-Chwatt, L.J. 1987. Malaria and is control: Present situation and future prospects. *Annual Review of Public Health*. **8**:75-110.

9. Cantorna, M.T. 2006. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. *Progress in Biophysics and Molecular Biology*. **92**(1):60-64.

10. Cantorna, M.T., Hullett, D.A., Redaelli, C., Brandt, C.R., Humpal-Winter, J., Sollinger, H.W. and DeLuca, H.F. 1998. 1,25-dihydroxyvitamin D3 prolongs graft survival without compromising host resistance to infection or bone mineral density. *Transplantation*. **66**(7):828-831.

11. Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigário, A.M., Silva, S., Leirião, P., Carapau, D., Armas-Portela, R., Comoglio, P.M., Rodriguez, A. and Mota, M.M. 2003. Hepatocyte growth factor and its receptor are required for malaria infection. *Nature Medicine*. **9**(11):1363-1369.

12. Casals-Pascual, C. and Roberts, D.J. 2006. Severe malarial anaemia. *Current Molecular Medicine*. **6**(2):155-168.

13. Christakos, S., Dhawan, P, Liu, Y., Peng, X. and Porta, A. 2003. New insights into the mechanisms os vitamin D action. *Journal of Cellular Biochemistry*. **88**(4):695-705.

14. Clark, I.A., Cowden, W.B. And Rockett, K.A. 1994. The pathogenesis of human cerebral malaria. *Parasitology Today*. **10**(11):417-418.

15. Clark, I.A. and Rockett, K.A. 1994.The cytokine theory of human cerebral malaria. *Parasitology Today*. **10**(10):410-412.

16. Cogswell, F.B. 1992. The hypnozoite and relapse in primate malaria. *Clinical Microbiology Reviews*. **5**(1):26-35.

17. Cunha-Rodrigues, M., Prudêncio, M., Mota, M.M. and Haas, W. 2006. Antimalarial drugs – host targets (re)visited. *Biotechnology Journal*.**1**(3):321-332.

18. Darlington, G.J., Bernhard, H.P., Miller, R.A. And Ruddle, F.H. 1980. Expression of liver phenotypes in cultured mouse hepatoma cells. *Journal of the National Cancer Institute*. **64**(4):809-819.

19. Demay, M.B. 2006. Mechanism of vitamin D receptor action. *Annals of the New York Academy of Sciences*. **1068**:204-213.

20. del Portillo, H.A., Lanzer, M., Rodriguez;Malaga, S., Zavala, F. And Fernandez;Bacerra, C. 2004. Variant genes and the spleen in Plasmodium vivax malaria. *International Journal of Parasitology*. **34**(13;14):1547;1554.

21. DeLuca, H.F. and Cantorna, M.T. 2001. Vitamin D: its role and uses in immunology. *FASEB*. **15**(14):2579-2585.

22. Eelen, G, Verlinden, L., De Clercq, P., Vandewalle, M., Bouillon, R. and Verstuyf, A. 2006. Vitamin D analogs and coactivators. *Anticancer Research*. **26**(4A):2717-2721.

23. Etkin, L. 2003. The Co-evolution of people, plants and parasites: biological and cultural adaptation to malaria. *Proceedings of Nutrition Society*. **62**:311-317.

24. Feldman, D., Krishnan, A., Moreno, J., Swami, S., Peehl, D.M. and Srinivas, S. 2007. Vitamin D inhibition of the prostaglandin pathways as therapy for prostate cancer. *Nutrition Reviews*.**65**(8 Pt2): S113-115.

25. Franke-Fayard, B., Trueman, H., Ramesar, J. Mendoza, J., van der Keur, M., na der Linden, R., Wanters, A.P. And Janse, C.J. 2004. A *Plasmodium berghei* reference line that constitutively express GFP at a high level throughout the complete life cycle. *Molecular and Biochemical Parasitology.***137**(1):23-33.

26. Frevert, U. 2004. Sneaking in through the back entrance: the biology of malaria liver stages. *Trends in Parasitology* **. 20**(9):417-424.

27. Frevert, U., Englemann, S., Zougbédé, S., Stamge, J., Ng, B., Matuschewski, K., Liebes, L. and Yee, H. 2005. Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. *PloS.* **3**(6):e 192.

28. Froicu, M., Weaver, V., Wynn, T.A., McDowell, M.A., Welsh, J.E and Cantorna,M.T. 2003. A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases. *Molecular Endocrinology*. **17**(12):2386:2392.

29. Garnham, P.C. 1951. Patterns of exoerythrocytic schizogony. *British Medical Bulletin*. **8**(1):10-15.

30. Grant, W.B. and Holick, M.F. 2005. Benefits and requirements of vitamin D for optimal health a review. *Alternative Medicine Reviews*. **10**(2):94;111.

31. Grau, G.E., Piguet, P.F., Vassali, P. And Lambert, P.H. 1989. Tumor-necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. *Immunological Reviews*. **112**:49-70.

32. Grau, G.E, Fajardo, L.F., Piguet, P.F., Allet, B., Lambert, P.H. and Vassalli, P. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science*. **237**(4819):1210-2.

33. Greenwood, B. and Mutabingwa, T. 2002. Malaria in 2002. *Nature*.

415(6872):670-672.

34. Griffin, M.D, Xing, N. and Kumar, R. 2003. Vitamin D and its analogs as regulators of Immune activation and antigen presentation. *Annual Review of Nutrition*. **23**:117-145.

35. Hadley, T.J. 1986 Invasion of erythrocytes by malaria parasites: a cellular and molecular overview. *Annual Review of Microbiology*. **40**:451-477.

36. Harrison, T., Samuel, B.U., Akompong, T., Hamm, H., Mohandas, N., Lomasney, J.W. and Haldar, K. 2003. Erythrocyte G protein-couple receptor signalling in malarial infection. *Science*. **301**(5640):1734-1736.

37. Hay, S.I., Guerra, C.A., Tatem, A.J., Noor, A.M. and Snow, R.W. 2004. The global distribution and population at risk of malaria: past, present, and future. *The Lancet Infectious Diseases*. **4**(6): 327-336.

38. Holick, M.F. 2006. Resurrection of vitamin D deficiency and rickets. *Journal of Clinical Investigation.* **116**(8): 2062-2072.

39. Holick, M.F. 2005. Vitamin D: important for prevention of osteoporosis, cardiovascular heart disease, type 1 diabetes, autoimmune diseases, and some cancers. *Southern Medical Journal.* **98**(10):1024-1027.

40. Holick, M.F. 2003. Vitamin D: A millennium perspective. *Journal of Celular Biochemistry*. **88**(2):296-307.

41. Holick, M.F. 1995. Environmental factors that influence the cutaneous production of vitamin D. *American Journal of Clinical Nutrition*. **61**(3Suppl):638S-645S.

42. Holick, M.F., MacLaughlin, J.A. and Doppelt, S.H. 1981. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. *Science*. **211**(4482):590-593.

43. Hume, J., Lyons, E. and Day, K. 2003. Human migration, mosquitoes and the evolution of *Plasmodium falciparum*. *Trends in Parasitology.* **19**(3): 144-149.

44. Hunt, N.H., Golenser, J., Chan-Ling, T., Parekh, S., Potter, S., Medana, I.M., Miu, J. and Ball, H.J. 2006 Immunopathogenesis of cerebral malaria. *International Journal of Parasitology* **36**(5):569-582.

45. Joy, D., Feng, X., Mu, J., Furuya, T., Chotivanich, K., Krettli, A.U., Ho, M., Wang, A., White, N.J., Suh, E., Beerli, P. and Su, X.Z. 2004. Early origin and recent expansion of *Plasmodium falciparum*. *Science*. **300**(5617):318-321.

46. Kitua, Y.A., Smith, T.A., Alonso, P.L., Urassa, H., Masanja, H., Kimario,

J. and Tanner, M. 1997. The role of low level *Plasmodium falciparum* parasitaemia in anaemia among infants living in an area of intense and perennial transmission. *Tropical Medicine & International Heath*. **2**(4):325-33.

47. Kwiatkowski, D. 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. *The American Society of Human Genetics*. **77**:171-192.

48. Lemire, J.M. 1995. Immunomodulatory actions of 1,25-dihydroxyvitamin D3. *Journal of Steroid Biochemestry and Molecular Biology.* **53**(1-6):599-602.

49. Lemire, J.M., Archer, D.C., Beck, L. and Spiegelberg, H.L. 1995. Immunosupressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition on Th1 functions. *Journal of Nutrition*. **125**(6):1704S-1708S.

50. Lin, R. and White, J.H. 2004. The pleitropic actions of vitamin D. *Bioessays*. **26**(1):21-28.

51. Liu, P.T., Stenger, S., Li, H., Wenzel, L., Tan, B.H., Krutzik, S.R., Ochoa, M.T., Schauber, J., Wu, K., Meinken, C., Kamen, D.L., Wagner, M., Bals, R., Steinmeyer, A., Zügel, U., Gallo, R.L., Eisenberg, D., Hewison, M., Hollis, B.W., Adams, J.S., Bloom, B.R. and Modlin, R.L. 2005. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial

response. *Science*. **311**(5768):1770-1773.

52. Mackintosh, C.L., Beeson, J.G. and Marsh, K. 2004. Clinical features and pathogenesis of severe malaria. *Trends in Parasitology*. **20**(12):597-603.

53. Martineau, A.R., Honecker, F.U., Wilkinson, R.J. and Griffinths. 2007. Vitamin D in the treatment of pulmonary tuberculosis. *Journal of Steroid Biochemestry and Molecular Biology*. **103**(3-5): 793-798.

54. Martineau, A.R., Wilkinson, K.A., Newton, S.M., Floto, R.A., Norman, A.W., Skolimowska, K., Davidson, R.N., Sørensen, O.E., Kampmann, B., Griffiths, C.J. and Wilkinson, R.J. 2007. IFN-gamma-and TNF-indepemdemt vitamin D-inducible human suppression of mycobacteria: role of cathelicidin LL-37. *Journal of Immunology*. **178**(11):7190-7198.

55. Mathieu, C., van Etten, E., Decallonne, B., Guilietti, A., Gysemans, C., Bouillon, R. and Overbergh, L. 2004. Vitamin D 1,25-dihydroxyvitamin D3 as modulators in the immune system. *Journal of Steroid Biochemistry and Molecular Biology*. **89-90**(1-5):449-452.

56. Mathieu, C., Van Etten, E., Gysemans, C., Decallonne, B., Kato, S., Laureys, J., Depovere, J., Valckx, D., Verstuyf, A. and Bouillon, R. 2001. *In vitro* analysis of the immune system os vitamin D knockout mice. *Journal of bone and Mineral Research*. **16**(11):2057-2065.

57. Matuschewski, K. Getting infectious: formation and maturation of Plasmodium sporozoites in the Anopheles vector. *Cell Microbiology*. **8**(10):1547-1556.

58. Medana, I.M. and Turner, G.D. 2006. Human cerebral malaria and the blood-brain barrier. *International Journal of Parasitology*. **36**(5):555-568.

59. Medica, D.L. and Sinnis, P. 2005. Quantitative dynamics of *Plasmodium yoelii* sporozoites transmission by infected anopheline mosquitos. *Infection and Immunity*. **73**(7):4363-4369.

60. Menendez, C., Fleming, A.F. and Alonso, P.L. 2000. Malaria-related anaemia. *Parasitology Today*. **16**(11):469-476.

61. Miller, L.H., Baruch, D.I., Marsh, K. and Doumbo, O.K. 2002. The pathogenic basis of malaria. *Nature*. **415**(6872):673-679.

62. Miller, L.H., Good, M.F. and Milon, G. 1994. Malaria pathogenesis. *Science*. **264**(5167):1878-1883.

63. Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepfer, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Greinier, J.K., Carpenter, A.E., Foo, S.Y., Stewart, S.A., Stockwell, B.R. Hacohen, N., Hahn, W.C., Lander, E.S., Sabatini, D.M. And Root, D.E. 2006. A lentiviral library for human and mouse genes applied to an arrayed viral high-content screen.

Cell. **124**(6): 1283-1298.

64. Mota, M.M., Hafalla, J.C. and Rodriguez, A. 2002. Migration through host cells activates Plasmodium sporozoites for infection. *Nature Medicine*. **8**(11):1318-1322.

65. Mota, M.M., Pradel, G., Vanderberg, J.P., Hafalla, J.C., Frevert, U., Nussenzweig, R.S., Nussenzweig, V. and Rodriguez, A. 2001. Migration of *Plasmodium* sporozoites through cells before infection. *Science*. **291**(5501): 24-25.

66. Mota, M. M. and Rodriguez, A. 2004. Migration through host cells:the first steps of Plasmodium sporozoites in the mammalian host. *Cell Microbiology*. **6**(12):1113-1118.

67. Mota, M.M. and Rodriguez, A. 2001. Migration through host cells by apicomplexan parasites. *Microbes and Infection*. **3**(13):1123-1128.

68. Mulenga, M., Malunga, P., Bennett, S., Thuma, P.E., Shulman, C., Fielding, K. and Greenwood, B.M. 2005. Factors associates with severe anaemia In Zambian children admitted with *Plasmodium falciparum* malarial anaemia. *Annals of Tropical Paediatrics*. **25**(2):87-90.

69. Murphy, S.C., Harrison, T., Hamm, H., Mohadas, N. and Haldar, K. Trythrocyte G protein as a novel target for malarial chemotherapy. *PLoS*

Medicine. **3**(12):e528.

70. Mutabingwa, T.K. 2005. Artemisinin-based combination therapies (ACTs): best hope for malaria treatment but inaccessible to the needy! *Acta Tropica*. **95**(3):305-315.

71. Nabarro, D.N. and Tayler, E.M. 1998. The "roll back malaria" campaign. *Science*. **280**(5372):2067-2068.

72. Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. and Sato, J. 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Journal of Carcer research*. **42**(9):3858-3863.

73. Norman, A.W. 2006. Minireview: vitamin D receptor: new assignments for an already busy receptor. *Endocrinology*. **147**(12):5542-5548.

74. Novac, N. and Heinzel, T. 2004. Nuclear receptors: overview and classification. *Current Drug Targets/Alergy and Inflammation*. **3**(4):335-346.

75. Paddison, P.J., Caudy, A.A., Sachidanandam, R. and Hannon, G.J. 2004. Short hairpin activated gene silencing in mammalian cells. *Methods in Molecular Biology*. **265**:85-100.

76. Planche, T. and Krishna, S. Severe malaria: metabolic complications. *Current Molecular Medicine*. **6**(2):141-153.

77. Pradel, G. and Frevert, U, 2001. Malaria sporozoites actively enter and pass through rat Kupfer cells prior to hepatocytes invasion. *Hepatology*. **33**(5):1154-1165.

78. Prudencio, M., Rodriguez, A. and Mota, M.M. 2006. The silent path to thousands of merozoites: the *Plasmodium* liver stage. *Nature Reviews Microbiology*. **4**, 849-856.

79. Prudencio, M., Rodrigues, C.D., Ataide, R. and Mota, M.M. 2008. Dissecting *in vitro* host cell infection by *Plasmodium* sporozoites using flow cytometry. *Cellyular Microbiology*. **10**(1):218-224.

80. Rajapakse, R., Uring-Lambert, B., Andarawewa, K.L., Rajapakse, R.P., Abou-Bacar, A., Marcellin, L. and Candolfi, E. 2007. 1,25(OH)2D3 inhibits in vitro and in vivo intracellular growth of apicomplexan parasite Toxoplasma gondii. *Journal of Steroid Biochemistry and Molecular Biology*. **103**(3-5):811-814.

81. Rosenberg, R., Wirtz, R.A., Schneider, I. and Burge, R. 1990. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **84**(2):209-212.

82. Rochel, N. and Moras, D. 2006. Ligand binding domain of vitamin D receptors. *Current Topics in Medicinal Chemestry*. **6**(12): 1229-1241.

83. Schofield, L. and Grau, G.E. 2005. Immunological process in malaria pathogenesis. *Nature Reviews. Immunulogy.* **5**(9):722-735.

84. Sachs, J. and Malaney, P. 2002. The economic and social burden of malaria. *Nature*. **415**(6872):680-685.

85. Schofield, L. and Grau, G.E. 2005. Immunological processes in malaria pathogenesis. Nature Reviews Immunology. **5**(9):722-735.

86. Silva, M.E., Silva, M.E., Silva, M.E., Nicoli, J.R., Bambirra, E.A. and Vieira, E.C. 1993. Vitamin D overload and experimental Trypanossoma Cruzi infection: parasitological and histopathological aspects. *Comparative Biochemistry and Physiology*. **104**(1):175-181.

87. Silvie, O., Franetich, J.F., Boucheix, C., Rubinstein, E. and Mazier, D. 2006. Alternative invasion pathways for *Plamodium berghei* sporozoites. *International Journal of Parasitology*. **37**(2):173-182.

88. Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S, Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., Weinberg, R.A.

And Novida, C.D. 2003. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA*. **9**(4):493-501.

89. Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J.M., Menard, R. and Heussler, V.T. 2006. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*. **313**(5791):1245-1246.

90. Uchida,M., Ozono, K. And Pike, J.W. 1994. Activation of the human osteocalcin gene by 24R,25-dihydroxyvitamin D3 occurs through the vitamin d receptor and the vitamin D-responsive element. *Journal of Bone and Mineral Research.* **9**(12):1981-1987.

91. Uphoff, C.C. And Drexler, H.G. 2001. Prevention of mycoplasma contamination in leukemia-lyphoma cell lines. *Human Cell*.**14**(3):244-247.

92. Vanderberg, J.P. 1977. *Plasmodium berghei*: quantification of sporozoites injected by mosquitoes feeding on rodent hosts. *Experimental Parasitology*. **42**(1):169-181.

93. Vanderberg, J.P., Khan, Z.M. And Stewart, M.J. 1993. Induction of hepatic inflammatory response by *Plasmodium berghei* sporozoites protects BALB/c mice against chalange with *Plasmodium yoelii* sporozoites. *Journal of Parasitology.* **79**(5):763-767.

94. Van Etten, E. and Mathieu, C. 2005. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. *Journal of Steroid Biochemistry and Molecular Biology*. **97**(1-2):93-101.

95. Vial, H.J., Thuet, M.J. and Philippot, J.R. 1982. Inhibition of the in vitro growth of plasmodium falciparum by D vitamins and vitamin d-3 derivates. *Molecular and Biochemical Parasitology*. **5**(3):189-198.

96. Vernick, K.D. 2004. Gamete interrupts; a novel calcium-dependent kinase is essential for malaria sexual reproduction. *Cell*. **117**(4):503-514.

97. Weatherall, D.J., Miller, L.H., Baruch,D.I., Marsh, K., Doumbo, O.K., Casals-Pascual, C. and Roberts, D.J. 2002. Malara and the red cell. *Hematology*. (1):35-57.

98. White, N.J. 2004. Sharing malaria. *The Lancet*. **363**(9414):1006

99. Wiesenfeld, L. 1967. Stickle-cell trait in human biological and cultural evolution. *Science*. **157**(793):1134-40.

100. Wilkinson, R.J. 2007. IFN-γ and TNF-Independent Vitamin D-Inducible Human Supression of Mycobacteria: the role of cathelicidin LL-37. *Journal of Immunology*. **178**(11):7190-7198.

101. Yazdani, S.S., Mukeherjee, P., Chauhan, V.S. and Chitnis, C.F.

2003. Immune responses to asexual blood-stages of malaria parasites. *Current Molecular Medicine*. **6**(2):187-203.