Functional analysis of variant proteins in *Plasmodium vivax*: Implications in pathology

Maria Bernabeu Aznar

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Functional analysis of variant proteins in *Plasmodium vivax:* Implications in pathology

Memòria presentada per Maria Bernabeu Aznar per aspirar al títol de Doctora per la Universitat de Barcelona

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Dra. María del Carmen Fernández Becerra

Prof. Hernando A. del Portillo

Barcelona, abril del 2013

Al Lluís i als meus pares,

ARTICLES QUE CONSTITUEIXEN LA TESI DOCTORAL

1

Functional analysis of *Plasmodium vivax* VIR proteins reveals different subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor

Maria Bernabeu, Francisco Javier Lopez, Mireia Ferrer, Lorena Martin-Jaular, Alain Razaname, Giampietro Corradin, Alexander G. Maier, Hernando A. del Portillo, Carmen Fernandez-Becerra

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2

A new computational approach redefines the subtelomeric vir superfamily of *Plasmodium vivax.*

Francisco Javier Lopez, Maria Bernabeu, Carmen Fernandez Becerra, Hernando A. del Portillo

BMC Genomics, 2013 Jan 16;14:8

Factor d'impacte: 4.07
Quartil: 1 (Applied Microbiology)

3

***Plasmodium vivax* subtelomeric variant proteins and cytoadherence to the human spleen.**

Maria Bernabeu, Mireia Ferrer, Richard Thomson, Francisco Javier Lopez, Lorena Martin-Jaular, Aleix Elizalde, Stefanie CP. Lopes, Fabio TM. Costa, Marcus VG. Lacerda, Hernando A. del Portillo and Carmen Fernandez-Becerra

Treball no publicat

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Abbreviations

ACP: Acyl Carrier Protein

ACTs: Artemisinin-based Combination Therapies

ATS: Acidic Terminal Sequence

BC: Barrier Cells

bp: base pair

BSA: Bovine Serum Albumin

BSD: Blasticidin S deaminase

cDNA: Complementary Deoxyribonucleic Acid

CDUP: Cytosine Deaminase/ Uridyl Phosphoribosil transferase

CHO: Chinese Hamster Ovary cells

CIDR: Cysteine Inderdomain Rich Protein

CQ: Cloroquine

CR1:Complement Receptor 1

CRT: Chloroquine Resistance Transporter

CSA: Chondroitin Sulphate A

CSP: Circumsporozoite Surface Protein

CVCs: Caveola Vesicle Complexes

DAPI: 4'6- diamidino- 2 phenylindole

DBL: Duffy Binding Like

DHFR: human dihydrofolate reductase-thymidylate synthase (*h:* human, *Tg;* *Toxoplasma gondii)*

DIC: Differential Interference Contrast

DMEM: Dulbecco Modified Eagle Medium

DNA: Deoxyribonucleic Acid

EC: Erythrocyte Cytoplasm

EDTA: Ethylenediamine tetra-acetic Acid

EM grade: Electron Microscopy grade

EM: Erythrocyte Membrane

ET: Early Trophozoite

F: Forward

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FV: Food Vacuole

gDNA: genomic Deoxyribonucleic Acid

GFP: Green Fluorescence Protein

G6PD: Glucose-6-Phospate Deshydrogenase enzyme

HA: hemagglutinin

HB: Homology Block

HLEC: Human Lung Endothelial cells

HMM: Hidden Markov Model

HPLC: High Performance Liquid Chromatography

HRP: Horseradish Peroxidase

HSPs: High Scoring Segment Pairs

HSV-TK: *Herpes simplex* Virus Thymidine Kinase

ICAM-1: Intercellular Adhesion Molecule 1

IFA: Immunofluorescence Assay

IgG: Immunoglobulin G

IPT: Intermittent Preventive Treatment

iRBC: Infected Red Blood Cell

iRet: infected-Reticulocyte

KAHRP: Knob-Associated Histidine Rich Protein

kDa: kilodalton

KO: Knock Out

LP1 and LP2: Long Peptide 1 and 2

LT: Late Trophozoite

LUC: Luciferase

MØ: macrophages

MAAP: Malarial Adhesins and Adhesin-like proteins

MACS: Magnet-Activated Cell Sorting column

MALDI-TOF: Matrix Assisted Laser Desorption Ionization - Time of Flight

MCL: Markov Clustering Algorithm

MDR1: Multidrug Resistance 1 protein

MSP1: Merozoite Surface Protein 1

MSP1(19): 19-kDa subunit of *P. falciparum* Merozoite Surface Protein 1

Mz: Merozoite

Mz-AM: Merozoite Associated Material

Pa: Pascal

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PEXEL: *Plasmodium* Export Element

Pf: *Plasmodium falciparum*

PfEMP1: *P. falciparum* Eryntrocyte Membrane Protein 1

PIR: Plasmodium interspersed repeat s

PNEP: PEXEL-Negative Exported Proteins

PNG: Papua New Guinea

Pv: *Plasmodium vivax*

PV: Parasitophorous Vacuole

PVM: Parasitophorous Vacuole Membrane

R: Reverse

RBC: Red Blood Cell

RBP: Reticulocyte Binding Protein

RMA: Robust Multi-array average

RNA: Ribonucleic Acid

RT- PCR: Reverse Transcriptase- PCR

S: Schizont

Sal1: Salvador strain 1

SAM: Significance Analysis of Microarray

SBEC: Saimiri Brain Endothelial cells

SBP1: Skeleton Binding Protein 1

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: SDS Polyacrylamide Gel Electrophoresis

SP: Sulfadoxine-Pyrimitheamine

SP: Signal Peptide

TM: transmembrane

TP: transit Peptide

tRNA: Transfer Ribonucleic Acid

V: Venules

V. Volt

VCAM: Vascular Cell Adhesion Molecule

WHO: World Health Organization

µF: microfarad

Plasmodium Variant Multigene families mentioned in this thesis

P. vivax → Human malaria

vir: P. vivax variant genes

pvstp: P. vivax subtelomeric transmembrane protein

pv-fam-A: also known as PvTRAg (*P. vivax* Tryptophan Rich Antigen)

pv-fam-B: also known as PHIS (*Plasmodia* helical interspersed subtelomeric)

pv-fam-D

pv-fam-E also known as RADp

pv-fam-H

pvpirA

pvpirD

pvpirH

P. falciparum→ Human malaria

var encodes for PfEMP1 proteins

rif encodes for RIFIN proteins

stevor

pfmc-2tm

surf encodes for SURFIN proteins

P. knowlesi → Primate malaria

kir

SICAvar: Schizont-Infected Cell Agglutination-*var*

pk-fam

P. yoelii → Rodent malaria

yir

P. berghei → Rodent malaria

bir

pb-fam

P. chabaudi → Rodent malaria

cir

PIR: Plasmodium interspersed repeats multigene superfamily.

Comprises the *vir, kir, bir, cir* and *yir* genes multigene families.

Introduction

1. General introduction

1.1 An introduction to malaria:

Malaria is an ancient intracellular parasitic disease which is caused by apicomplexan protozoans from the genus *Plasmodium.* These parasites have been evolving alongside humans for a very long time (Garnham, 1966). References to the symptoms of malaria can be found throughout recorded history, as early as 2700 BC where Chinese medical writings describe the characteristic periodic fevers associated with this disease (Cox, 2002). As a consequence, malaria is a disease which has shaped world history and it is believed that it may have contributed to the decline of the Roman Empire and that it defeated significant historical figures such as Alexander the Great, Tutankhamun and Genghis Khan. The infectious agent was first discovered many years later by Dr. Laveran, within the red blood cells of soldiers who were suffering from paludic fevers (Laveran, 1881).

Plasmodium spp. does not only infect humans, but it also infects other animal species. There are more than 100 species of *Plasmodium* which are able to infect many animal species such as reptiles, birds, as well as various mammals, from mice to apes (Garnham, 1967, Escalante *et al.*, 1994, Cox, 2010). In humans there are four species of *Plasmodium* which account for the disease (*P. falciparum, P. vivax, P. ovale, P. malariae)* and a fifth species, *P. knowlesi,* which has been recently identified from a zoonotic phenomenon in Malaysian Borneo (Singh *et al.*, 2004). *Plasmodium spp.* are transmitted to humans thorough the bite of female mosquitoes from the genus *Anopheles* and the infection may result in a wide variety of symptoms, ranging from absent or very mild symptoms (flu-like fever, chills, headache, body aches, anemia and vomits) to severe disease (coma, acute respiratory distress, severe anemia, hypoglycemia) and even death.

1.2 Malaria epidemiology

Approximately half of the world's population is at risk of malaria infection. In 2010, there were about 219 million malaria cases (ranged from 154 to 294) with the estimated deaths of approximately 660.000 (WHO 2012). Despite the fact that increased prevention and control measures led to a reduction in global malaria mortality rates by more than 25% since the year 2000, malaria is still a huge global public health problem. Of the five species which can cause malaria, almost all clinical cases are due to infections of *P. falciparum* and *P. vivax.*

P. falciparum is transmitted within 87 countries (Guerra *et al.*, 2008) and it is considered that 2.57 billion people live in areas which are at any risk from this species. Of these, 1.44 billion people live in areas which present a risk of stable transmission of the disease, in Central, South and South-East Asia (46%) or Africa (52%), with a much smaller presence in the Americas (2%). Despite the fact that Asia accounts for nearly half of the population at a high risk, the greatest burden of the disease occurs in Africa due to the fact that these countries present technical and financial obstacles to effective disease control. The remaining 1.13 billion people at risk live in areas of unstable transmission where their chance of infection is very low. The vast majority of people at low risk live in Asia (91%), with a smaller proportion in the Americas (5%) and in Africa (4%) (Hay *et al.*, 2009) (Figure 1A).

P. vivax is the most widespread malaria, and is a potential cause of morbidity amongst the 2.85 billion people living at any risk of this particular infection (Guerra *et al.*, 2010) (Figure 1B). It is transmitted within 95 countries, and it presents with a much wider latitude range than *P. falciparum,* due to the fact that the parasite's sexual stage is active at lower temperatures. Consequently, the generation of sporozoites is possible at higher altitudes and more extreme latitudes.

Vivax accounts for approximately 25–40% of the total global malaria burden, and it is estimated that it causes between 132 and 391 million cases of Malaria per year (Price *et al.*, 2007). Most of the *P. vivax* burden (80%) lies in South and South East Asia, where this parasite accounts for up to 50% of malaria cases. On the other hand, only 5% of the global burden occurs in Central and South America but nearly all (70-81%) of the malaria cases in these countries are due to this parasite (Guerra *et al.*, 2010).

Questions remain regarding the presence of *P. vivax* in Africa. Historically, it has been widely accepted that this parasite is absent in Western and Central Africa, due to the absence, in the vast majority of the population, of the Duffy blood group that is considered indispensable for *P. vivax* invasion of reticulocytes (Miller *et al.*, 1976). However, increasing evidence of the circulation of this species in this part of Africa has been reported recently (Rubio *et al.*, 1999, Ryan *et al.*, 2006, Culleton *et al.*, 2009, Dhorda *et al.*, 2011, Culleton *et al.*, 2012), including episodes affecting Duffy negative individuals (Cavasini *et al.*, 2007, Rosenberg, 2007, Menard *et al.*, 2010, Mendes *et al.*, 2011, Wurtz *et al.*, 2011). On the one hand, this evidence could support the hypothesis that *P. vivax* has been often misdiagnosed as *P. ovale*. On the other hand, this may challenges the notion that this species has a single invasion mechanism, due to the fact that different Reticulocyte Binding Proteins (RBPs) have also been found in the *P. vivax* genome annotation (Carlton *et al.*, 2008).

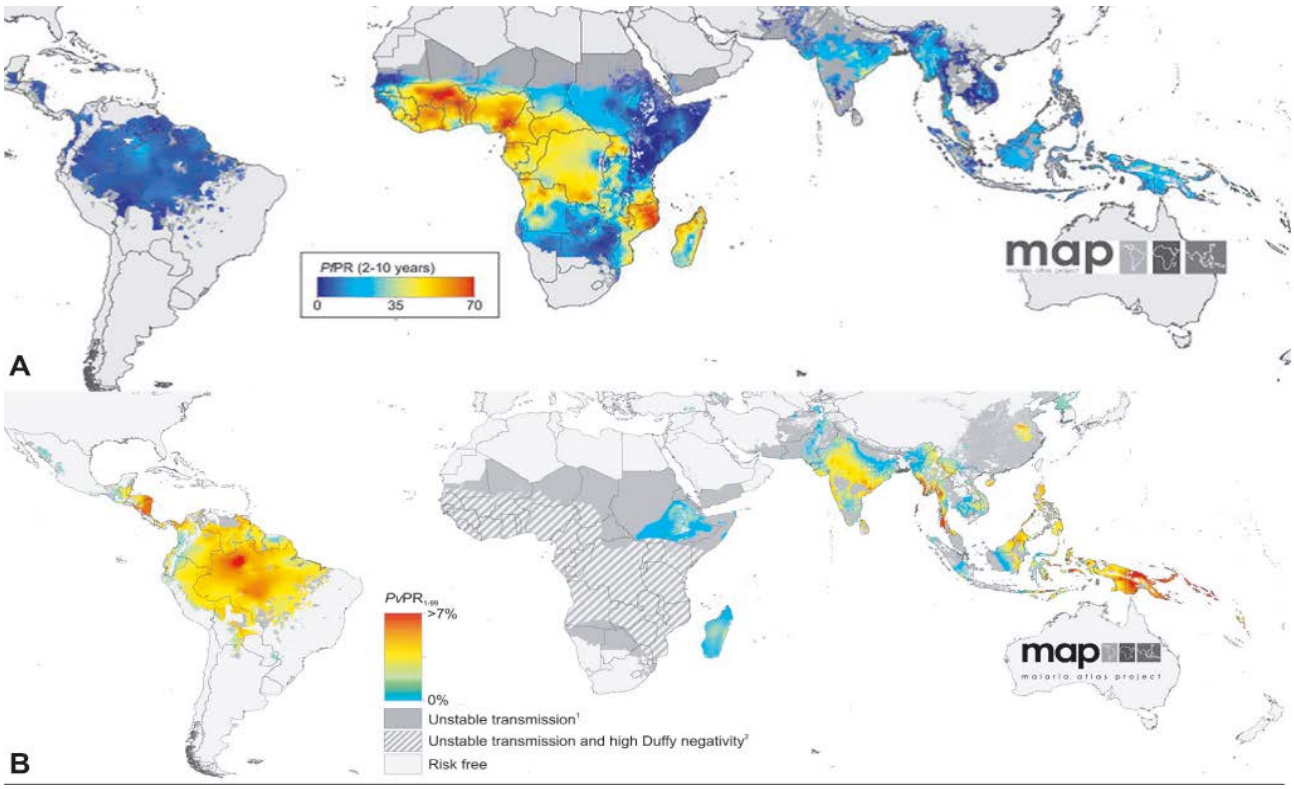

Figure 1. Global distribution of malaria in 2010. Endemicity of *Plasmodium falciparum* (A) *and Plasmodium vivax* (B). Colors represent the stability of transmission ranging from blue (low stability) to red (high stability). Areas in which Duffy negativity gene frequency is predicted to exceed 90% are hatched. Image source: A, (Gething *et al.*, 2011) and B, (Gething *et al.*, 2012)

1.3 Malaria control and prevention

Current strategies for malaria control are divided into direct intervention against the parasite and prevention against the vector. As a result of the implementation of these strategies, from 1945 to 2010, 79 countries have eliminated malaria (Feachem *et al.*, 2010). Vector control strategies include the widespread use of insecticide treated bed nets and indoor insecticide residual spraying. On the other hand, parasite direct interventions consist in chemotherapy and malaria prevention through Intermittent Preventive Treatment (IPT) and vaccination.

- *P. falciparum* chemotherapy

Despite there are research efforts being conducted to establish methods for parasite prophylaxis and prevention, chemotherapy against clinical cases can still be considered the most powerful tool. Unfortunately, the constant appearance of isolates resistant to the currently available drugs is threatening the fight against malaria. In 1955, the World Health Organization (WHO) implemented chloroquine (CQ) chemotherapy to complement the initial vector control measures against *P. falciparum*. Unfortunately, in the 1960s, the first isolates resistant to chloroquine appeared in Southeast Asia and South America (Najera *et al.*, 2011) (Figure 2). The subsequent spread of chloroquine resistant *P. falciparum* to Africa led to a 2- to 3-fold increase in malaria-related deaths in the 1980s (Trape, 2001). By that time, the only viable alternative to chloroquine was sulfadoxine–pyrimethamine (SP), although resistance to SP appeared only one year after its implementation (Clyde *et al.*, 1957). Later, other drugs including mefloquine, amodiaquine and quinine were used as monotherapy, and also resulted in the selection of resistant parasites, at least in some parts of the world (Cowman *et al.*, 1994, Sa *et al.*, 2009, Meng *et al.*, 2010, Briolant *et al.*, 2011). At the present time, the most effective treatments for malaria are artemisinin-based combination therapies (ACTs), which combine a semi-synthetic derivative of artemisinin (a chemical compound isolated from the plant *Artemisia annua)*, with a partner drug of a distinct chemical class (Egan *et al.*, 2007). At present, despite the fact that some parasites are being cleared from circulation more slowly than before by ACTs (Dondorp *et al.*, 2009), there has been only a few reports that clearly confirmed artemisinin-resistant malaria (Jambou *et al.*, 2005, Noedl *et al.*, 2008). Together, these observations encourage the development of scientific programs to avoid the

development of *P. falciparum* superbugs in Southeast Asia (reviewed in (Petersen *et al.*, 2011, Meshnick, 2012, Mita *et al.*, 2012)).

- *P. vivax* chemotherapy

Chloroquine is still the first-line therapy for the cure of vivax malaria. It is coadministrated with primaquine to prevent relapses from dormant liver-stages despite the fact that the first *P. vivax* chloroquine resistance cases were reported in Papua New Guinea (PNG) in 1989. It should be noted that this appeared 30 years later than the first appearance of *P. falciparum* resistances probably due to the lower parasite biomass associated to this infection and/or to the presence of asymptomatic patients (Rieckmann *et al.*, 1989). As happened in the case of *P. falciparum* (Trape *et al.*, 1998), the appearance of chloroquine resistant isolates could be associated with an increase in severe malaria (Russell *et al.*, 2008). The molecular process underlying chloroquine resistance in *P. vivax* has been associated with a polymorphism in the *pvmdr1* gene (Y976) *in vitro* (Suwanarusk *et al.*, 2007) and do not appear to be associated with polymorphism in *pvcrt,* as has been demonstrated in *P. falciparum* (Nomura *et al.*, 2001, Suwanarusk *et al.*, 2007, Barnadas *et al.*, 2008). Today, high levels of *P. vivax* chloroquine resistant strains have been found in PNG (Karunajeewa *et al.*, 2008) and Indonesia (Baird *et al.*, 1996, Fryauff *et al.*, 1998, Fryauff *et al.*, 2002, Sumawinata *et al.*, 2003, Ratcliff *et al.*, 2007, Sutanto *et al.*, 2009). Low-level chloroquine resistant strains are also present in Myanmar (Guthmann *et al.*, 2008), Vietnam (Taylor *et al.*, 2000, Phan *et al.*, 2002), South Korea (Lee *et al.*, 2009), Turkey (Kurcer *et al.*, 2006), Ethiopia (Teka *et al.*, 2008), Madagascar (Barnadas *et al.*, 2008), Brazil (de Santana Filho *et al.*, 2007) and Colombia (Soto *et al.*, 2001) (Figure 2). Despite the use of artemisinin-combination treatments (ACTs) in many countries to treat mixed infections against *P. falciparum* and *P. vivax* infections, few ACTs have been thoroughly tested for their safety and effectiveness against *P. vivax.*

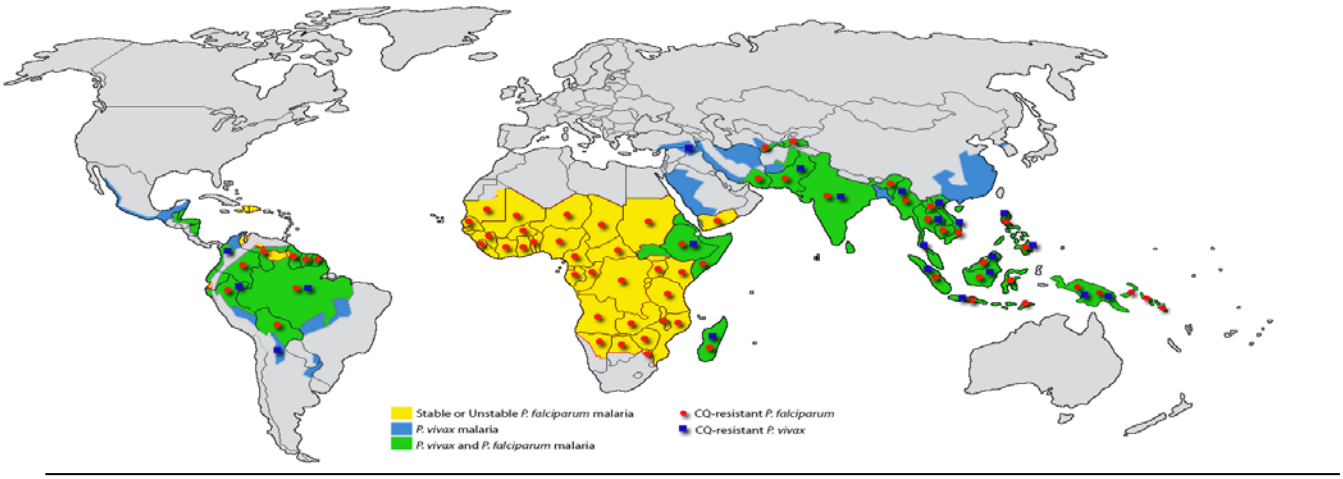

Figure 2. Global distribution of chloroquine resistant cases.

P. falciparum chloroquine resistances are depicted in a red point, *P. vivax* chloroquine resistances are depicted in a blue square. Yellow: Stable or unstable *P. falciparum* malaria; yellow: *P. vivax* malaria; Green: *P.* vivax and *P. falciparum* malaria. Image source: Carolina Bustamante

- Malaria prevention

Due to the fact that *P. vivax* has been historically considered a benign infection, past and present research efforts have been mostly directed towards *P. falciparum* malaria. In parallel to clinical chemotherapeutic treatment, the WHO is establishing a policy of recommending Intermittent Preventive Treatment (IPT) in infancy and pregnant women based upon the administration of 2 to 3 doses of SP (WHO 2010). Despite IPT is being associated with an increase in resistance to SP, the effectiveness of this intervention has not been decreased so far (Mayor *et al.*, 2008). On the other hand, to overcome the limitations of the control strategies mentioned above, many research efforts are being conducted towards a potentially effective vaccine against Malaria. Today, the RTS,S vaccine against the Circumsporozoite Surface Protein (CSP) of *P. falciparum* is in an ongoing phase III trial. This vaccine has already demonstrated an efficacy rate of approximately 50% in children aged from 5 to 17 months (Agnandji *et al.*, 2011) and of 30% in children aged from 6 to 12 weeks (Agnandji *et al.*, 2012).

Much remains to be done if malaria eradication is to be achieved. Without doubt, further research on the biology of both *P. falciparum* and *P. vivax*, as well as on host-pathogen interactions will be indispensable in the development of successful malaria control tools in the future.

2. Biological and clinical aspects of *P. vivax*

Because *P. falciparum* accounts for more than 90% of all malaria deaths in the African Region, the majority of research has been conducted in this species. *P. vivax* has been historically treated as a benign infection, but the recent appearance of chloroquine resistant strains as well as the documentation of an increase in severe cases have put this parasite in the spotlight (Mueller *et al.*, 2009). Today, research undertaken to understand the unique pathology and biology of this parasite is still difficult and challenging, due in most part to the lack of an *in vitro* long-term continuous culture for blood stages, as well as the low parasitaemias associated with natural infections. Certainly, if malaria eradication is to be achieved, *P. vivax* has to be considered a major public health problem, and further research must be encouraged.

2.1 *P. vivax* life cycle

Plasmodium vivax infective sporozoites are inoculated in the skin by *Anopheles* mosquitoes where they reach the bloodstream. Those blood circulating sporozoites finally reach the liver, invade hepatocytes and initiate the exoerythrocytic stage. Upon invasion, the parasite matures into hepatic schizonts that induce the formation of a parasitophorous vacuole (PV) that supports the development of thousands infective hepatic merozoites. In the meantime, *P. vivax* can remain dormant in the liver, in a stage called hypnozoite, that upon activation after months or years can cause clinical relapses (Krotoski, 1985). Active hepatic schizonts release merozoites to the circulatory system through budding derived vesicles called merosomes that bulge into liver sinusoids and appear to act as shuttles that ensure the release of living merozoites directly into the circulation (this phenomenon was well described in *P. berghei* by (Sturm *et al.*, 2006)). The ~ 48 h red blood cell asexual developments is complex and is initiated by merozoite invasion of reticulocytes, with three successive morphological stages . The first phase is termed the 'ring stage', where parasite proteins are exported into the host cell. The second phase, the 'trophozoite stage', is characterized by rapid parasite growth and hemoglobin digestion that is transformed into hemozoin inside the food vacuole (FV). Later, the parasite enters the 'schizont stage' that generates up to 20 daughter merozoites that, after rupture of the host cell, will invade new reticulocytes. Finally, a proportion of asexual parasites will convert to gametocytes which transmit the infection to female *Anopheles* mosquitoes, thus completing the malaria life cycle. The *P. vivax* life cycle is depicted in figure 3 (Mueller *et al.*, 2009).

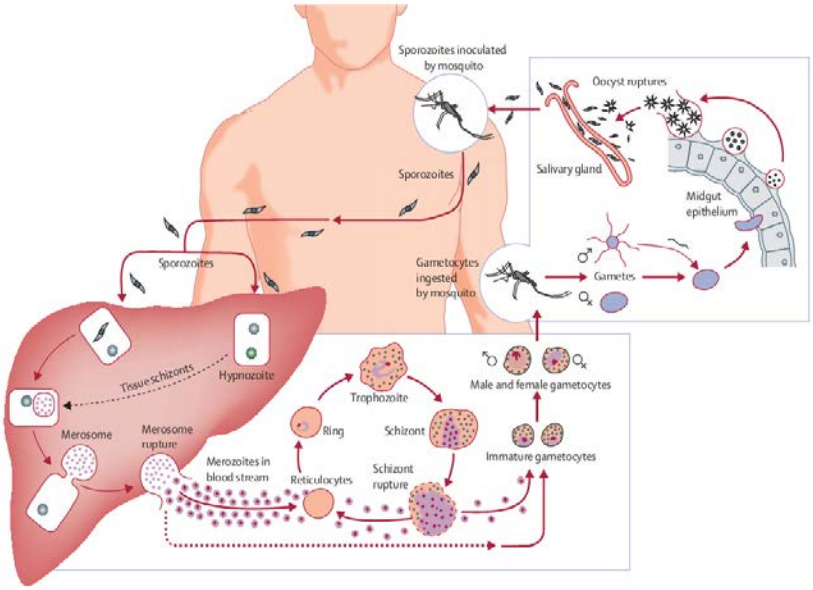

Figure 3. *Plasmodium vivax* life cycle. Image source: (Mueller *et al.*, 2009)

2.2 *P. vivax* unique biological features

P. vivax has different biological features that distinguish it from other *Plasmodium sp.* (Figure 4). The most obvious features include: First, the presence of hypnozoites that remain dormant in the liver and cause subsequent clinical relapses weeks or months after the first infection (Krotoski, 1985). This phenomenon might help the parasite to ensure transmission and propagation in temperate zones where the presence of the mosquito is seasonal. However, the trigger for the activation of hypnozoites is not understood, although stress seems to play a part. Second, once merozoites are released to the bloodstream, they invade preferably (if not exclusively) reticulocytes (Kitchen, 1938). This could be a parasitic adaptation to limit hyperparasitaemias and/or associated virulence. Alternatively, the reticulocyte might offer a special microenvironment necessary to support the growth of this species. Third, after reticulocyte invasion, *P. vivax* induces a pitted membrane surface with the formation of numerous flask-shaped indentations, called caveolae, in association with vesicles, termed caveola–vesicle complexes (CVCs) (Aikawa, 1988). These structures physically accumulate Giemsa stain resulting in the reddish dotted appearance in *P. vivax* smears, known as Schüffner's dots. In contrast, electron dense 'knob' protrusions are the predominant feature at the surface of *P. falciparum* infected Red Blood Cells (iRBCs) and are an excellent scaffold for parasite ligands that mediate adhesion. On the contrary, the function of the CVCs surface structures still remains unknown. Fourth, unlike *P. falciparum,* all blood-stage forms of *P. vivax* are found in the peripheral circulation and deformability of infected reticulocytes is dramatically increased (Handayani *et al.*, 2009). Fifth, *P. falciparum* presents "banana-shaped" gametocytes. On the other hand, *P. vivax* sexual stages, like most *Plasmodium* species, look circular in Giemsa-stained blood smears (Boyd and Kitchen, 1937). Remarkably, they appear in blood before clinical symptoms occur, thus favouring a successful transmission to mosquitoes. Sixth, mosquito vectors from areas where *P. vivax* is more prevalent are outdoor biting, thus limiting the value of measures based on insecticide impregnated bed nets (Mueller *et al.*, 2009).

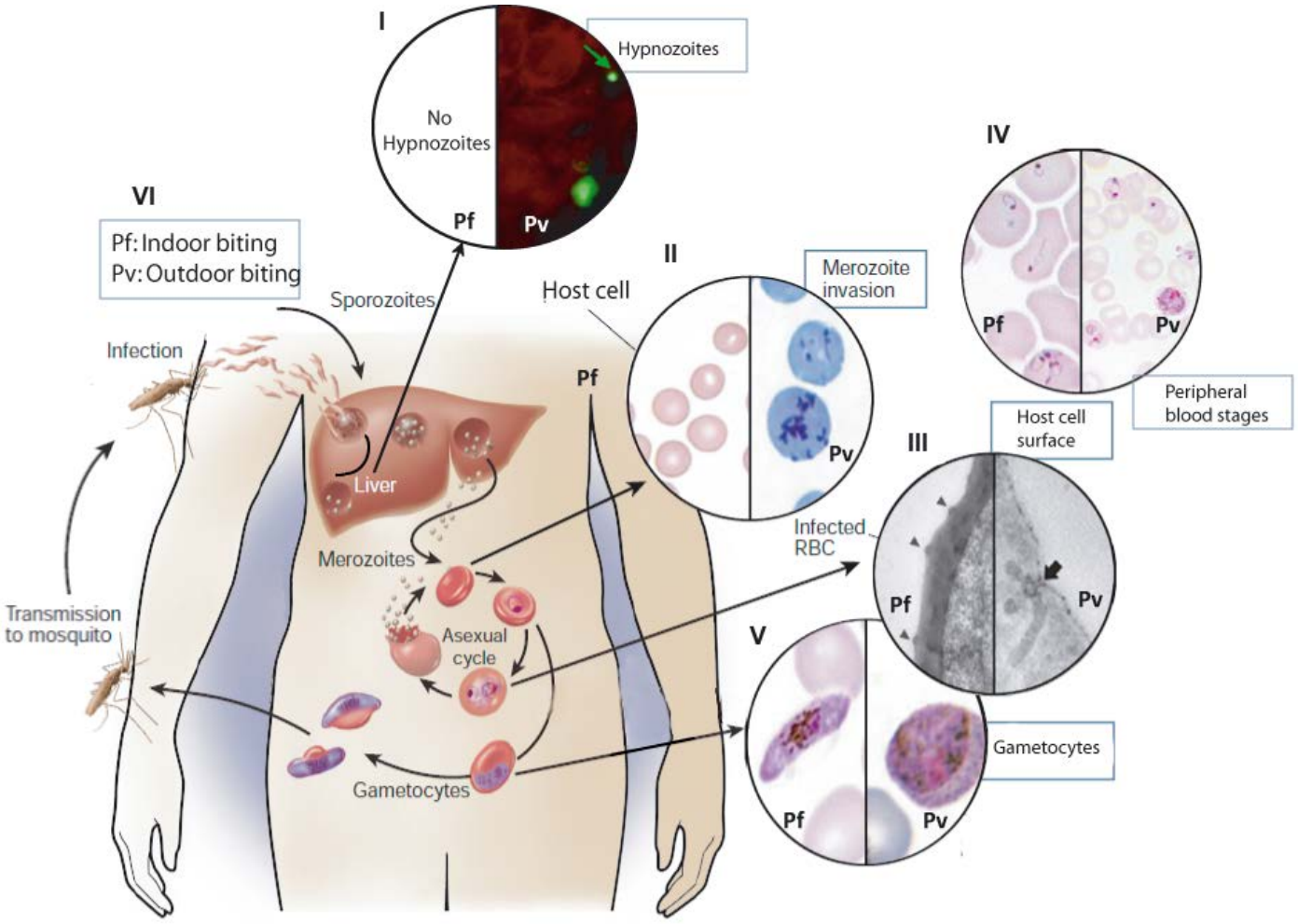

Figure 4. Main differences between *Plasmodium falciparum* (Pf) *and Plasmodium vivax* (Pv) depicted in a *Plasmodium* life cycle: Image extracted and modified from (Miller *et al.*, 2002).

I. *P. vivax* hypnozoites dormant stages (Chattopadhyay *et al.*, 2010). II. Host cells: *P. falciparum* invades erythrocytes while *P. vivax* invades exclusively reticulocytes. III. Host cell surface modification: Knobs are found in the surface of infected *P. falciparum* erythrocytes (image source (Rug *et al.*, 2006)) and caveola–vesicle complexes appear in the surface of the *P. vivax* infected reticulocytes (image source (Akinyi *et al.*, 2012)). IV. Only early stages of the parasite are found in the peripheral blood of *P. falciparum* infected patients while all stages are found in *P. vivax* patients. V. *P. falciparum* "banana-shaped" gametocytes and *P. vivax* round gametocytes. VI. Vector behaviour.

2.3 Clinical manifestations

Until recently, *P. vivax* had been considered a benign infection, when compared with the severe manifestations observed in *P. falciparum.* However, this widely accepted view of vivax malaria is now being challenged. Recent evidences revealed that *P. vivax* accounts for up to 40% of patients hospitalized with malaria in vivax-endemic areas were falciparum and vivax malaria coexist (Anstey *et al.*, 2012). Moreover, case reports of *P. vivax* presenting severe symptoms have been published recently (Kochar *et al.*, 2005). Finally, similar mortality rates in *P. vivax* and in *P. falciparum* hospitalized patients were found in countries such as Indonesian Papua (Barcus *et al.*, 2007, Tjitra *et al.*, 2008, Price *et al.*, 2009), India (Kochar *et al.*, 2010a) and Brazil (Lanca *et al.*, 2012). Moreover, *P. vivax* has a lower pyrogenic threshold than *P. falciparum* (the level of parasitaemia associated with fever) and inflammatory response is greater (Anstey *et al.*, 2009, Anstey *et al.*, 2012) and the parasitaemia of the patient do not easily reflect the symptomatology of the disease.

P. vivax has been shown to cause severe anemia, respiratory distress and acute lung injury, hepatic dysfunction and jaundice, acute kidney injury, spleen rupture, malnutrition, and possibly coma (Kochar *et al.*, 2005, Price *et al.*, 2007, Mueller *et al.*, 2009, Price *et al.*, 2009, Anstey *et al.*, 2012). Principal complications derived of *P. vivax* infection are depicted in Table 1. Of them, only severe anemia (Price *et al.*, 2009), acute respiratory syndrome (Tan *et al.*, 2008, Valecha *et al.*, 2009) and acute kidney injury (Chung *et al.*, 2008, Kute *et al.*, 2012a, Kute *et al.*, 2012b, Kute *et al.*, 2012c, Sinha *et al.*, 2012) have presented the most convincing evidence for *P. vivax* being a major contributory factor. The *P. vivax* severity was well exemplified in a study presented by Lacerda and collaborators in which they described that *P. vivax* was the most plausible cause of death for 13 out of 17 death patients with a clinical diagnosis of this parasite. Acute respiratory distress syndrome (6 cases) was the most frequent complication while the second causes of death were spleen rupture (3 cases) and multiorgan dysfunction syndrome (3 cases) (Lacerda *et al.*, 2012).

Clinical manifestations	Comments	Country	References
Severe anaemia	Commonest manifestation of severe vivax disease Common in children and in adults, specially post-pubescent females Twofold higher risk in pregnant woman infected with vivax malaria than pregnant woman without vivax infection	Papua India Thailand Brazil Venezuela	(Barcus *et al.*, 2007, Poespoprodjo *et al.*, 2008, Tjitra *et al.*, 2008) (Kochar *et al.*, 2009) (Nosten *et al.*, 1999) (Andrade *et al.*, 2010) (Rodriguez-Morales *et al.*, 2006)
Acute lung injury and respiratory distress	Occurring as part of multiple organ dysfunction/ failure It presents high case-fatality rate	India Brazil	(Kochar *et al.*, 2005, Kochar *et al.*, 2009) (Alexandre *et al.*, 2010, Andrade *et al.*, 2010, Lacerda *et al.*, 2012)
Coma	23 times less frequently than in falciparum malaria. Risk 12, 5-fold less than that with *P. falciparum.*	India Papua Brazil	(Beg *et al.*, 2002, Kochar *et al.*, 2005, Thapa *et al.*, 2007, Sarkar *et al.*, 2008, Harish *et al.*, 2009, Kasliwal *et al.*, 2009, Kochar *et al.*, 2009, Parakh *et al.*, 2009, Thapa *et al.*, 2009, Kochar *et al.*, 2010b) (Lampah *et al.*, 2011) (Lacerda *et al.*, 2012)
Acute kidney injury	Associated with multiorgan dysfunction/ failure and with pre-existing co-morbidities	Korea India Brazil	(Chung *et al.*, 2008) (Prakash *et al.*, 2003, Kochar *et al.*, 2005, Kochar *et al.*, 2009, Kute *et al.*, 2012a, Kute *et al.*, 2012b, Sinha *et al.*, 2012) (Alexandre *et al.*, 2010, Andrade *et al.*, 2010, Lacerda *et al.*, 2012)
Multiorgan dysfunction/failure	Possibly due to a bacterial co-infection	Brazil Korea Malaysia	(Alexandre *et al.*, 2010) (Song *et al.*, 2007) (Barber *et al.*, 2013)
Splenic rupture	Presents high mortality rates	Brazil ---	(Lacerda *et al.*, 2012) (Kapland *et al.*, 1946, Lubitz, 1949, Moses *et al.*, 1997, Imbert *et al.*, 2010)
Splenic infarction	Rare in vivax malaria	India	(Gupta *et al.*, 2010)
Thrombocytopenia	Occurs in 24-94% of patients with vivax malaria	---	(Tan *et al.*, 2008, Kochar *et al.*, 2009, Lacerda *et al.*, 2012)
Jaundice	Present in 36-57% of adults with severe vivax	India Brazil	(Kasliwal *et al.*, 2009, Parakh *et al.*, 2009) (Alexandre *et al.*, 2010, Andrade *et al.*, 2010)

Table 1. *Plasmodium vivax* clinical manifestations

3. Variant proteins and pathology in *P. vivax*

Ongoing researches into the mechanisms that mediate pathogenesis in *P. falciparum* have revealed new ways that could help towards future control of the disease. Current understanding of *P. vivax* pathobiology is far less than the knowledge we have about molecular events which mediate *P. falciparum* pathology. Because many biological differences keep away the behaviour of these two species, many more research efforts are required to fill the knowledge gaps in *P. vivax.*

3.1 *P. falciparum:* pathology and multigene families

One of the key features of *P. falciparum* malaria is the cytoadhesive properties of mature trophozoites and schizonts (Miller *et al.*, 2002) to i) endothelial cells (Newbold *et al.*, 1999), ii) uninfected erythrocytes (rosetting) (Udomsangpetch *et al.*, 1989), iii) other iRBC through platelets (clumping) (Pain *et al.*, 2001a) or iv) the placenta syncytiotrophoblast (Walter *et al.*, 1982). It is widely believed that cytoadhesion is the mechanism used by the parasite to avoid spleen clearance, as splenectomised infected patients with *P. falciparum* present an impairment in parasite tissue sequestration and in consequence, mature stages are found in peripheral blood circulation (Demar *et al.*, 2004, Bachmann *et al.*, 2009). Parasite accumulation in organs such as brain, lung or placenta produces blood flow obstruction, hypoxia and tissue damage that can lead to organic failure and fatal malaria (Miller *et al.*, 2002).

P. falciparum cytoadheres to receptors such as CD36 (Barnwell *et al.*, 1989), intercellular adhesion molecule 1 (ICAM-1) (Berendt *et al.*, 1989), Chondroitin Sulphate A (CSA) (Rogerson *et al.*, 1995), Complement Receptor 1 (CR1) (Rowe *et al.*, 1997) or gC1qR (Biswas *et al.*, 2007). gC1qR is involved in parasite clumping and formation of agglutinates (Biswas *et al.*, 2007), while rosette formation is linked to the expression of the CR1 receptor in the surface of the iRBC (Rowe *et al.*, 1997). Placental malaria is due to parasite adhesion to CSA (Fried *et al.*, 1996) and cerebral malaria is involved with sequestration of parasites in the brain and may involve, at least partially the ICAM-1 receptor (Newbold *et al.*, 1999, Avril *et al.*, 2012, Claessens *et al.*, 2012, Lavstsen *et al.*, 2012, Bengtsson *et al.*, 2013). Finally, CD36 is the parasite receptor located in the inner microvasculature and severity associated to CD36 cytoadhesion is under debate. Although CD36 adhesion is common in malaria uncomplicated isolates (Rogerson *et al.*, 1999), a nonsense mutation in CD36 is associated with protection from severe malaria (Pain *et al.*, 2001b).

The main parasite cytoadhesion ligand is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Baruch *et al.*, 1995). PfEMP1 is expressed at the surface of the iRBC and is anchored in knobs that display focal aggregates of the parasite ligand and provide a platform for adherence (Crabb *et al.*, 1997). PfEMP1 proteins are codified by a subtelomeric multigene family termed *var* (Su *et al.*, 1995), that is composed by 60 different genes per

haploid genome in the 3D7 reference strain (Gardner *et al.*, 2002). Despite the big repertoire of genes that compose the subtelomeric family, each mature parasite within a iRBC expresses a single *var* gene in a process known as antigenic variation (Scherf *et al.*, 1998) in which the parasite has the ability to switch the protein expressed in the surface (Smith *et al.*, 1995, Scherf *et al.*, 1998). As a direct result of antigenic variation, parasites are able to escape the host immunity that develops slowly and even after many exposures, humans are not refractory to malaria parasites although they develop clinical immunity that prevents symptomatic disease (Miller *et al.*, 2002). PfEMP1 members are high molecular weight proteins that ranged from 200 to 350 KDa (Pasternak *et al.*, 2009) and *var* genes present a 6 to 13 Kb classical structure in two exons. The first long exon (3,5-9 Kb), that codifies for the highly polymorphic extracellular region, is highly variable as a result of different combinations of adhesive domains "Duffy Binding Like" (DBL) and "Cysteine Interdomain Rich Protein" (CIDR) as well as a N-terminal segment (NTS) (Smith *et al.*, 2000). On the other hand, the second exon codifies for the highly conserved cytoplasmic domain, known as "Acidic Terminal Sequence" (ATS), which is essential for PfEMP1 anchoring to the knobs (Figure 5).

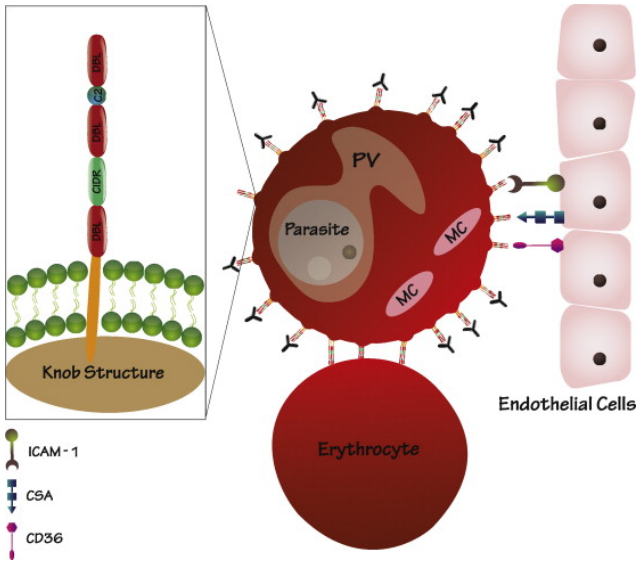

Figure 5. PfEMP1 mediates adhesion to endothelial cells and uninfected erythrocytes. PfEMP1 is anchored to knobs in the surface of the infected erythrocytes and the variable extracellular DBLs and CIDR regions mediate binding to receptors such as ICAM-1, CSA and CD36. (PV: Parasitophorous Vacuole; MC: Maurer's Clefts). Image source: (Pasternak *et al.*, 2009)

Apart from *var* genes, *P. falciparum* subtelomeric domains contain clusters of hypervariable multigene families, such as *rif, stevor* and *Pfmc-2TM* (Box 2, Figure 8). These multigene families are considered members of the 2TM superfamily and encode for exported proteins with a predicted two-transmembrane topology and an intermediate hypervariable loop, which is assumed to be surface exposed. Despite the fact that no functional role can be established for these proteins thus far, it has been hypothesized that RIFIN and STEVOR might play a role in antigenic variation and immune evasion (Petter *et al.*, 2007, Niang *et al.*, 2009, Khattab *et al.*, 2011, Bachmann *et al.*, 2012), whereas members of the PfMC-2TM protein family have been implicated as transporters, channels or receptors (Sam-Yellowe *et al.*, 2004). In addition, it has been recently described that RIFIN and STEVOR might play a role in membrane deformability of the iRBC (Templeton, 2009, Sanyal *et al.*, 2012). Furthermore, *surf* is a multigene family located within or close to the subtelomeric regions and have been associated with putative functions involved in erythrocyte invasion (Winter *et al.*, 2005, Mphande *et al.*, 2008).

3.2 *P. vivax* multigene families

3.2.1 The *vir* multigene family

To identify genes encoding variant antigens in *P. vivax,* a representative genomic library of *P. vivax* was constructed in a yeast artificial chromosomes (YAC) (Camargo *et al.*, 1997). The sequence of a subtelomeric YAC revealed the existence of a *P. vivax* specific multigene family termed *vir* (*P. vivax* variant genes) (del Portillo *et al.*, 2001) that comprised 31 *vir* genes grouped into 6 different subfamilies, termed A to F (Figure 6A). At that time, it was estimated that 600-1000 copies were present in the *P. vivax* genome. The *vir* genes presented a similar structure with a short first exon, a second exon containing a transmembrane domain and a third exon of uniform length (Figure 6B). Their variant nature and subtelomeric location led to the suggestion that VIR proteins were mainly involved in antigenic variation. Moreover, the subcellular localization in the reticulocyte membrane (Figure 6C) and their immunogenicity in natural infections also pointed in that direction.

The first study of expression in natural infections revealed that *vir* genes were not clonally expressed (Fernandez-Becerra *et al.*, 2005). Single cell RT-PCR revealed that at least, two different subfamilies were transcribed concomitantly and immunofluorescence assays showed co-expression in the same parasite of proteins pertaining to different subfamilies (Figure 6D) (Fernandez-Becerra *et al.*, 2005, Oliveira *et al.*, 2006). Moreover, there was no significant difference in the recognition of VIR-GST tagged proteins by sera of first-infected patients compared with sera of multiple-infected patients. Altogether, these data indicate that despite these proteins might have a role in immunogenic evasion, they are inconsistent with a predominant role in antigenic variation through a strict sense allelic exclusion.

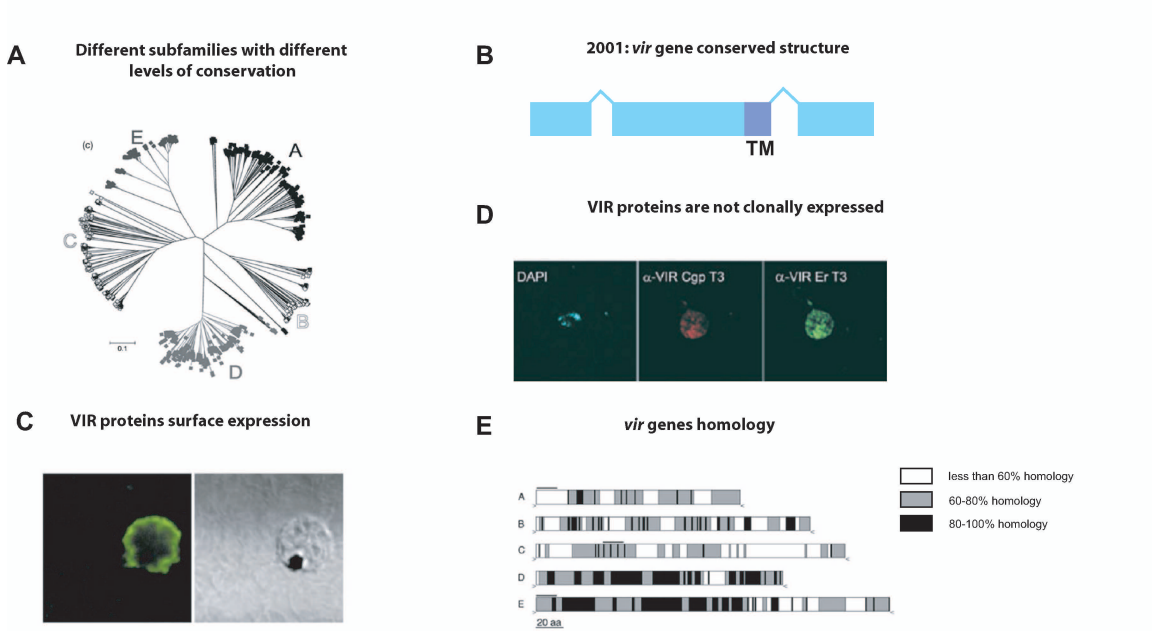

Figure 6. The *vir* multigene family. A. Bootstrap consensus tree of amino acid sequences of VIR subfamilies (A-E) from *Plasmodium vivax* patients (Merino *et al.*, 2006). B. Representation of a consensus *vir* gene showing a three exon structure and the predicted transmembrane domain (TM, purple). C. Immunofluorescence assay using antibodies against subfamily C demonstrated that VIR proteins are express on the surface of the infected reticulocyte (del Portillo *et al.*, 2001). D. Immunofluorescence assay and western blot revealed that VIR proteins are not clonally expressed (Fernandez-Becerra *et al.*, 2005). E. *vir* second exons sequence homology differences amongst subfamilies. White boxes: less than 60% homology; Grey boxes: 60-80% homology; Black boxes: 80-100% homology. (Fernandez-Becerra *et al.*, 2005)

In the absence of a continuous *in vitro P. vivax* culture, analysis of *vir* gene sequences has contributed to a large extend to the knowledge that we have about this family. Before the *P. vivax* annotation was published in 2008, an analysis of sequences, comprising the second variable *vir* exon, from natural parasite populations confirmed the existence of VIR subfamilies that varied in size and allele polymorphisms. Interestingly, different levels of conservation were found in the different subfamilies: subfamilies A-C were highly variable, subfamily D showed two large terminal highly conserved blocks and a central variable block whereas subfamily E presented a large conserved N-terminus block an a polymorphic C-terminus block (Figure 6E) (Fernandez-Becerra *et al.*, 2005, Merino *et al.*, 2006). Later, anotation of the genome of *P.vivax*, Salvador 1 strain (Sal1), revealed that the number of *vir* genes is lower than the initial prediction (Carlton *et al.*, 2008): 346 *vir* genes and 80 pseudogenes were anotated and new algorithms added 6 new subfamilies (G-L) to the previous identified A-F. Yet, it still is the largest multigen family in human malarias. Moreover, *vir* appeared to be more complex than the three exon consensus structure as different numbers of exons (1-5) and widely different sizes (156bp-3434bp) were found (Figure 7A) . Furthermore, probabilistic modelling and predictions of protein and gene structures revelead the existance of motifs conserved among VIR proteins (Figure 7B). In addition, *in situ* hybridization analysis showed that *P. vivax* chromosome ends localize to the nuclear perhipery, where ectopic recombination favours the generation of new gene variants and, it was hypothesized that *P. vivax* uses chromosomal exchange as a mechanism to generate diversity.

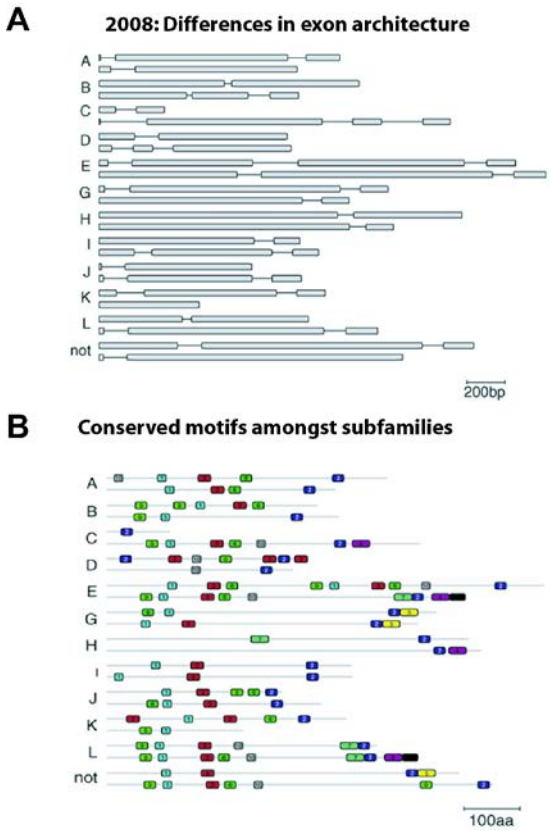

Figure 7. Complexity of the *P. vivax vir* multigene family

A. Representation of a variable *vir* gene structure from different subfamilies. (Fernandez-Becerra *et al.*, 2009). Exons are depicted as boxes an introns as lines. B. VIR proteins motifs conserved amongst different subfamilies (Fernandez-Becerra *et al.*, 2009).

Finally, the analysis of the entire *vir* repertoire gave clues regarding VIR proteins function. Analysis of protein domains and secondary structures from parasite sequences revealed that VIR subfamily A is related to SURFIN (Merino *et al.*, 2006, Carlton *et al.*, 2008), which is located at the surface of iRBC and merozoites (Gardner *et al.*, 2002, Winter *et al.*, 2005), whereas VIR subfamily D contains 2TM domains similar to the P*fmc-2tm* multigene family, located at the Maurer's Clefts (Sam-Yellowe *et al.*, 2004)), a convoluted set of membranes that lie within the erythrocyte's cytoplasm (Wickert *et al.*, 2004). Besides that, approximately only one-half of the VIR repertoire contain a PEXEL-like motif necessary to export proteins to the RBC cytoplasm (Walter *et al.*, 1982, Crabb *et al.*, 1997). Together, these data generate the hypothesis that VIR proteins from different subfamilies might have different sub-cellular localizations and might exert different functions, including immune evasion.

BOX 1: The *Plasmodium* interpersed repeats gene superfamily (PIR)

The *P. vivax vir* multigenic family is encompassed in the *Plasmodium* interspersed repeats (PIR) multigene superfamily. This superfamily is the largest family to date in *Plasmodium spp.* And initially comprised homologous genes in *P. falciparum* (*rif/ stevor*), *P. vivax* (*vir*), *P. knowlesi* (*kir*), *P. berghei* (*bir*), *P. chabaudi* (*cir*) and *P. yoelii* (*yir*) (Janssen *et al.*, 2002, Janssen *et al.*, 2004). The homology of the superfamily was based on gene copy number, intra-family sequence conservation, genome location, secondary structure, expression pattern and gene size and amino-acid similarities range from 30% to 50% between the rodent malarias and from 20% to 30% between *cir* and *vir* multigene families.

It is considered that most members contain a conserved three-exon structure with one transmembrane domain (Janssen *et al.*, 2004). However, as mentioned before a more complex gene structure has been described in most multigenic families. Sub-telomeric genomic location is present in *P. vivax, P. falciparum, P. berghei* and *P. chabaudi*. However in *P. yoelii,* both sub-telomeric and non-subtelomeric family members are found (Fonager *et al.*, 2007) and more random chromosomal positions are occupied in *P. knowlesi* (Pain *et al.*, 2008). Despite *rif* and *stevor* were considered ancestral members of this gene family (Janssen *et al.*, 2004), the low amino acid similarity and the lack of a conserved gene structure is challenging an evolutionary relationship between the RIF/ STEVOR and the PIR superfamily (Cunningham *et al.*, 2010).

3.2.2 New subtelomeric families

Apart from *vir* genes, eight new multigene subfamilies were identified in subtelomeric regions in the Sal1 *P. vivax* genome annotation (Box 2, Figure 8). Therefore the subtelomeric chromosome ends of *P. vivax* remain the most divers among all human malaria parasites. These multigene families were termed from *pv-fam-A* to *pv-fam-E*, and *pv-fam-G* to *pv-fam-I* (Carlton *et al.*, 2008). At present, little research has been conducted on them. Multigene families Pv-FAM-B (PHIST: Plasmodia helical interspersed subtelomeric) (Sargeant *et al.*, 2006), Pv-FAM-E (RAD) and Pv-FAM-H are predicted to be exported (Westenberger *et al.*, 2010). Interestingly, in the transcriptome analysis done by Westenberger and collaborators, while *vir* genes showed lower levels of expression overall, families *pv-fam-A*, *pv-fam-B*, *pv-fam-D* and *pv-fam-E* showed a dramatic upregulation in one patient sample (Westenberger *et al.*, 2010). Additionally, members of family *pv-fam-A* (PvTRAG: Tryptophan Rich Antigen) also present two non-overlapping waves of expression and one member of this family encodes for a protein located in the caveola-vesicle complex and has been shown to elicit a humoral immune response during the course of natural infections (Jalah *et al.*, 2005). Finally, immunofluorescence and immunoelectron assays using antibodies raised against a member of the Pv-FAM-B (PHIST) family revealed a CVC localization pattern of the orthologous in *P. cynomolgi* (Akinyi *et al.*, 2012).

BOX 2

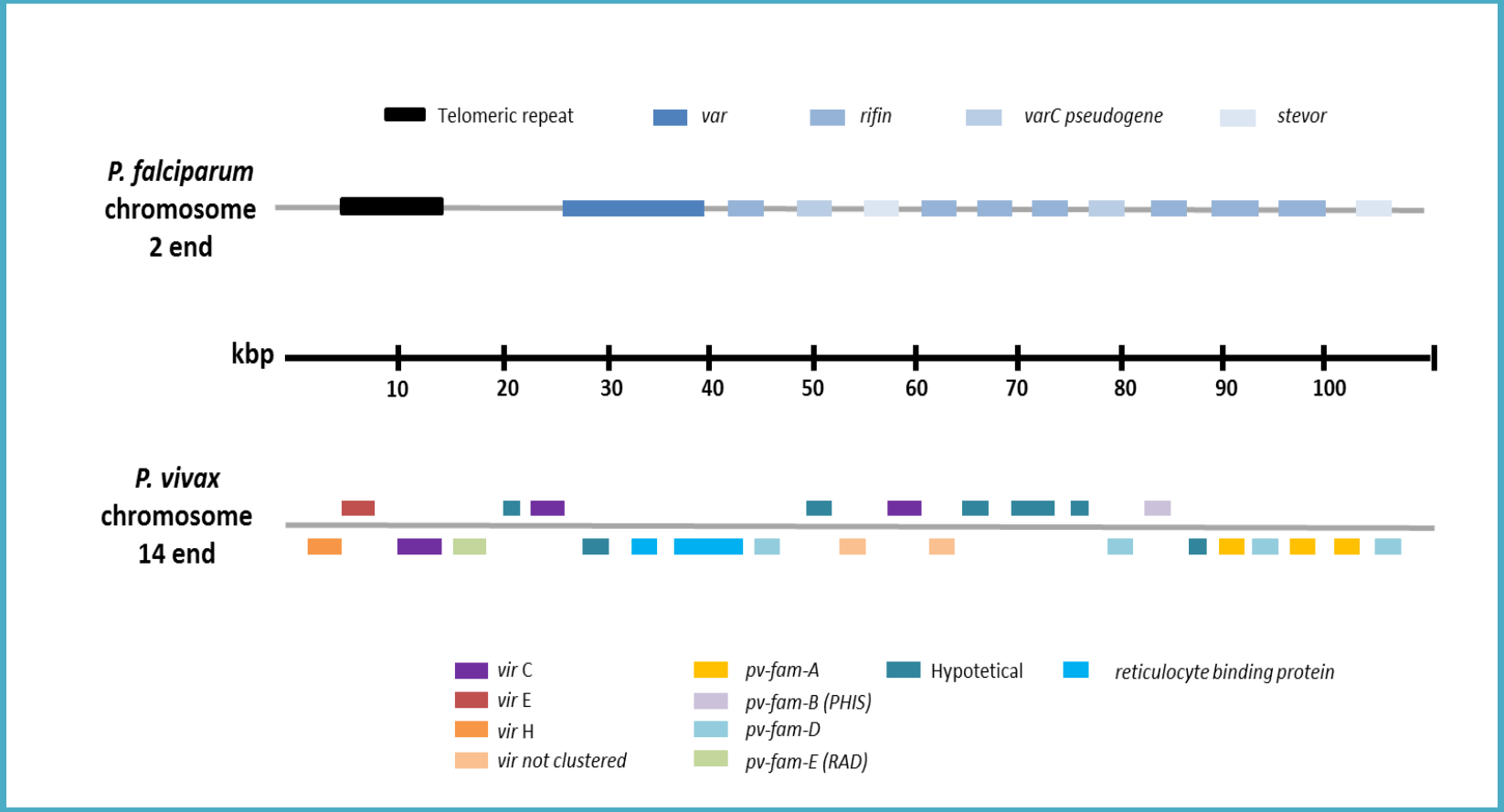

Figure 8. Two representative subtelomeric chromosome ends of *P. vivax* and *P. falciparum* comprising variant multigene families

3.3 *P. vivax* pathogenesis

Despite the fact that most of the symptoms associated to *P. vivax* are believed to be a consequence of a greater inflammatory response, the role of *P. vivax* rosetting and cytoadhesion in pathogenesis is still a matter of debate.

Rosette formation, was first reported in Thailand in 1995 (Udomsanpetch *et al.*, 1995). To date, only four studies have described rosetting in *P. vivax* in the Asia-Pacific region (Udomsanpetch *et al.*, 1995, Chotivanich *et al.*, 1998, Russell *et al.*, 2011, Chotivanich *et al.*, 2012) and even though almost all isolates analyzed formed rosettes, its role in vivax pathophysiology is still unknown. However, a recent study conducted in the Brazilian amazon confirmed that rosetting was a frequent event and that it usually appears associated to an increasing risk of anaemia (Marin-Menendez *et al.*, 2013).

Because mature stages of *P. vivax* are found in peripheral blood of infected patients, it has been widely accepted that there is no cytoadherence of infected reticulocytes. This was due to the fact that autopsy reports in *P. vivax* malaria have shown, historically, overall little evidence of parasite accumulation in the inner organs (Billings and Post, 1915, Bruestsch, 1932, Clark and Tomlinson, 1949) (McGready *et al.*, 2004, Valecha *et al.*, 2009, Lacerda *et al.*, 2012). However, recent articles are challenging the lack of sequestration of *P. vivax* (Figure 9). First, in 2007, Anstey and collaborators described an impairment of the pulmonary capillary vascular changes in physiological pulmonary parameters in *P. vivax* infected patients that pointed to a possible parasite sequestration within the pulmonary microvasculature (Anstey *et al.*, 2007). Later, Carvalho and collaborators performed the most complete cytoadhesion report until the moment in *P. vivax* natural infections from the Brazilian Amazon. They showed cytoadhesion of *P. vivax* to i) endothelial pulmonary cells (HLEC; Human Lung Endothelial Cells), ii) brain cells (SBEC; Saimiri Brain Endothelial Cells) iii) placenta cryosections and iv) CHO cells expressing ICAM-1 under both static and flow conditions. Although *P. vivax* infected reticulocytes adhesion levels were lower than those observed in *P. falciparum,* the strength of the interaction in flow conditions was similar (Carvalho *et al.*, 2010). Moreover, cytoadhesion to HLEC cells was partially inhibited by sera raised against VIR peptides. Recently, two reports have studied cytoadhesion to the

placenta. The first one was a study conducted in Thailand that described binding to the known receptors that mediate adhesion of *P. falciparum* to the placenta, HA and CSA as well as placenta cryosections (Chotivanich *et al.*, 2012). The second one was conducted in Papua New Guinea by Mayor and collaborators and showed the presence of *P. vivax* by PCR in the placenta of women that did not present parasites in peripheral circulation. Despite, placental presence of *P. vivax* was found in the intervillous space of some placentas, direct cytoadhesion evidence was not shown in this study (Mayor *et al.*, 2012). Finally, the presence of large number of parasites in the red pulp of the spleen of a 19-year-old man that suffered a traumatic spleen rupture in the course of an acute untreated no severe infection with *P. vivax*, suggested parasite cytoadhesion into this organ (Machado Siqueira *et al.*, 2012).

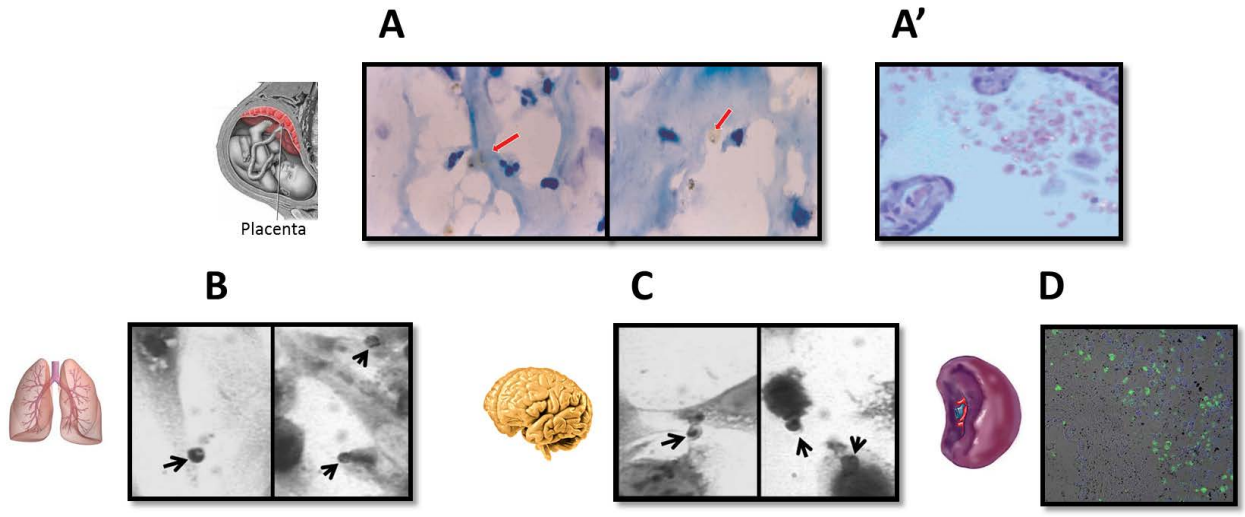

Figure 9. Microphotographies showing *P. vivax* adhesion. A-A'. Placenta criosections (Chotivanich *et al.*, 2012, Mayor *et al.*, 2012); B. Human Lung Endothelial Cells (Carvalho *et al.*, 2010); C. Saimiri Brain Endothelial Cells (Carvalho *et al.*, 2010). D. Spleen section immunofluorescence against *P. vivax* VIR proteins (Machado Siqueira *et al.*, 2012).

3.4 Role of the spleen in malaria

It is widely accepted that *P. falciparum* escapes spleen clearance by cytoadherence of the mature stages of the parasite in the deep capillaries of the inner organs through adhesion mediated by the *var* multigene family. On the contrary, a question remains as to whether *P. vivax* is able to escape spleen clearance to establish chronic infections? Do the VIR proteins play a role in spleen escape?

The spleen is an organ with a complex architecture composed by three different compartments: the white pulp, the red pulp and the marginal zone that lies between the two others. The white pulp, formed by T cell zones and B cell follicles, has an important role in active immune response through humoral and cell-mediated pathways. The red pulp and the marginal zone have been adapted to the filtration and destruction of senescent red blood cells and the elimination by specialized macrophages of inert particles, bacteria, viruses and parasites (Bowdler, 2002).

Infections by malaria parasite induce a dramatic splenic response mostly characterized by variable levels of splenomegaly. This is due to the fact that the spleen plays an important and dual role in malaria. At the same time that it destructs iRBC, it is able to modulate expression of parasite antigens and variant proteins in the surface of iRBC, thus generating pathology. The later phenomenon was first described in splenectomized monkeys infected with *P. knowlesi* that presented a gradual decrease in parasite agglutination in the presence of immune sera, suggesting downregulation of expression variation of SICAvar antigens. After several passages in monkeys without spleen, immune sera failed into agglutinating *P. knowlesi* iRBCs and expression of variant proteins was restored upon reinfection in monkeys with an intact spleen (Barnwell *et al.*, 1983). Afterwards, similar observations were made in *P. falciparum, P. fragile* and *P. chabaudi* (David *et al.*, 1983, Hommel *et al.*, 1983, Handunnetti *et al.*, 1987, Gilks *et al.*, 1990). In splenectomized patients infected with *P. falciparum,* parasites present a lower expression of variant proteins in the surface of the iRBC linked to the appearance of mature stages of the parasites in peripheral blood due to an impairment of parasite tissue sequestration (Demar *et al.*, 2004, Bachmann *et al.*, 2009). Finally, in both immune (Bachmann *et al.*,

2009) and non-immune individuals (Looareesuwan *et al.*, 1993, Chotivanich *et al.*, 2000), the absence of the spleen increases the severity of the disease.

Mouse and monkey models have revealed most of our knowledge of the role of this organ in malaria infections, due to the ethical impediments associated with working with the human spleen. Remodeling of the spleen during murine malarias infection was widely studied by Weiss and collaborators: upon infection with *P. yoelii* non-lethal strain 17X, the "open" circulation of the spleen changed to a "closed" circulation due to the reorganization of highly active contractile fibroblasts (termed barrier cells) that form a physical barrier (Weiss, 1989, Weiss, 1990, Weiss, 1991) whose number increased significantly in both human and mouse pathogenic spleens (Tablin *et al.,* 2002). Besides that, it has been documented that in different murine pathological conditions, the spleen has an important role in erythropoiesis (Palitzsch *et al.*, 1987, Beguin *et al.*, 1989, Bowdler, 2002). For this reason, Weiss hypothesized that barrier cells physically surround nascent erythrocytes providing a safe environment for newly formed reticulocytes (Weiss *et al.*, 1986). Indeed, our group described a few years ago the appearance of longitudinal structures expressing FGF8, a fibroblast growth factor, only in the spleens of animals infected with a *P. yoelii* reticulocyte prone non-lethal strain (17X). Apparently, the reorganization of the spleen caused a loss of directionality and the reduction of the velocity of circulating parasites. In addition 25% of non-lethal parasites were found associated to this structure and a lower number of iRBCs were found within macrophages (Martin-Jaular *et al.*, 2011). As a way to escape spleen clearance, we hypothesized that *P. vivax* infected reticulocytes specifically cytoadhere to barrier cells, through the binding of VIR proteins (or other ligands) exposed in the surface of the infected reticulocyte. As a result, binding to spleen barrier cells will avoid direct contact of infected cells with spleen macrophages and at the same time, it will facilitate reticulocyte encounter upon releasing of new merozoites (del Portillo *et al.*, 2004, Fernandez-Becerra *et al.*, 2009).

4. Functional gene analysis in *P. vivax*

One of the major obstacles to the development of new strategies for malaria eradication is the complexity of the parasites and their interactions with human hosts and insect vectors. *P. falciparum* continuous culture has provided great advances in the research of the biology of this parasite. Genetic tools, such as transfection, provide the opportunity to specifically alter the parasite genome to explore its biology and gain new insights into gene function. Due to the absence of a long term *in vitro* culture, research in *P. vivax* is often restricted to obtaining clinical isolates, often with very low parasitaemias, or to the infection of non-human primates with an adapted strain of the parasite. Under this scenario, it is important to develop alternative strategies to shed light on the biology and pathology of this parasite.

4.1 *Plasmodium* transfection

Historically, the fight against malaria had been focused on empirical approaches. However on the last 20 years, research based on molecular techniques have gained acceptance. Since the complete genome sequences of *P. falciparum, P, vivax, P. knowlesi, P. berghei* and *P. yoelii* (Carlton *et al.*, 2002, Gardner *et al.*, 2002, Hall *et al.*, 2005, Carlton *et al.*, 2008, Pain *et al.*, 2008) are now available, the challenge remains to translate all this information, to understand the biology of the parasite. Transfection techniques allow the alteration of malaria genes to decipher its function and gene tagging, allelic replacement and gene deletion have helped to elucidate key parasite strategies such as host-parasite interactions, pathogenesis, drug resistance or immune evasion. The great advances achieved in deciphering *Plasmodium* biology through reverse genetics can be exemplified in the publication by Rug and collaborators, in which they generated stable transgenic *P. falciparum* strains expressing mutant versions of KAHRP (Rug *et al.*, 2006). As a result, the mutant parasites presented impairment in knob formation and differences in adhesive properties and membrane elasticity, thus revealing the important role that knobs exert in the *P. falciparum* erythrocyte.

Present available tools to modify the *P. falciparum* genome as well as the elements that compose expression vectors present are showed in Table 2. The standard mode of DNA delivery into *Plasmodium* is electroporation. Transfection in *P. falciparum,* is a long and tedious process because it presents a low transfection efficiency in the range of one cell out of 10^6 (O'Donnell *et al.*, 2002) due to the great challenge to target DNA across four membranes and maintain, at the same time, the integrity of the host cell (de Koning-Ward *et al.*, 2000b). Nowadays, *P. falciparum* is routinely transfected either by direct electroporation of ring stage parasites within the red blood cells (Wu *et al.*, 1995) or by purification of mature blood stages of the parasite that will invade plasmid-electroporated red blood cells (Deitsch *et al.*, 2001). Transfection technology was first reported in *Plasmodium* spp. using plasmids that remained as episomes (Wu *et al.*, 1995, Crabb *et al.*, 1996). Today, the identification of *P. falciparum* resistant genes (Table 3) has allowed the integration of plasmids into the haploid genome (Crabb *et al.*, 1996, Wu *et al.*, 1996). However, the selection of *P. falciparum* integrated transfectants is still a time consuming process that can take approximately 3-4 months.

Component	Gene reporter and drug selection	*Confers resistance to (in the case of selection genes)*	*Comments*
Backbone	Contains the origin of replication		
	Contains the Bacterial positive selection drug	Ampicillin, chloramphenicol, kanamycin and tetracycline	Used during plasmid construction and cloning
***Plasmodium* positive selection cassette**	Contains the positive selection gene:		In KO constructs the positive selection gene is flanked by two homologous recombinant regions. Disruption of gene is achieved by introducing a positive selection marker into its open reading frame by double cross-over recombination.
	- TgDHFR-TS (Donald *et al.*, 1993)	WR99210	
	- *h*DHFR (de Koning-Ward *et al.*, 2000a)	WR99210	Most frequently used
	- BSD	Blasticidin S	
	- NEO (Mamoun *et al.*, 1999)	Geneticin (G418)	Less frequently used
	- Puro (de Koning-Ward *et al.*, 2001)	Puromycin	Less frequently used
Expression cassette or targeting sequence	Contains the protein that is being studies. In case of expression cassettes, proteins are usually fused to tags		
	- CAT (Wu *et al.*, 1995) - LUC (Goonewardene *et al.*, 1993)		First reporters used. Frequently used to investigate control of gene expression
	- Green Fluorescence Protein (GFP)		Advantages: Allows visualization of parasites in vivo. Disadvantages: Large size of the tag (27 KDa) and faint fluorescence in some subcellular localizations
	- myc - hemagglutininine (HA)		Smaller tag. Allow both pull down and localization studies Triple repeats of the tag are used to enhance antigenicity
Negative selection cassette	For KO generation. Contains a gene that codifies for an enzyme that converts a non-toxic pro-drug in a potent drug.		
	- HSV-TK (Duraisingh *et al.*, 2002)	Ganciclovir	
	- CDUP (Maier *et al.*, 2006)	5-Fluorocytosine	Most commonly used

Table 2. Components found in an archetypical *P. falciparum* transfection plasmid. Purple: KO requirements. Full gene names are found in the abbreviations section

4.2 *Plasmodium vivax* culture and transfection.

Our understanding in the *P. falciparum* biology has been mostly achieved by the establishment of long-term parasite culture techniques and reverse genetics modifications tools. On the contrary, establishment of a stable and continuous *P. vivax* line in culture has not yet been achieved. Unlike *P. falciparum,* which invades both reticulocytes and normocytes, *P. vivax* invades preferently (if not exclusively) reticulocytes (Kitchen et al., 1938). To overcome the parasite tropism, many research efforts have been focused in finding a reticulocyte enriched blood, such as hemochromatosis blood (Golenda *et al.*, 1997), cord blood enriched in reticulocytes (Udomsangpetch *et al.*, 2007) and reticulocytes derived from hematopoietic stem cells (Panichakul *et al.*, 2007). Despite many efforts are being invested in this direction (reviewed in Table 3 and in (Udomsangpetch *et al.*, 2008, Noulin *et al.*, 2013)), only parasitaemias under 0,015% have been maintained over 80 days (Panichakul *et al.*, 2007). Undoubtedly, the acquisition of the *P. vivax* long term *in vitro* culture will help into bypassing the bottleneck that today presents this parasite in molecular and cellular research.

Consequently to the lack of an efficient *P. vivax* culture, the parasite stable transfection is still unavailable. As an alternative, Pfahler and collaborators succeed in the development of *P. vivax* transient transfection using *Saimiri boliviensis boliviensis* demonstrating that *P. falciparum* transcription signals are recognized by *P. vivax* (Pfahler *et al.*, 2006). As a safeguard against the lack of a *P. vivax* long-term effective culture and stable transfection, our group developed heterologous transfection of *P. vivax* genes to render the parasite more amenable to molecular investigation (Sa *et al.*, 2006). Because promoter regions of *P. vivax* are poorly or not recognized by *P. falciparum* (Azevedo *et al.*, 2007) a *P. berghei* intergenic region was used to drive the expression of both the hDHFR resistance gene and the pvcrt-o reporter gene (Sa *et al.*, 2006). Remarkably, overexpression of the *pvcrt-o* gene in *P. falciparum* 3D7 strain increased chloroquine resistance 2.3-fold and revealed colocalization of *Pv*CRT-o with the endogenous *Pf*CRT protein at the digestive vacuole. Finally, other groups have been successful in assessing PvDHFR-TS interactions with new antifolates using heterologous transfection (O'Neil *et al.*, 2007, Auliff *et al.*, 2010).

Year	*P. vivax* source	Reticulocyte source	Cultivation media	Days in culture	Parasite growth	References
1979	*Aotus* monkeys adapted strains and infected patients (Philippines)	Monkey RBCs fraction	RPMI 1640	8 days	Increased until day 5 and then decreased	(Siddiqui, 1979)
1985	Infected patients (Thailand)	Human RBCs	SCMI 612	6 days	Low parasite density, poor reinvasion	(Brockelman *et al.*, 1985)
1992	*Aotus* monkeys adapted strain	Human blood enriched in reticulocytes by Percoll gradient	RPMI 1640	22 days	Parasite density decreased from the beginning	(Lanners, 1992)
1997	*Aotus* monkeys adapted strain	Hemochromatosis blood enriched in reticulocytes by homologous plasma	McCoy's 5A	15 days	Parasite density remained stable doubling every cycle	(Golenda *et al.*, 1997)
2001	Infected patients (Thailand)	None	RPMI 1640	10 days	Parasitaemia readily decreased (no reticulocytes added)	(Chotivanich *et al.*, 2001)
2007	Infected patients	Cord blood and hemochromatosis blood	RPMI 1640	30-40 days	Low parasitaemia, no linear growth	(Udomsangpetch *et al.*, 2007)
2007	Infected patients	Derived from hematopoietic stem cells	McCoy's 5A	85 days	Very low parasitaemia 0,015%	(Panichakul *et al.*, 2007)
2011	Infected patients	Cord Blood Reticulocytes	McCoy's 5A	Just invasion assays	22,3% maximal parasite density post invasion	(Russell *et al.*, 2011)
2012	Cryopreserved infected patients (Thailand)	Cord Blood enriched in reticulocytes by Percoll gradient	McCoy's 5A	10 days	Low parasitaemia	(Borlon *et al.*, 2012)

Table 3. List of publications and methods used in *P. vivax in vitro* culture attempts. Reviewed in (Udomsangpetch *et al.*, 2008, Noulin *et al.*, 2013)

Bibliography

Hypothesis

vir genes encode the largest subtelomeric multigene family of human malarial parasites. We hypothesize that VIR proteins have different sub-cellular localizations and hence different functions. In addition, we also hypothesize that VIR proteins mediate parasite adherence to the human spleen. Proving these hypotheses correct, will challenge the present view that cytoadherence is exclusive of *P. falciparum* and that the spleen is the evolutionary driven force for cytoadhesion of malarial parasites in other organs.

Objectives

The main objective of this thesis is to establish a deeper understanding of the *vir* multigene family in order to gain insights into the role that it might play in pathology. In the absence of a continuous *P. vivax in vitro* culture, heterologous transfection in *P. falciparum,* studies on wild isolates, and an *in silico* redefinition of this family will be presented in this thesis.

Specific objectives

- To validate the heterologous expression approach for determining sub-cellular location of *P. vivax* proteins by expressing a putatively targeted apicoplast protein.

- To express a selected subset of VIR proteins in *P. falciparum* and determine the sub-cellular localization of these proteins in this system.

- To determine if different sub-cellular localizations of VIR proteins are observed in *P. vivax* wild isolates.

- To biologically validate a new computational approach that redefines the *vir* muligene family.

- To study the adhesion properties of the VIR proteins expressed in the *P. falciparum* transgenic lines to human endothelial receptors.

- To determine the adhesion capacity of the *P. falciparum* transgenic lines expressing VIR proteins and *P. vivax* wild isolates to spleen fibroblasts.

Results

Results are presented as a compendium of articles and a report. The materials and methods section is included in every article and report

1

Functional analysis of *Plasmodium vivax* VIR proteins reveals different subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor

Maria Bernabeu, Francisco Javier Lopez, Mireia Ferrer, Lorena Martin-Jaular, Alain Razaname, Giampietro Corradin, Alexander G. Maier, Hernando A. del Portillo, Carmen Fernandez-Becerra

Cellullar Microbiology, 2012 Mar;14(3):386-400

2

A new computational approach redefines the subtelomeric vir superfamily of *Plasmodium vivax.*

Francisco Javier Lopez, Maria Bernabeu, Carmen Fernandez Becerra, Hernando A. del Portillo

BMC Genomics, 2013 Jan 16;14:8

3

***Plasmodium vivax* subtelomeric variant proteins and cytoadherence to the human spleen.**

Maria Bernabeu, Mireia Ferrer, Richard Thomson, Francisco Javier Lopez, Lorena Martin-Jaular, Aleix Elizalde, Stefanie CP. Lopes, Fabio TM. Costa, Marcus VG. Lacerda, Hernando A. del Portillo and Carmen Fernandez-Becerra

Unpublished work

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Functional analysis of *Plasmodium vivax* VIR proteins reveals different subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor

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Summary

The subcellular localization and function of variant subtelomeric multigene families in *Plasmodium vivax* remain vastly unknown. Among them, the *vir* superfamily is putatively involved in antigenic variation and in mediating adherence to endothelial receptors. In the absence of a continuous *in vitro* culture system for *P. vivax*, we have generated *P. falciparum* transgenic lines expressing VIR proteins to infer location and function. We chose three proteins pertaining to subfamilies A (VIR17), C (VIR14) and D (VIR10), with domains and secondary structures that predictably traffic these proteins to different subcellular compartments. Here, we showed that VIR17 remained inside the parasite and around merozoites, whereas VIR14 and VIR10 were exported to the membrane of infected red blood cells (iRBCs) in an apparent independent pathway of Maurer's clefts. Remarkably, VIR14 was exposed at the surface of iRBCs and mediated adherence to different endothelial receptors expressed in CHO cells under static conditions. Under physiological flow conditions, however, cytoadherence was only observed to ICAM-1, which was the only receptor whose adherence was specifically and significantly inhibited by antibodies against conserved motifs of VIR proteins. Immunofluorescence studies using these antibodies also showed different subcellular localizations of VIR proteins in *P. vivax*-infected reticulocytes from natural infections. These data suggest that VIR proteins are trafficked to different cellular compartments and functionally demonstrates that VIR proteins can specifically mediate cytoadherence to the ICAM-1 endothelial receptor.

Introduction

Plasmodium vivax remains the most widely distributed human malaria parasite with 2.85 billion people living at risk of infection (Guerra *et al.*, 2010). Noticeably, the number of yearly clinical cases seems to be increasing from 70–80 million (Mendis *et al.*, 2001) to 132–391 million cases (Hay *et al.*, 2004), including severe disease and death exclusively associated with *P. vivax* (Kochar *et al.*, 2005; 2010). Unfortunately, research in this species has been largely neglected due to the absence of a continuous *in vitro* culture system for blood stages and the low parasitemias associated with natural human infections. Yet, experts agree that present tools to control *P. falciparum*, the most virulent species, are inadequate to prevent *P. vivax* (Mueller *et al.*, 2009). Therefore, understanding the biology, pathology and epidemiology of *P. vivax* is a priority if control, elimination and eventually eradication of human malaria are to be achieved.

Sequence analysis of a chromosome end from a *P. vivax* isolate revealed the presence of a subtelomeric multigene variant superfamily termed *vir* (*P. vivax* variant genes) likely involved in virulence (del Portillo *et al.*, 2001). The recent annotation of the complete genome sequence of the *P. vivax* Salvador 1 strain (Sal1) revealed the entire *vir* gene repertoire comprising 346 *vir* genes with different numbers of exons (1–5) and widely different sizes (156–3434bp) (Carlton *et al.*, 2008). Moreover, probabilistic modelling and predictions of protein and

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gene structures revealed the existence of several different subfamilies (A-L) and conserved motifs that shuffle among them (Carlton *et al*., 2008). Based on confocal images of wild isolates using an affinity-purified anti-VIRC antibody from a human patient, VIR proteins were thought originally to be exclusively located at the surface of infected reticulocytes (del Portillo *et al*., 2001). Confocal images using other antibodies on single *P. vivax*-infected reticulocytes, however, later demonstrated that there was no clonal expression of VIR proteins and that they could be found inside infected reticulocytes (Fernandez-Becerra *et al*., 2005). Moreover, motif analysis of the complete *vir* gene repertoire revealed that only 160 *vir* genes contain a motif similar to the *Plasmodium* export element/vacuolar transport signal sequence (PEXEL/VTS), linked to export of proteins to the erythrocyte surface and cytosol (Hiller *et al*., 2004; Marti *et al*., 2004). Furthermore, only a subset of VIR D proteins contains a classical PEXEL motif (Sargeant *et al*., 2006). In addition, *in silico* analyses of protein domains and secondary structures (Merino *et al*., 2006) revealed that subfamily A is related to the *P. falciparum* SURFIN proteins found at the surface of merozoites and infected erythrocytes (Winter *et al*., 2005). Also, subfamily D members contain 2 transmembrane (2TM) domains similar to the Pfmc-2TM multigene family located at Maurer's clefts (Sam-Yellowe *et al*., 2004). All together, these data strongly suggest that VIR proteins can have distinct subcellular localizations exerting different functions. These different functions have been speculated to involve antigenic variation (del Portillo *et al*., 2001), spleen cytoadherence (del Portillo *et al*., 2004), and adherence to the lungs (Anstey *et al*., 2009). Noticeably, the first evidence of *in vitro* adherence of *P. vivax*-infected reticulocytes to endothelial cell receptors, partly mediated by VIR proteins, has been recently reported (Carvalho *et al*., 2010).

Previous studies have demonstrated the feasibility of using heterologous transfections in *P. falciparum* to address subcellular localization and function of *P. vivax* proteins (Sa *et al*., 2006). Thus, overexpression of the *pvcrt-o* gene in *P. falciparum* 3D7 strain increased chloroquine resistance 2.3-fold and revealed colocalization of *Pv*CRT-o with *Pf*CRT at the digestive vacuole. Moreover, it has been recently shown that this strategy has been successful in assessing PvDHFR-TS interactions with new antifolates (O'Neil *et al*., 2007; Auliff *et al*., 2010). In this study, we generated *P. falciparum* transfectant parasites expressing HA-tagged or GFP-tagged VIR proteins belonging to subfamilies A (VIR17-like), C (putative VIR14) and D (VIR10 related), and demonstrated that, when expressed in *P. falciparum*, they trafficked to different subcellular compartments. Moreover, using polyclonal antibodies against conserved motifs of VIR proteins, different subcellular localization of VIR proteins was observed in *P. vivax*-infected reticulocytes. Remarkably, phenotypical analysis revealed that the transgenic line exposing VIR14 at the surface of iRBC, specifically mediated cytoadherence to different endothelial receptors under static conditions but only to ICAM-1 under flow physiological conditions.

Results

Expression of P. vivax vir *genes in* P. falciparum

To determine whether *vir* genes could have different subcellular localizations, we selected three *vir* genes whose deduced amino acid sequences and/or motifs were similar to *P. falciparum* proteins located in different cellular compartments. Thus, *vir17-like* (PVX_112645) from subfamily A has sequence similarities to *P. falciparum* SURFIN proteins located at the surface of iRBC and merozoites (Winter *et al*., 2005); *putative vir14* (PVX_108770) from subfamily C contains a J-domain recently proposed to traffic proteins through the cytosol independent of Maurer's clefts (Kulzer *et al*., 2010); *vir10* related (PVX_102635) from subfamily D has a single exon structure with 2 TM domains and as shown in PlasmoDB ORTHO-MCL predictions it has a *P. falciparum* exported protein orthologue (PF11_0038). In addition, VIR14 and VIR10 contain a pentameric PEXEL-like motif linked to export of proteins to the erythrocyte surface and cytosol *albeit* in different positions (Fig. 1A and Table S1) (Hiller *et al*., 2004; Marti *et al*., 2004). As a control, we selected the *P. vivax acyl carrier-protein 4* (*PvACP4*) (PVX_003880), which contains a bipartite signal that should traffic this protein to the apicoplast (Foth *et al*., 2003).

The three *vir* genes and *pvacp4* were amplified from Sal 1 genomic DNA and cloned into the plasmid pARL1a– under the control of the *pfcrt* promoter region (Crabb *et al*., 2004) and modified to have either a triple haemagglutinin (3HA)-tag or a GFP-tag (Fig. 1B). Following transfection into 3D7 parasites and drug selection with WR99210, four different transgenic lines were obtained, 3D7_vir17-GFP, 3D7_vir14-3HA and 3D7_vir10-3HA and 3D7_ACP4-GFP. Transcription of *P. vivax* genes was assessed by RT-PCR. Of interest, mature and processed transcripts of predicted sizes were detected in the transgenic lines (Fig. 1C). In addition, Western blot analyses using anti-HA or anti-GFP antibodies confirmed that all parasite transgenic lines expressed *P. vivax* fusion proteins of expected sizes *albeit* to different levels (Fig. 1D). These data demonstrate that *P. vivax* genes, with different AT-content and number of introns (Table S1), can be transcribed, processed and translated by *P. falciparum*.

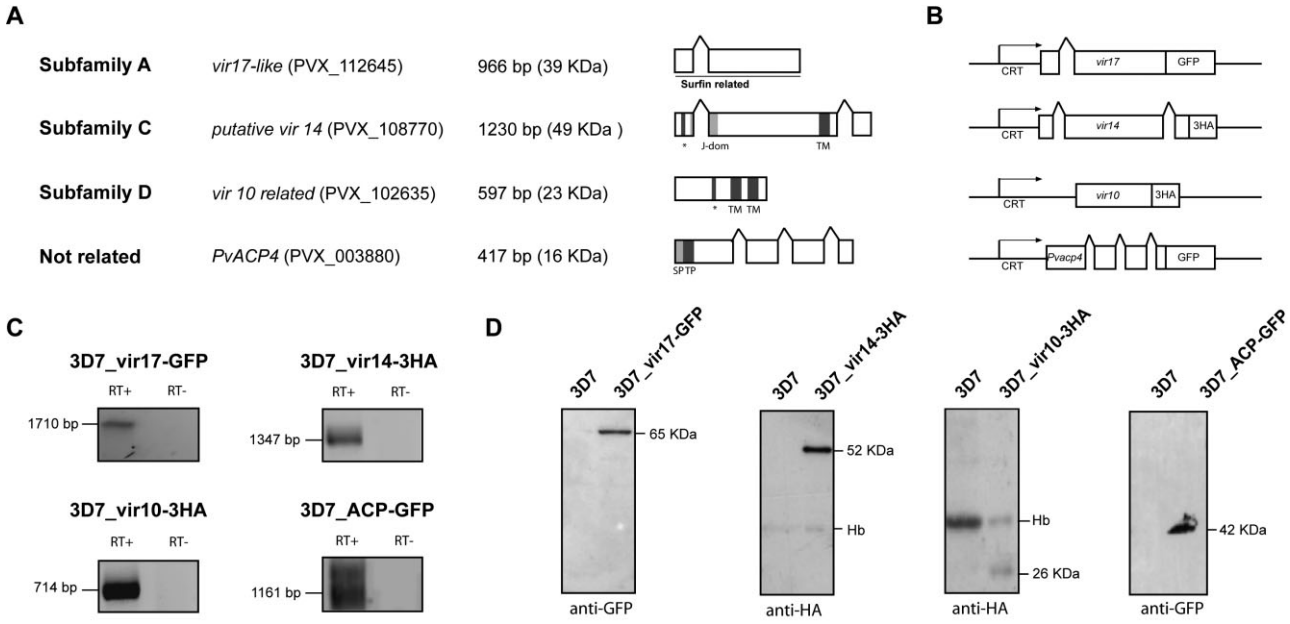

Fig. 1. Schematic representation of *P. vivax* genes and expression in *P. falciparum.*
A. General structure of the *P. vivax* genes expressed in *P. falciparum.* Molecular weights of each gene coding sequence and protein are given in base pair (bp) and kiloDaltons (kDa). Gene representation: exons as boxes and introns as linking lines; asterisk (*): PEXEL-like motif; TM: Transmembrane domain; J-dom: J-domain; SP: Signal Peptide and TP: Transit Peptide.
B. Diagram of the expression cassette of the transgenic lines.
C. RT-PCR expression of *P. vivax* genes in transgenic *P. falciparum.* 3D7_vir17-GFP (upper-left panel), 3D7_vir14-3HA (upper-right panel), 3D7_vir10-3HA (lower left panel) and 3D7_ACP-GFP (lower right panel). Molecular weights are given in base pair (bp). RT+ indicates cDNA treated with reverse transcriptase and RT– not treated.
D. Western blot expression analysis of *P. vivax* proteins in transgenic *P. falciparum.* Wild-type *P. falciparum* 3D7 as well as *P. falciparum* transgenic lines 3D7_vir17-GFP, 3D7_vir14-3HA, 3D7_vir10-3HA and 3D7_ACP-GFP were subjected to western analysis and probed with anti-HA (middle panels) and anti-GFP (right and left panel). Molecular weights of *P. vivax* proteins fused to the tags are given in kiloDaltons (kDa).

Immunofluorescence analysis reveals different subcellular localization of VIR proteins

To determine the subcellular localization of the different *P. vivax* proteins in the *P. falciparum* transgenic lines, immunofluorescence assays (IFAs) were carried out with antibodies raised against HA or GFP (Fig. 2). In early trophozoites stages, all three proteins were found inside the parasite body. Noticeably, VIR17 was still located in the parasite body at late trophozoites stages and around the merozoites in schizonts (Fig. 2A, upper panels). In contrast, VIR14 was found inside the parasite close to the parasitophorous vacuole membrane (PVM) and VIR10 within the erythrocyte cytosol in trophozoite stages and yet both proteins were later found in the erythrocyte membrane during mature schizont stages (Fig. 2A, middle and lower panels). As expected, PvACP4 colocalized with the *P. falciparum* ACP at the apicoplast (Fig. 2B).

To address the exact localization of VIR17, we performed co-immunofluorescence assays using anti-GFP and anti-PfMSP1(19) antibodies using mature schizonts as well as free merozoites (Fig. 3A). Results from these experiments showed that VIR17 remains at the apex and spread around the merozoite and that there is not clear colocalization of both proteins at the surface of merozoites. These results were confirmed by immunoelectron microscopy using anti-GFP antibody (Fig. 3B). To exclude the possibility that the GFP tag does not determine VIR17 location, we generated a stable transgenic line expressing VIR17 fused to 3xHA. Similar results as those obtained with VIR17 fused to GFP were observed (Fig. S1).

To further demonstrate the different subcellular localization of VIR proteins in these transgenic lines, we generated polyclonal antibodies against two long synthetic peptides (LP1 and LP2) representing conserved VIR motifs. These motifs, corresponding to globular domains, TM domains and PEXEL sequences were predicted using MEME models (Carlton *et al.*, 2008). For this study, we synthesized two long peptides, LP1 (74 aa) containing central core conserved motifs and LP2 (112 aa) containing C-terminus conserved motifs (Fig. 4A). The sequences of these long synthetic peptides are described in the experimental procedures section. Guinea pigs were immunized with these peptides and anti-LP1 and anti-LP2

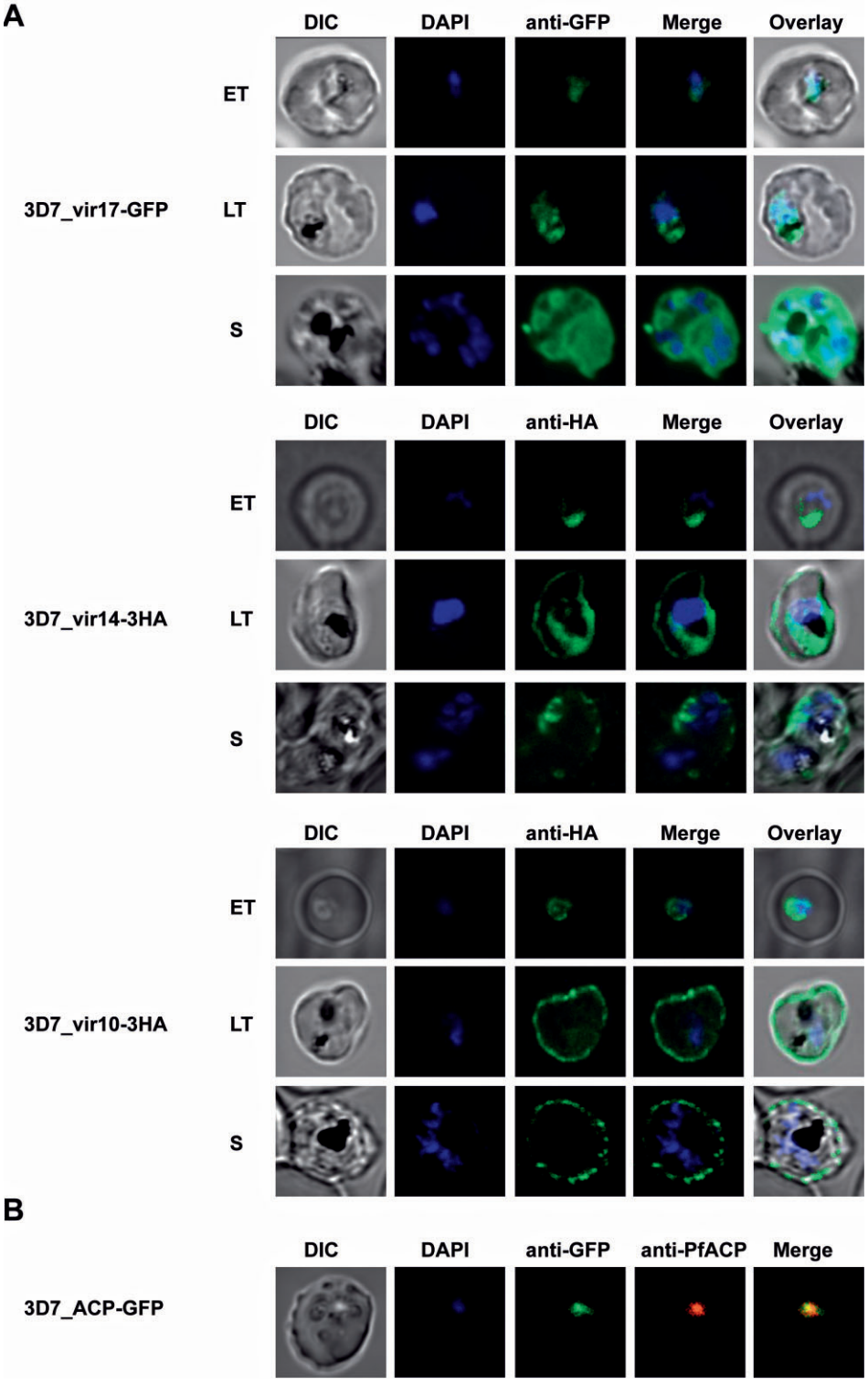

Fig. 2. Subcellular localization of *P. vivax* proteins expressed in the *P. falciparum* transgenic lines.
A. Immunofluorescence images of transgenic iRBCs fixed with paraformaldehyde-glutaraldehyde. 3D7_vir17-GFP (upper panels), 3D7_vir14-3HA (middle panels) and 3D7_vir10-3HA (lower panels). Parasites were labelled with anti-GFP or anti-HA (green) and DAPI for nuclear staining (blue). The first column represents differential interference contrast (DIC) and the fifth column the overlay of all images. For each VIR transgenic line early trophozoites (ET), late trophozoites (LT) and schizonts (S) are shown. Immunofluorescence assays were repeated more than five times and we always observed the same pattern (for VIR17 > 95%, VIR14 > 88% and VIR10 > 90%).
B. 3D7_ACP-GFP parasites fixed with paraformaldehyde-glutaraldehyde were labelled with anti-GFP (green), anti-PfACP (red) and DAPI for nuclear staining (blue). The first column represents DIC and the fifth column the merge of the GFP and the PfACP signal.

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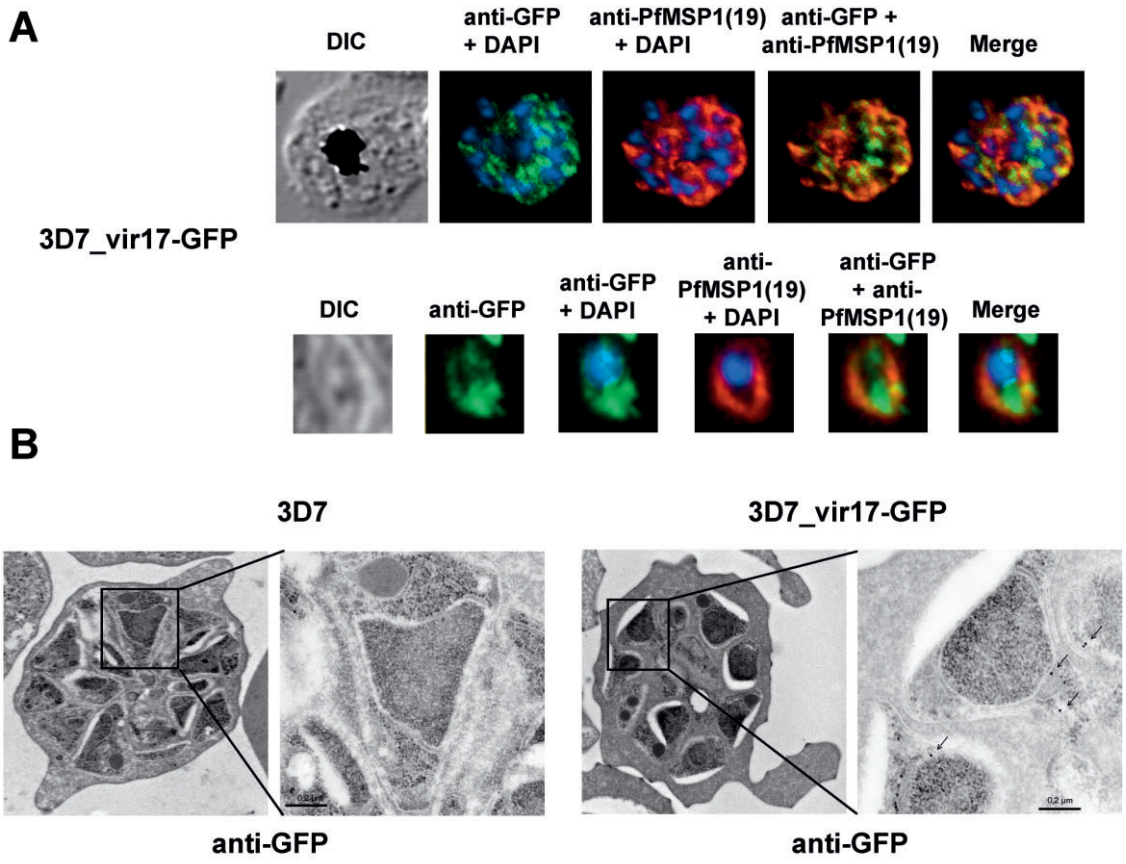

Fig. 3. Subcellular localization of VIR17 expressed in mature stages of *P. falciparum* transgenic line 3D7_vir17-GFP.
A. Co-immunofluorescence images of acetone/methanol fixed schizonts (upper row) and merozoites (lower row) of transgenic line 3D7_vir17-GFP. Parasites were labelled with anti-GFP (green), anti-PfMSP1(19) (red) and DAPI for nuclear staining (blue). The first column represents DIC.
B. Immunoelectron microscopy on 3D7 (left panels) and 3D7_vir17-GFP (right panels) late schizonts. VIR17 was detected with anti-GFP. Gold immunoparticles are indicated by arrows. No gold labelling can be observed in 3D7 nascent merozoites.

antisera were obtained. IFA analysis using these antibodies revealed similar subcellular localizations as those detected against HA and GFP tags (Fig. S2A). The distribution of the conserved motifs in the 3 VIR selected proteins is represented in Fig. S2B.

To address whether VIR proteins could have different subcellular localizations in natural infections, we performed immunofluorescence assays with parasites obtained from a human patient using the anti-LP1 and anti-LP2 antibodies. As shown in Fig. 4B and C, a rim of fluorescence was detected at the membrane of infected reticulocytes containing ring stages (upper panels) whereas distinct fluorescence patterns inside the cytoplasm and parasite were detected in trophozoites (lower panels). Different locations of VIR proteins in the cytosol of ring stages were also observed in *P. vivax*-infected reticulocytes obtained from a second patient (Fig. S3). All together, these results showed that, when expressed in *P. falciparum*, VIR14 and VIR10 are exported to the membrane of iRBC whereas VIR17 remains inside the parasite and as merozoite-associated material. Moreover, they suggest that VIR proteins have different subcellular localizations in natural infections.

VIR14 and VIR10 are exported to the iRBC membrane and are not associated with Maurer's clefts

To address whether VIR14 and VIR10 are exposed at the surface of iRBCs, as opposed to the internal plasma membrane, we performed immunofluorescence staining of live parasites using anti-HA and anti-VIR peptide antibodies. As shown in Fig. 5A, a typical punctuate staining at the surface of iRBCs was readily detected in the transgenic line expressing VIR14 but not VIR10 using either antibody. These results were confirmed by flow cytometry assays of live parasites treated or not with trypsin and stained with anti-VIR peptide antibodies (Fig. S4). Moreover, as different trafficking mechanisms are known to export proteins to the surface of iRBC in *P. falciparum*, we carried out co-immunofluorescence assays using antibodies raised

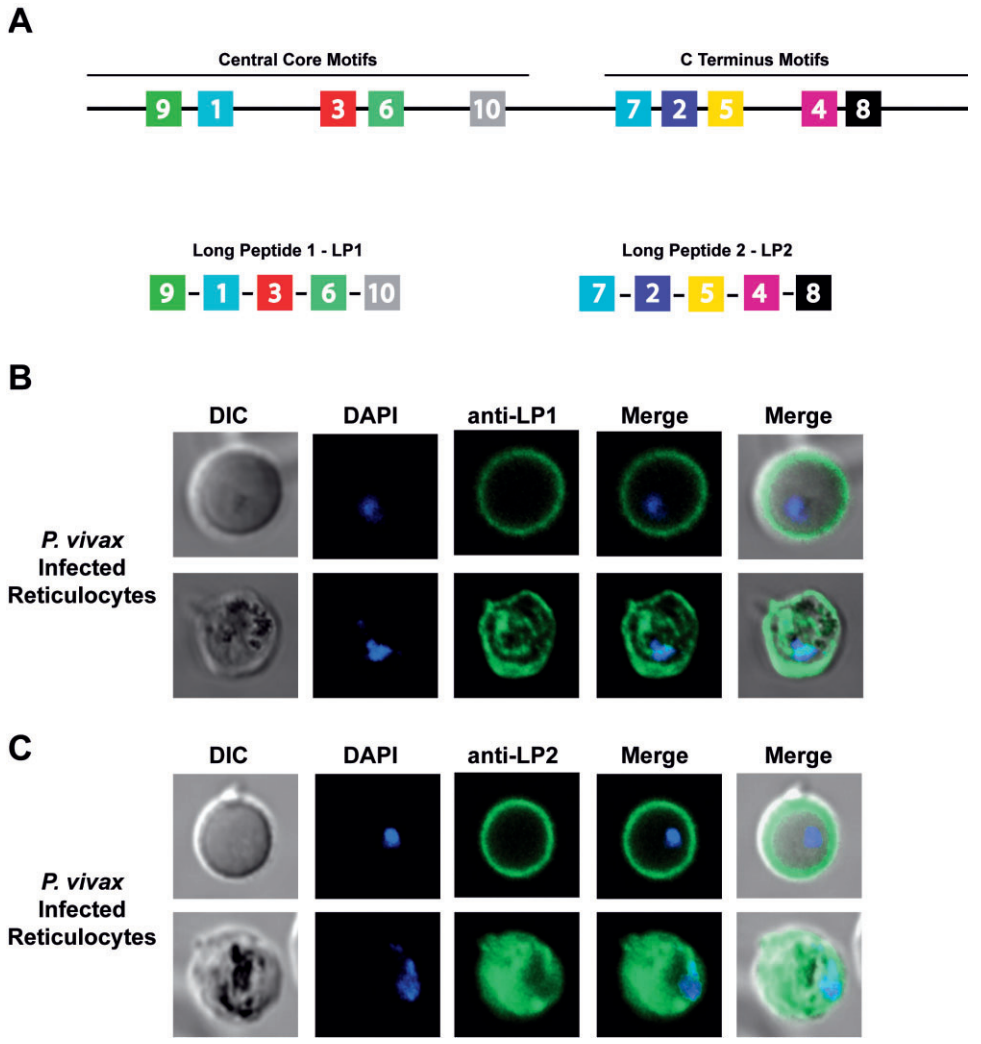

Fig. 4. Anti-LP1 and anti-LP2 recognize VIR proteins in *P. vivax* natural infections.
A. Archetypical distribution of conserved motifs of VIR proteins (upper) and schematic representation of the peptides designed to generate anti-LP1 and anti-LP2 antibodies (lower).
B and C. Immunofluorescence assay of individually infected *P. vivax* reticulocytes fixed with paraformaldehyde-glutaraldehyde. Different parasites stages from *P. vivax* wild isolates were labelled with anti-LP1 (B, green) and anti-LP2 (C, green) and DAPI for nuclear staining (blue). The first column represents DIC and the fifth column the merge of all three images.

against SBP1 (skeleton-binding protein 1), a well characterized *P. falciparum* protein exclusively associated with Maurer's clefts (Blisnick *et al.*, 2000), and against the acidic terminal sequences (ATS) of the PfEMP1 protein which is known to be trafficked through the clefts (Cooke *et al.*, 2006). As shown in Fig. 5, VIR14 and VIR10 showed a dot-like pattern within the iRBC cytoplasm in immature parasites; yet, there was no colocalization of VIR proteins and SBP1 (Fig. 5B), nor of VIR proteins and PfEMP1 even though PfEMP1 seemed in closer proximity to VIR proteins (Fig. 5C). These results indicate that VIR14 and VIR10 are exported to the membrane of iRBCs independently from Maurer's clefts and that only VIR14 is exposed at the surface of the membrane.

Adhesion of VIR14 to different endothelial receptors under static and flow conditions

Recently, cytoadherence under static and flow conditions of *P. vivax*-infected reticulocytes has been described (Carvalho *et al.*, 2010). To determine the potential role of VIR14 as a parasite ligand of endothelial receptors, we first determined the cytoadherence capacity of 3D7_vir14-3HA, 3D7_vir10-3HA and 3D7 to CHO cell lines expressing different endothelial receptors (CD36, ICAM-1, VCAM and E-selectine) under static conditions. In these experiments, the parental strain 3D7 as well as the transgenic line expressing VIR10 served as stringent controls as none of them showed any significant adherence to these

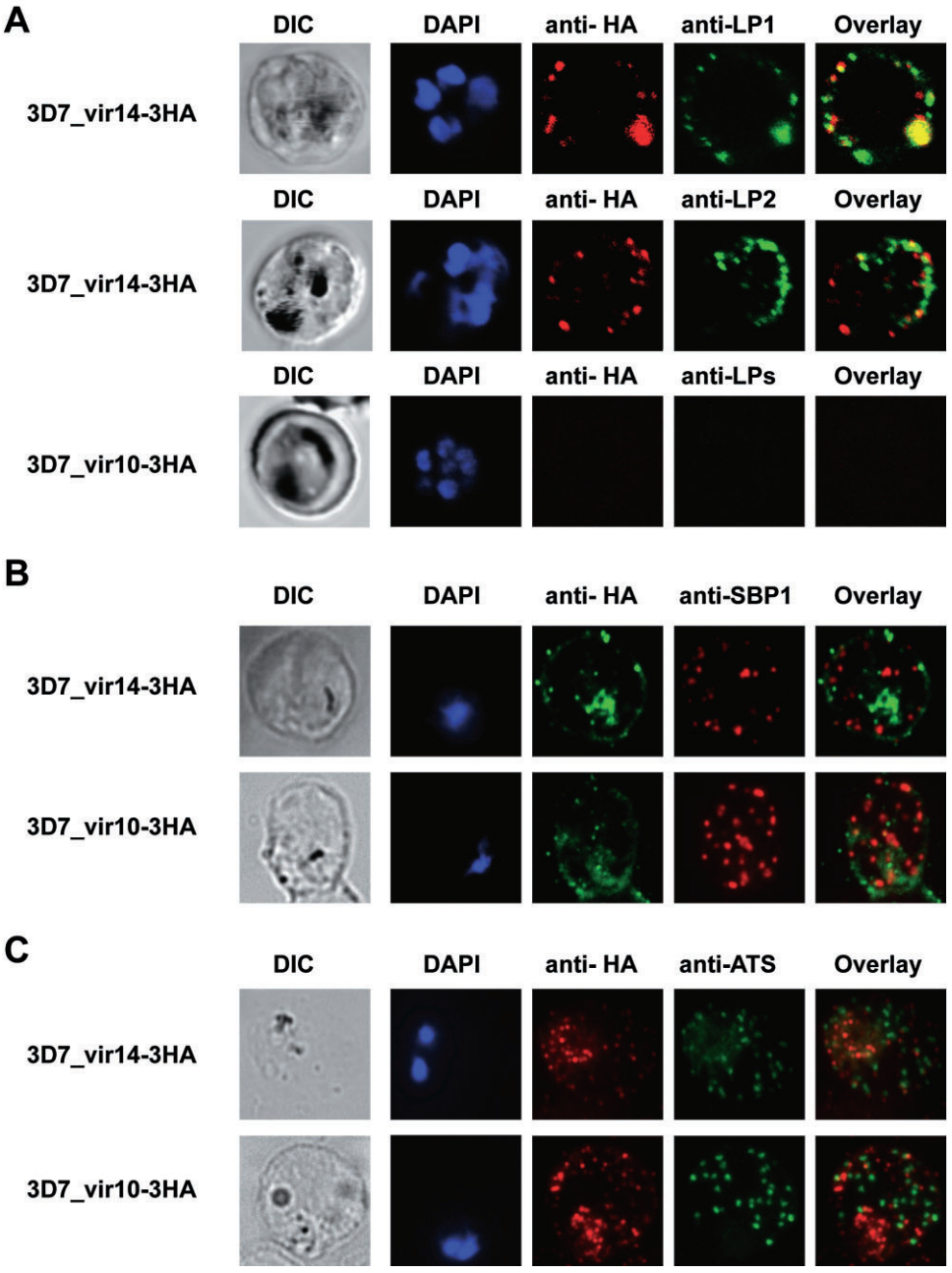

Fig. 5. Colocalization studies in live and fixed transgenic lines.
A. Live immunofluorescence assay. All antibodies anti-HA and anti-LP1/LP2 recognized 3D7_vir14-3HA transgenic line (upper and middle rows) and did not recognize 3D7_vir10-3HA transgenic line (lower row). Parasites were labelled with anti-HA (red) or anti-LP1/LP2 (green) and DAPI for nuclear staining (blue). The first column represents DIC and the fifth column represents the merge of anti-HA and anti-LPs.
B and C. Co-immunofluorescence analysis of methanol/acetone fixed transgenic lines 3D7_vir14-3HA and 3D7_vir10-3HA using either anti-SBP1 (B, red) or anti-ATS (C, green) and anti-HA (B, green; C, red). The merge of the two stains is shown in the fifth column. The first column represents DIC and the second column DAPI nuclear staining (blue).

receptors (less than 15 iRBC/100 CHO cells) (Fig. 6A). In contrast, the transgenic line expressing VIR14 cytoadhered to all CHO cells (from 30 to 100 iRBC/100 CHO cells) (Fig. 6A) but cytoadherence to ICAM1 was the only one significantly and specifically inhibited by anti-VIR LP1/LP2 antibodies or rabbit anti-HA antibodies (Fig. 6B and C, Fig. S5A). Specificity of these results was further demonstrated by using guinea pig anti-PvMSP1 (19) antibodies (Fig. S5A). Next, we performed cytoadhesion assays under physiological flow conditions. Remarkably, only the transgenic line expressing VIR14 remained cytoadhered to CHO-cells expressing the ICAM-1

receptor (Fig. 6D and Movie S1) even under high flow conditions (Fig. S5B). Together, these results demonstrate that VIR14, exposed at the surface of iRBCs, specifically mediates cytoadherence to ICAM-1.

Discussion

Here, to assess subcellular localization and function of VIR proteins, we have generated three transgenic lines in *P. falciparum* that express VIR17 (Subfamily A), VIR14 (Subfamily C) and VIR10 (Subfamily D) proteins. These proteins have homologies, motifs and/or structures similar to *P. falciparum* proteins exported to different cellular compartments where they exert different functions (Marti *et al*., 2004; Sam-Yellowe *et al*., 2004; Winter *et al*., 2005). Our results demonstrated that VIR17 proteins remained inside the parasite whereas VIR14 and VIR10 proteins were exported to the membrane of infected red blood cells (iRBCs). Of note, different subcellular localization of VIR proteins was confirmed in *P. vivax*-infected reticulocytes. Remarkably, VIR14 proteins, exposed at the surface of iRBCs, specifically mediated cytoadherence to ICAM-1 under flow physiological conditions.

Promoter control has been implicated in correct subcellular localization of reporter constructs in *P. falciparum* (Rug *et al*., 2004). As *P. vivax* promoter regions are poorly or not recognized by *P. falciparum* (Azevedo and del Portillo, 2007), we chose the pARL1− vector, which uses the *pfcrt* promoter suitable in determining subcellular localization and trafficking of GFP-tagged proteins in live *P. falciparum*-infected erythrocytes (Crabb *et al*., 2004). In fact, the low expression of proteins driven by this promoter has been successful in avoiding cytotoxic levels of GFP expression. We also modified the pARL1a− vector to study HA-tagged proteins as it has been recently shown that the HA-tag can be interchanged by GFP-tag without altering subcellular trafficking (Kulzer *et al*., 2010). To validate the use of plasmid pARL1a−, we generated a transgenic line expressing the *P. vivax* Acyl Carrier Protein 4 (PvACP4) containing the bipartite sequence that predicts the protein to be exported to the apicoplast (Foth *et al*., 2003). The colocalization of PvACP and PfACP at the apicoplast (Fig. 2B) validates the use of plasmid pARL1a− to determine subcellular localization of *P. vivax* proteins in *P. falciparum* heterologous transfection assays.

All three VIR proteins expressed in *P. falciparum* showed different subcellular localizations. VIR17 is related to the *P. falciparum* SURFIN multigene family located at the surface of iRBC, as well at the PVM and surrounding merozoites (Winter *et al*., 2005; Mphande *et al*., 2008). Of note, one member of this family, $SURFIN_{4.1}$, has been recently found to be present in the parasitophorous vacuole (PV) and on the released merozoites, but not in the surface of infected erythrocytes (Mphande *et al*., 2008). Moreover, $SURFIN_{4.1}$ is only expressed at later trophozoite and/or schizont stages. The IFA pattern as well as the results obtained with the immunoelectron microscopy with the transgenic lines 3D7_VIR17-GFP and 3D7_VIR17-3HA revealed that, similar to $SURFIN_{4.1}$, VIR17 was localized surrounding the nascent merozoites in the mature schizonts but not at the surface of iRBCs (Figs 2 and 3, Figs S1 and S2). In addition, expression data from *P. vivax* isolates revealed that VIR17 is only transcribed during the late asexual blood stages (Bozdech *et al*., 2008). These data suggest that VIR17, similar to what has been previously suggested for $SURFIN_{4.1}$ and others members of the SURFINs family, could play a role in erythrocyte invasion or in antigenic variation in free merozoites. In the absence of functional evidence this function remains to be demonstrated.

In contrast to VIR17, transgenic lines expressing VIR14 and VIR10 showed fluorescence confined to the parasite body at early stages (Fig. 2A) whereas during parasite maturation they were exported to the erythrocyte cytosol and to the membrane of iRBCs in dot like structures (Figs 2 and 5). Export of *P. falciparum* proteins to iRBCs has three main types of secretory pathways (i) the N-terminal PEXEL/VTS motif, a pentameric sequence (RxLxQ/E) located ~20 amino acids downstream of a hydrophobic ER signal (Hiller *et al*., 2004; Marti *et al*., 2004); (ii) PEXEL-negative exported proteins (PNEPs) associated with Maurer's clefts (Spielmann and Gilberger, 2010); and (iii) PfEMP1 proteins containing the N-terminus PEXEL motif but lacking of a signal/hydrophobic sequence (Marti *et al*., 2004; Knuepfer *et al*., 2005). Strikingly, colocalization studies using SBP1 and PfEMP1 as molecular markers of Maurer's clefts and of proteins trafficked to the erythrocyte cytosol through Maurer's clefts (Cooke *et al*., 2006; D'Ombrain *et al*., 2007; Maier *et al*., 2007) failed to show colocalization of either of them with VIR14 or VIR10. Based on Hidden Markov models and the presence of two TM domains, VIR10 was related to the *P. falciparum Maurer's clefts multigene family* Pfmc-2TM (Carlton *et al*., 2008). Our data, however, functionally argue against this relation. Moreover, the genome sequence of *P. vivax* failed to detect homologues of *P. falciparum* proteins associated with Maurer's clefts (Carlton *et al*., 2008). Yet, VIR14 blast searches against the genome of *P. falciparum* revealed homologies with the J-Domain of a *P. falciparum* protein (PF14_0013, score 30.4, e-value 0.00002) pertaining to a family of heat shock proteins that trafficking parasite proteins in a novel pathway (Kulzer *et al*., 2010). These data thus suggest that export of VIR14 and VIR10 in *P. falciparum* is not associated with Maurer's clefts but the exact trafficking pathways of VIR proteins in these transgenic lines remains to be further investigated.

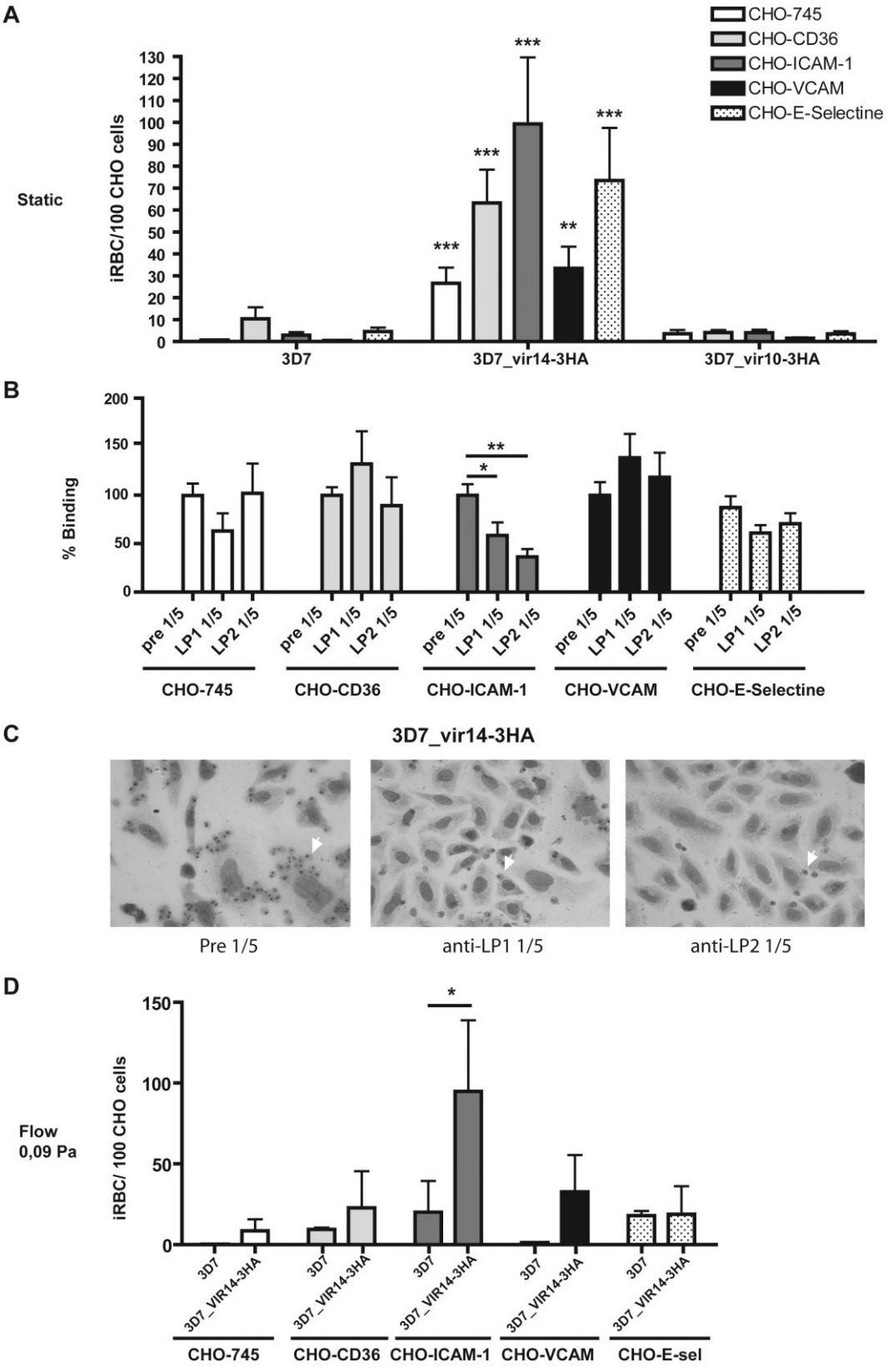
A
CHO-745
CHO-CD36
CHO-ICAM-1
CHO-VCAM
CHO-E-Selectine
Static
iRBC/100 CHO cells
130
120
110
100
90
80
70
60
50
40
30
20
10
0
***
***
***
**
***
3D7
3D7_vir14-3HA
3D7_vir10-3HA
B
% Binding
200
150
100
50
0
**
*
pre 1/5
LP1 1/5
LP2 1/5
CHO-745
CHO-CD36
CHO-ICAM-1
CHO-VCAM
CHO-E-Selectine
C
3D7_vir14-3HA
Pre 1/5
anti-LP1 1/5
anti-LP2 1/5
D
Flow
0,09 Pa
iRBC/ 100 CHO cells
150
100
50
0
*
3D7
3D7_VIR14-3HA
CHO-745
CHO-CD36
CHO-ICAM-1
CHO-VCAM
CHO-E-sel

Fig. 6. Cytoadhesion assays of *P. falciparum* transgenic lines expressing VIR proteins.
A. Cytoadherence of the *P. falciparum* transgenic lines expressing VIR proteins to CHO cells expressing human endothelial receptors in static conditions. Cytoadherence was expressed as iRBC per 100 CHO cells. Transgenic lines that significantly adhered to a specific receptor, compared with 3D7 parental strain, are marked with an asterisk (Mann–Whitney *U*-test) (**$P < 0.01$; ***$P < 0.005$). Data are shown as mean of the binding ± standard error of the means of 3–5 experiments. White, CHO-745; light grey, CHO-CD36; dark grey, CHO-ICAM-1; black, CHO-VCAM; dotted, CHO-E-Selectine.
B and C. Cytoadhesion inhibition assay by anti-LP1 and anti-LP2 antisera. (B) Cytoadhesion inhibition assay of transgenic line 3D7_vir14-3HA to CHO cells expressing human endothelial receptors. Results are shown as % of binding. For these assays, iRBCs treated with pre-immune serum were considered to have a 100% of cytoadhesion. Statistically significant differences in cytoadhesion are shown with an asterisk (*$P < 0.05$; **$P < 0.01$) (Mann–Whitney *U*-test). Mean ± standard error of three independent experiments are shown. (C) Stained image of the cytoadhesion of transgenic line 3D7_vir14-3HA to CHO cells expressing ICAM-1 in the presence of pre-immune sera (left), anti-LP1 (middle) and anti-LP2 (right) at 1/5 dilution. Arrows show adherent iRBC.
D. Cytoadhesion under flow conditions of 3D7 and transgenic line 3D7_vir14-3HA to CHO cells expressing human endothelial receptors at a wall shear stress of 0.09 Pa (two-way ANOVA Bonferroni post test) (*$P < 0.05$). Data are shown as mean of the binding ± standard error of the means of two experiments.

The topology of VIR14 at the membrane of iRBCs is presently unknown even though it is predicted to be a type 1 transmembrane protein. An unsolved issue thus remains why antibodies against conserved VIR motifs and anti-HA antibodies are capable of specifically inhibiting adherence of VIR14 to ICAM-1. Using different TM predictors, we detected the presence of a highly hydrophobic region one hundred amino acids before the predicted TM domain in VIR14 protein. It is thus tempting to speculate that regions N- and C-terminal containing, respectively, VIR conserved motifs and the HA-tag are both exposed explaining the inhibition properties of these antibodies. Alternatively, depending on the topology of VIR14 motifs at the surface of iRBCs, anti-LP1/LP2 antibodies are capable of cross-reacting.

Questions also remain as to the extent that results presented in here can be extrapolated to natural infections. To attempt demonstrating different subcellular locations of VIR proteins in wild isolates, we raised polyclonal antibodies against long-synthetic peptides representing conserved VIR protein motifs. Using these antibodies, we demonstrated that VIR proteins in *P. vivax*-infected reticulocytes can have different subcellular localizations during different phases of the intraerythrocytic developmental cycle. Of interest, unlike PfEMP1 proteins, we readily detected a membrane rim like pattern of fluorescence in ring stages of *P. vivax* parasites from one patient whereas internal labelling in these same stages was observed in *P. vivax*-infected reticulocytes obtained from a second patient (Fig. S3). Unfortunately, due to the low parasitemias in these natural infections (< 0.1%), quantification of different subcellular localizations as well as the use of FACS analysis precluded a more precise definition of these different subcellular localizations. Further studies in wild isolates are thus needed to understand the implications of this expression and exact localizations; yet, these data indicate that VIR proteins are trafficked to different cellular compartments in infected reticulocytes in natural infections.

Life IFA assays of mature trophozoites of the transgenic lines exporting VIR proteins to the iRBCs revealed that only VIR14 was exposed at the surface of the membrane. Unexpectedly, life confocal images of HA-tag staining and either LP1 or LP2 staining showed non-colocalizing patterns of punctated surface fluorescence (Fig. 5A). Similar results have been reported in life IFA colocalization studies of one particular PfEMP1 protein using antibodies against different domains of this PfEMP1 variant (Joergensen *et al*., 2010). Thus, it seems that topological phenomena or artefacts of cross-linking of primary via secondary species-specific antibodies are also the likely explanation for our results (Joergensen *et al*., 2010).

The 3D7_VIR14-3HA transgenic line offered the unique opportunity to functionally demonstrate VIR-mediated cytoadherence to endothelial receptors. Under static conditions, 3D7_VIR14-3HA adhered to all endothelial receptors tested but only the binding to ICAM-1 was significantly and specifically inhibited by antibodies against VIR conserved domains. As several reports have highlighted the importance of performing these assays under flow physiological conditions (reviewed in Cooke and Coppel, 1995; Carvalho *et al*., 2010; Fernandez *et al*., 2010), we allowed 3D7 parasites and transgenic 3D7 parasites expressing VIR14 to directly adhere under flow (0.09 Pa) to CHO-cells expressing different receptors. Remarkably, under these flow physiological conditions, 3D7_VIR14-3HA cytoadhered only and significantly ($P < 0.05$) to ICAM-1 (Fig. 6D). We excluded the possibility that these results were due to expression of *var* genes coding for ligands of ICAM1 (Golnitz *et al*., 2008) by performing real-time PCR analysis of these particular genes in 3D7_VIR14-3HA (Fig. S6). ICAM-1 is widely expressed in organs such as brain, lung, liver and kidney and *P. falciparum* adhesion to ICAM-1 has been associated to several malaria symptoms. Moreover, ICAM-1 has been recently implicated in the cytoadhesion of *P. vivax* parasites (Carvalho *et al*., 2010). Whether cytoadhesion of *P. vivax*-infected reticulocytes to ICAM-1 is related to severe disease remains to be determined.

The specificity of cytoadherence of VIR14 to ICAM-1 prompted us to further computational approaches to search for adhesion properties of this particular VIR14 protein. Of notice, the distribution of conserved motifs in VIR14 was very similar to some members of VIR subfam-

ily C and E, recently predicted to be adhesins using Malarial adhesins and adhesin-like proteins predictors (MAAP) (Ansari *et al*., 2008; Fig. S7). It is thus likely that VIR14 is functionally related to VIR E subfamily members, previously suggested to be implicated in *P. vivax* cytoadherence (del Portillo *et al*., 2004).

In summary, these data reinforce the view that VIR proteins can have different subcellular localizations and functions. Most relevant, it functionally and stringently demonstrates that VIR14, a protein pertaining to the highly variant subfamily C of the *vir* multigene superfamily, is a ligand of the endothelial ICAM-1 receptor. Adhesion of PfEMP1 to ICAM-1 is associated with severe disease in *P. falciparum* and this endothelial receptor has been recently implicated in the cytoadhesion of *P. vivax* parasites (Carvalho *et al*., 2010). Whether *P. vivax* can sequester *in vivo* and be directly involved in pathology is presently under investigation.

Experimental procedures

Human subjects and ethical approval

Informed consent was sought and granted from the two patients that participated in this study and were present at the Hospital Clinic in Barcelona. The procedures were approved by the local Ethics Committee Board (2009/5148).

Parasite culture, plasmid constructs and parasite transfection

Plasmodium falciparum parasites were cultured with human erythrocytes (3% haematocrit) in RPMI media (Sigma) supplemented with 10% AB^+ human plasma using standard methods (Trager and Jensen, 1976). Plasmid pARL1a– (kindly donated by Dr Tobias Spielmann) was modified by adding either a triple haemagglutinine tag (3xHA) or GFP on the KpnI/XhoI site. The 3xHA was generated by designing two overlapping primers, F-HA: TACCACTAGTCCCGGGCTGCAGTACCCATA CGACGTCCCAGACTACGCTTACCCATACGACGTCCCAGACT ACGCT and R-HA: CTCGAGTTATTAAGCGTAGTCTGG GACGTCGTATGGGTAAGCGTAGTCTGGGACGTCGTATGGG TAAGCGTAGTCTGGGACGTCGTATGGGTA. GFP was amplified from the plasmid containing the mutant 3b with primers, F-GFP: GGTACCACTAGTCTGCAGATGAGTAAAGGAGAAGA ACTTTTC and R-GFP: CTCGAGTTATTATTTGTATAGTTCATC CATGCC. Genes *vir17-like* (PVX_112645), putative *vir14* (PVX_107880), *vir10-related* (PVX_102635) and *PvACP4* (PVX_003880) were amplified from *P. vivax* Sal1 gDNA using primers; F-VIR17: GGTACCATGGACGATTCTGATATG; R-VIR17: CTGCAGTCGATTATGGGAGTTCCGTA; F-VIR14: GGATCCGGTACCATGTTCGATCTGGAAGG; R-VIR14: GGAT CCCTGCAGATAATCCAATGTGGAAGCG; F-VIR10; GGATC-CGGTACCATGAAAGATAACATCAAG; R-VIR10: GGATCCCTG-CAGATCTTTACAAAAATGATAA; F-ACP4: GGTACCATGATGA AGGCCATTCTGCTTTG; R-ACP4: CTGCAGCGCATCCGATT TTTTATTTTTCTC. *P. vivax* genes were cloned in the KpnI–PstI site of transfection vectors pARL1a-3HA or pARL1a-GFP. *P. falciparum* 3D7 parasites were transfected as described previously (Deitsch *et al*., 2001). Briefly, 150 μg of each plasmid was used to electroporate (310 V, 950 μF) 600 μl of uninfected red blood cells and this mix was added to about 10^7 parasites. Transfected parasites were selected on 2.5 nM of WR99210 drug and resistant parasites appeared in culture from 30 to 55 days after drug application.

Western blotting

Trophozoite stage parasites were harvested at 5–6% parasitaemia with gelatine flotation and resuspended in PBS with protease inhibitor cocktail (Roche). Samples were boiled and separated on a 10–12% SDS-PAGE, transferred on to Hybond-C nitrocellulose membrane (Amersham) and blocked in blocking buffer (1 × PBS, 0.1% Tween-20, 5% milk powder) overnight. The blot was washed and incubated for 1 h with primary antibody [rat anti-HA (1:250, Roche) or rabbit anti-GFP antibody (1:250, Molecular Probes)] in dilution buffer (1 × PBS, 0.1% Tween-20, 1% milk powder). Subsequently, the blots were washed and incubated for 1 h with secondary antibody conjugated to HRP [anti-rabbit or anti-rat IgG antibody (1:1500, Molecular Probes)]. Bands were visualized by using ECL detection kit (Amersham).

RNA preparation and transcriptional analysis

Trophozoite stage parasites were harvested at 5–6% parasitaemia, treated with 0.15% saponine and lysed in TRIzol reagent (Invitrogen). RNA was extracted using manufacturer's instruction. To minimize the risk of DNA contamination, RNA was DNase treated (Invitrogen). First-strand cDNA synthesis was performed using 1 μg of total RNA and the Superscript-II pre-amplification system (Invitrogen) with random hexamer primers. For RT-PCR, specific forward primers of *P. vivax* genes and specific reverse primers of tags HA and GFP were used.

Production of long synthetic VIR peptides

The polypeptides were synthesized by solid-phase Fmoc chemistry (Atherton and Sheppard, 1989) using an Applied Biosystem synthesizer 433A (Foster City, CA, USA). Derivatized diethylene glycol (DEG, Merck Chemicals, Nottingham, UK) was inserted in between the different individual segments (Corradin *et al*., 2010). The resulting constructs were HPLC purified and the purity (> 80%) was confirmed by analytic C18 HPLC and mass spectrometry (MALDI-TOF; Applied Biosystem). All reagents used were purchased from Fluka (Buchs, Switzerland) and Novabiochem (Laufelfingen, Switzerland).

Peptide sequences:

(LP1)
VKELCKKLVRNLKKIS—DEG—CIYLNYWLYDQ—DEG—KERK DLHDYFKNYDTIKC—DEG—CEKYCTYVTYIKSLYE—DEG—Y DPKDLLSKLDC

(LP2)
IADSPGTLGTVHEELDSNFFRNIIM—DEG—VVGVMMTFFFLYK FT—DEG—VGAFFRGGRGRVHRIPRSFHGQFPG—DEG—KR KGKIFEHNYYEEYEKELAMYGSE—DEG—FLDSQMDRYYLNY QPDQDSYY

Production of antibodies against long synthetic VIR peptides

For the production of antibodies against VIR long peptides LP1 and LP2, 400 g female Dunkin-Hartley guinea-pigs were injected at multiple (2–5) subcutaneous sites with 150 μg immunogen diluted to 0.125 ml with sterile saline and combined with same volume of complete Freund's adjuvant at primary immunization. Previous to immunizations, pre-immune samples were taken from all animals. For subsequent boosts, intraperitoneal injections of 75 μg of antigen with incomplete Freund's adjuvant were given at three-week intervals. All animals received at least three boosts and blood samples were taken 10 days after the last boost. Blood was stored at 4°C overnight and the serum separated after centrifugation (2500 *g* for 10 min), aliquoted and then stored at −20°C.

Indirect immunofluorescence assays

Cultured *P. falciparum* transgenic lines and *P. vivax* isolates were washed in PBS and then fixed with 4% EM grade paraformaldehyde and 0.075% EM grade glutaraldehyde in PBS (Tonkin *et al*., 2004). Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 h at room temperature in 3% PBS-Bovine Serum Albumin (PBS-BSA). Samples were incubated overnight with primary antibody [rabbit anti-HA (1:50, Molecular Probes) or rabbit anti-GFP (1:100, Molecular Probes) or guinea pig anti-LP1 or anti-LP2 (1:200) or rabbit anti-PfACP (1:100 kindly donated by Dr Geoff McFadden)] diluted in 3% PBS-BSA followed by 1 h incubation with secondary antibody [anti-rabbit or anti-guinea pig IgG conjugated with Alexa Fluor 488 (1:200, Molecular Probes)] diluted in 3% PBS-BSA. Nuclei were stained for 10 min with 4,6-diaminido-2-phenylindole (DAPI, 2 μg ml^{-1} diluted in PBS). Confocal microscopy was performed using a laser scanning confocal microscope (TCS-SP5; Leica Microsystems), at microscopy scientific and technical services of Universitat de Barcelona. A second procedure of IFA was done on acetone/methanol (90%/10%) fixed smears with asynchronous parasites. After 1 h of blocking in 3% PBS-BSA, slides were probed with primary antibodies [mouse anti-PfMSP1(19) (1:50) and rabbit anti-GFP (1:50, Molecular Probes), mouse anti-ATS (1:50) or rabbit anti-SBP1 (1:750) and mouse anti-HA (1:1000, 12CA5; Roche) or rabbit anti-HA (1:25, Genscript)] followed by a secondary incubation with anti-mouse or anti-rabbit conjugated to either Alexa Fluor 488 or to Alexa Fluor 594 (1:500, Molecular Probes). All antibodies were diluted in a 3% PBS-BSA solution. Microscopy was performed using a UPlanSApo 100× 1.4 oil objective on a Olympus IX81 Live Cell Imaging Inverted Microscope equipped with an Olympus F-View camera and primarily processed with analogSIS LS Research software package. Images were processed using ImageJ image browser software.

Immunoelectron microscopy

For immunoelectron microscopy the protocol described by Winter *et al*. (2005) was followed with some modifications. Mature parasites were purified on a magnet-activated cell sorting column (MACS) by magnetic separation, purified and fixed in 4% paraformaldehyde/0.1 M phosphate buffer overnight at room temperature. After dehydration and polymerization, embedded cells pellets were cut into thin sections, placed on gold grids, blocked in 1% PBS-BSA, and incubated with anti-GFP (1:10, Molecular Probes) primary antibody for 2 h. The grids were rinsed in PBS and incubated with anti–rabbit IgG antibodies conjugated with 15 nm gold particles for 1 h at room temperature. Samples were examined at 80 kV in a transmission electron microscope Jeol JEM 1010 equipped with a Bioscan Model 792 camera (Gatan) at scientific and technical services of Universitat de Barcelona.

Live immunofluorescence assays

Cultured *P. falciparum* transgenic lines were washed in RPMI. Samples were incubated for 1 h with rabbit anti-HA (1:50, Molecular Probes) and guinea pig anti-LP1 or anti-LP2 (1:200) diluted in RPMI. Followed by 1 h incubation with secondary antibody anti-guinea pig IgG conjugated with Alexa Fluor 488 and anti-rabbit conjugated to Alexa Fluor 594 (1:100, Molecular Probes) diluted in RPMI. Nuclei were stained for 10 min with DAPI (2 μg ml^{-1}). Confocal microscopy was performed using a laser scanning confocal microscope (TCS-SP5; Leica Microsystems) and images were processes using ImageJ image browser software.

Flow cytometry assays

Cultured *P. falciparum* mature stages were purified on a MACS column. Mature stages were resuspended in 1 mg ml^{-1} Trypsin-EDTA solution (Gibco) for 10 min at 37°C. Then, the trypsin reaction was stopped by addition of 10% FBS (Gibco) for another 5 min and parasites were washed three times in RPMI. Tripsinized and non-tripsinized samples were incubated for 2 h with guinea pig anti-LP1 or anti-LP2 (1:25) primary antibody diluted in RPMI. Followed by 30 min incubation with ethidium bromide and secondary antibody [anti-guinea pig conjugated to Alexa Fluor 488 (1:100, Molecular Probes)]. Samples were analysed using LSRFortessa flow cytometer. The gate was set to include infected cells (EtBr+ cells).

Static and flow cytoadhesion and inhibition assays

Static binding assays were performed as described previously (Marsh *et al*., 1988). Briefly, 5×10^4 CHO cells (CHO-746, CHO-CD36, CHO-ICAM-1, CHO-VCAM and CHO-E-Selectine kindly donated by Dr Artur Scherf) (Buffet *et al*., 1999) were seeded in 24 wells with coverslips (Nunc) and left to attach for 2 days. Cells were washed with binding medium and 1×10^6 *P. falciparum* transgenic lines culture were added to each coverslips, each experiment was run in triplicate. Cells plus iRBCs were incubated for 1 h at 37°C and unbound cells were washed by dipping coverslips in binding medium.

For flow cytoadhesion assays, coverslips seeded with CHO cells were mounted in a Cell Adhesion Flow Chamber. The system was connected to a precise infusion/withdrawal pump (model KDS120, IBIDI) to control the flow of the iRBCs suspension or cell-free medium through the perfusion chamber. The outlet of the perfusion chamber was connected to reservoirs containing the iRBCs suspension or binding medium. 1×10^7 iRBC were flowed over for a total of 30 min, and then binding buffer was flowed over for 10 min to

remove unbound cells. The flow rate yielded a wall shear stress of 0.09 Pa, which mimics wall shear stresses in the microvasculature.

For cytoadhesion inhibition assays, transgenic line 3D7_vir14-3HA was incubated for 1 h at 37°C with guinea pig pre-immune sera, anti-LP1, anti-LP2, anti-PvMSP1(19) and anti-HA (1:5 dilution in binding medium) before incubation with CHO cells. To analyse the results, coverslips were fixed with 100% methanol and stained with 10% Giemsa. For each cell line, 1000 cells and the adhering parasites were counted in separate areas of the coverslip. Cytoadherence was expressed as iRBC per 100 CHO cells or % binding rate (inhibition assay). Two to five assays were done in different days. Data were analysed using GraphPad Prism 4 software.

Real-time PCR

Total RNA from 25 h post-infection 3D7 and 3D7_vir14-3HA synchronized cultures were retro-transcribed and cDNA was subjected to real-time PCR. The relative copy number of target genes was determined in an ABI Prism 7500 Real-Time system (Applied Biosystems), using primers specific for the selected *var* genes described previously (Salanti *et al*., 2003) and for Putative-*vir14*; F: GGGTTGTTCTTACGTATCCTGGTAAT; R: GCTGCTCTTTTGCCTTTACA. Reactions were performed in a final volume of 20 µl, including 5 µl of cDNA and 10 µl of Power SYBR Green Master mix (Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Seryl-tRNA synthetase (*seryl-tRS*, PF07_0073) was used as the endogenous control. All runs were done in duplicate and yielded virtually identical Ct (cycle threshold) values. The ΔCt for each individual target gene was determined by subtracting the measured Ct value from the Ct value of the control seryl-tRNA synthetase (PF07_0073). Relative copy numbers were then calculated relative to the expression of its calibrator ($\Delta\Delta C_T$ method).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Subcellular localization of VIR17 expressed in mature schizonts and free merozoites stages of *P. falciparum* transgenic line 3D7_vir17-3HA.
A. General structure of the *vir17* gene expressed in *P. falciparum* and diagram of the expression cassette of the transgenic line 3D7_vir17-3HA.
B. Immunofluorescence images of acetone/methanol fixed transgenic line 3D7_vir17-3HA (schizont, upper row; merozoites, lower row). Parasites were labelled with anti-HA (green) and DAPI for nuclear staining (blue). The first column represents DIC and the fifth column the overlay of all images.
Fig. S2. Anti-LP1 and anti-LP2 recognize VIR proteins in *P. falciparum* transgenic lines.
A. *P. falciparum* paraformaldehyde-glutaraldehyde fixed transgenic lines 3D7_vir17-GFP; 3D7_vir14-3HA; 3D7_vir10-3HA and the parental strain 3D7 were labelled with either anti-LP1 or anti-LP2 (green) and DAPI for nuclear staining. The fourth column represents the merge of the two stains. The first column represents DIC and the fifth column the overlay of all three images.
B. Schematic representation of conserved motifs of VIR17-like (PVX_112645), Putative VIR14 (PVX_108770) and VIR10 Related (PVX_102635).
Fig. S3. Anti-LP1 and anti-LP2 recognize VIR proteins in *P. vivax* natural infections. Immunofluorescence assay of individually paraformaldehyde-glutaraldehyde fixed *P. vivax* infected reticulocytes from a second patient were labelled with anti-LP1 (A, green) and anti-LP2 (B, green) and DAPI for nuclear staining (blue). The first column represents DIC and the fifth column the overlay of all three images.
Fig. S4. Anti-LP1 and anti-LP2 antisera recognize VIR14 at the surface of 3D7_vir14-3HA iRBC in flow cytometry assays. Surface reactivity of 3D7 (white bars), 3D7_vir10-3HA (grey bars) and 3D7_vir14-3HA (black bars) to anti-LP1 and anti-LP2 antisera measured by flow cytometry. Results are shown as the mean ± standard error ratio between the percentages of positive trypsin untreated cells to the positive trypsin treated cells of two independent experiments.
Fig. S5. Cytoadhesion assays of 3D7_vir14-3HA transgenic line.
A. Cytoadhesion inhibition assay by anti-HA and anti-PvMSP1(19) antisera of transgenic line 3D7_vir14-3HA to CHO cells expressing human endothelial receptors. Results are shown as % of cytoadhesion. For these assays, iRBCs treated with pre-immune serum were considered to have a 100% of cytoadhesion. Statistically significant differences in cytoadhesion are shown with an asterisk ($*P < 0.05$) (Mann–Whitney *U*-test). Mean of two independent experiments are shown.
B. Resistance of binding of transgenic line 3D7_vir14-3HA. Enriched 3D7_vir14-3HA transgenic line (1×10^6 iRBC) was allowed to adhere to CHO-745 and CHO-ICAM-1 cells during 1h incubation at 37°C. Coverslips were mounted in a flow chamber system, and binding medium was flowed through at a wall shear stress of 0.09 and 0.36 Pa for 10 min and 5 min respectively. The iRBC that still remain bound at the end of the flow period were counted in 12 randomly selected fields. Mean of two independent experiments are shown.
Fig. S6. Real-time transcription assays of transgenic line 3D7_vir14-3HA.
A. Transcription levels of *var* genes (PFD0615c, PFD0625c, PFD995c and PFD995c/1000c) were measured by real-time PCR using specific primers in 3D7 (white bars) and in the transgenic line 3D7_vir14-3HA (black bars). Values are presented as relative copy numbers, calibrated to the housekeeping gene seryl-tRNA synthetase (PF07_0073). *Var* genes relative copy number was calculated using 3D7 as the calibrator sample.
B. Transcription levels of putative *vir14* (PVX_108770) in 3D7 and transgenic line 3D7_vir14-3HA putative. Values are presented as relative copy numbers, calibrated to the housekeeping gene seryl-tRNA synthetase (PF07_0073). Putative *vir14* was calculated using 3D7_vir14-3HA transgenic line as the calibrator sample.
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Table S1. *Plasmodium vivax* genes expressed in *P. falciparum*.
Movie S1. Representative time-lapse microscopy of the flow adhesion experiment at a wall shear stress of 0.09 Pa of transgenic line 3D7_vir14-3HA to CHO-ICAM-1. The movie was taken from minutes 10 to 15. Circles in the last frame represent adherent iRBCs. Quick-time. S1 (10 MB, 22 s).

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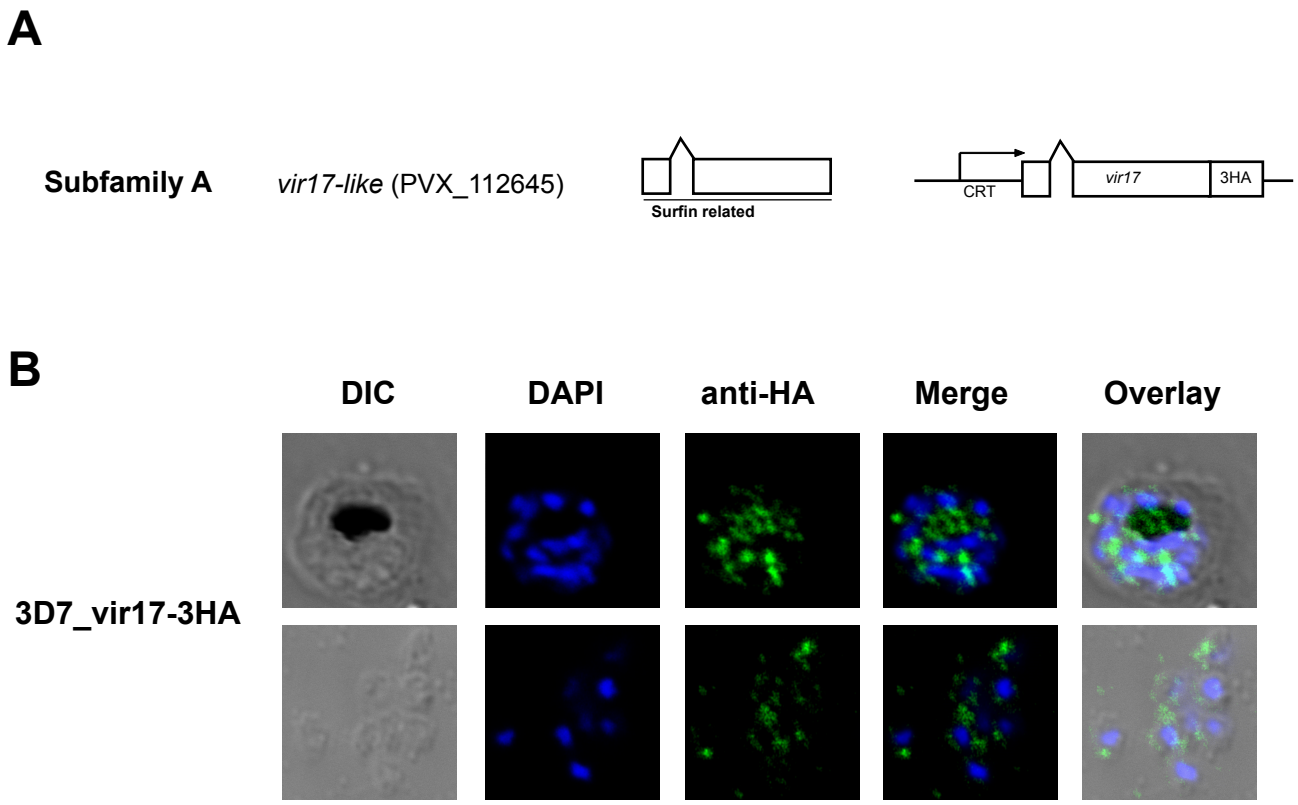

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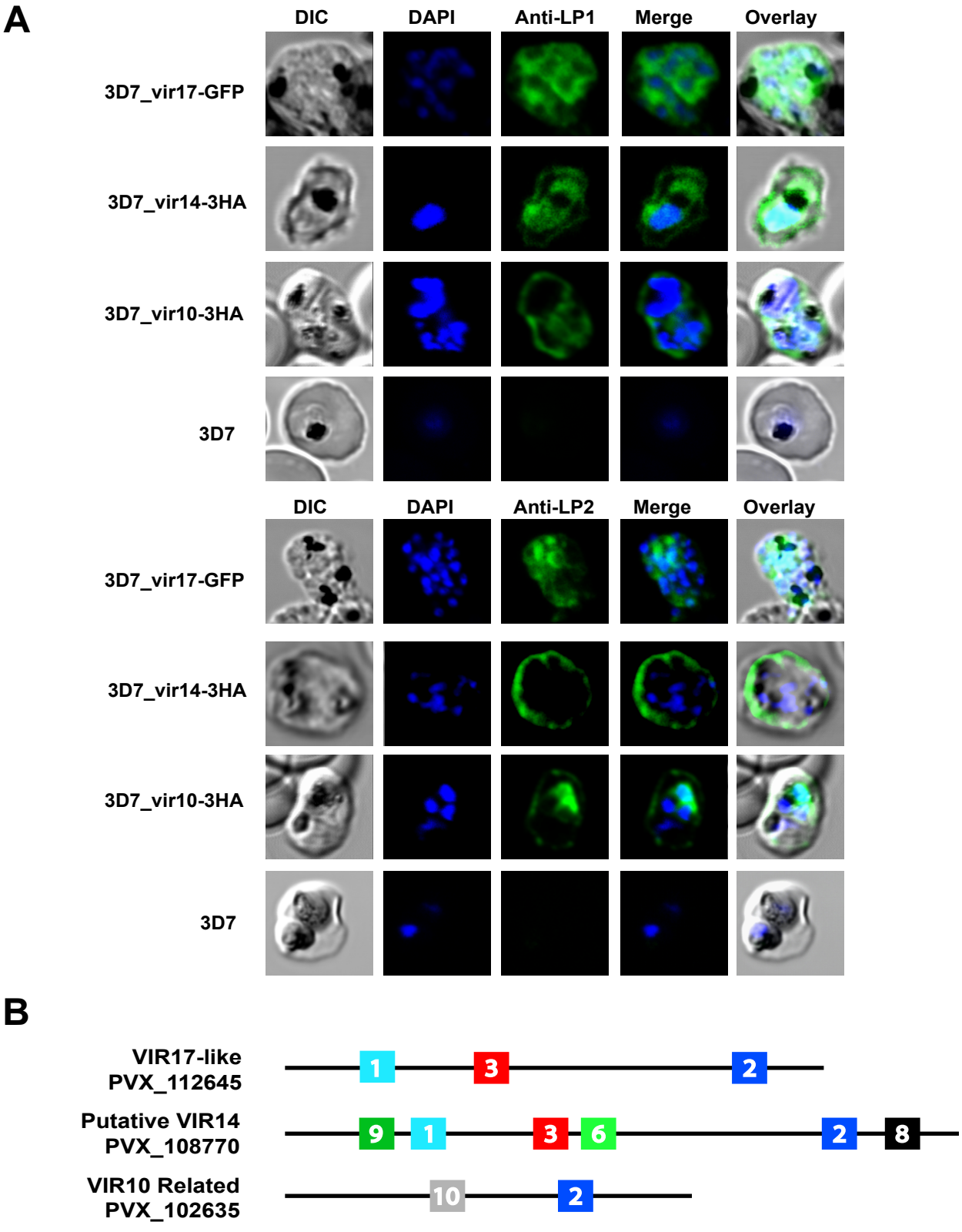

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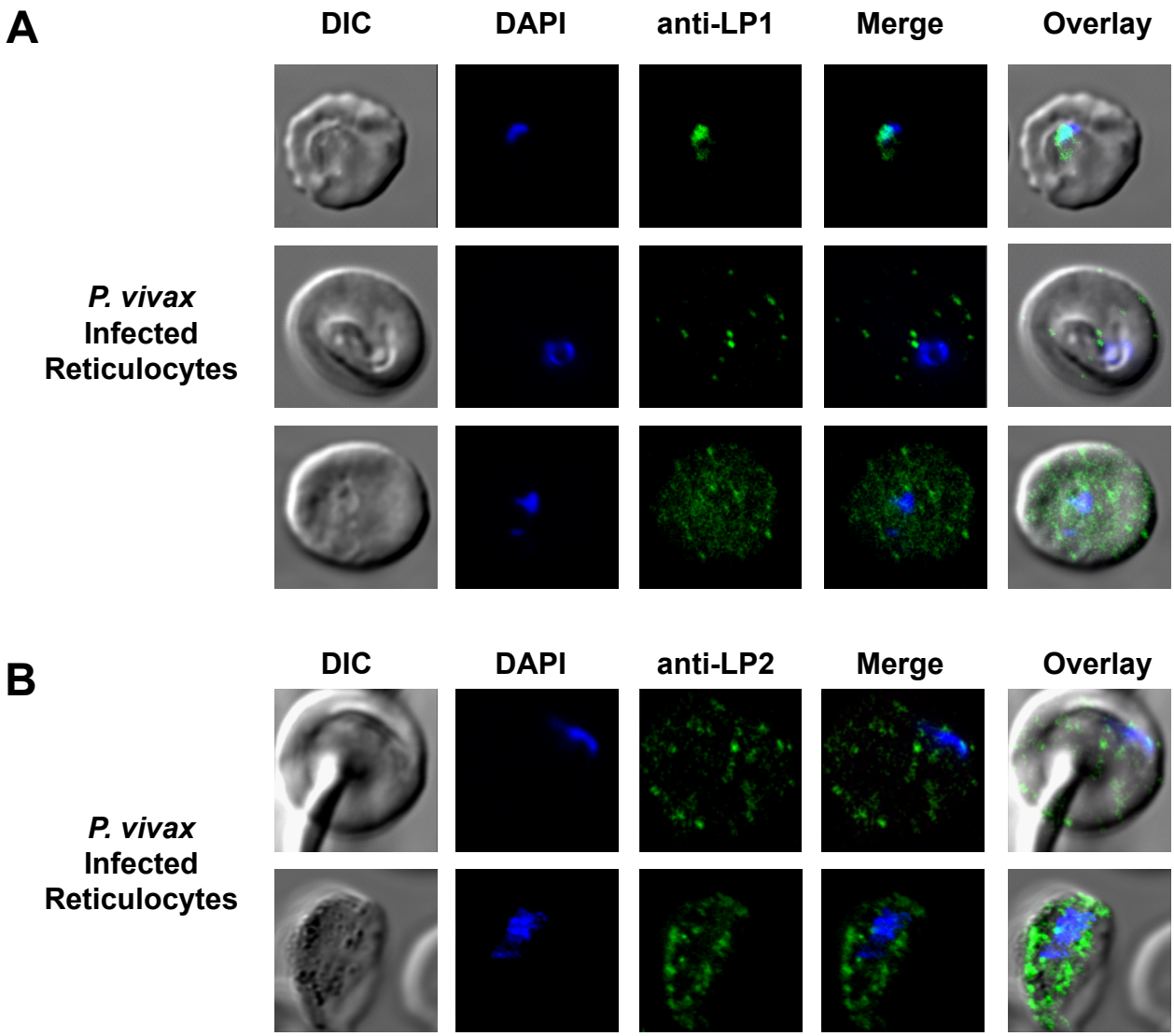

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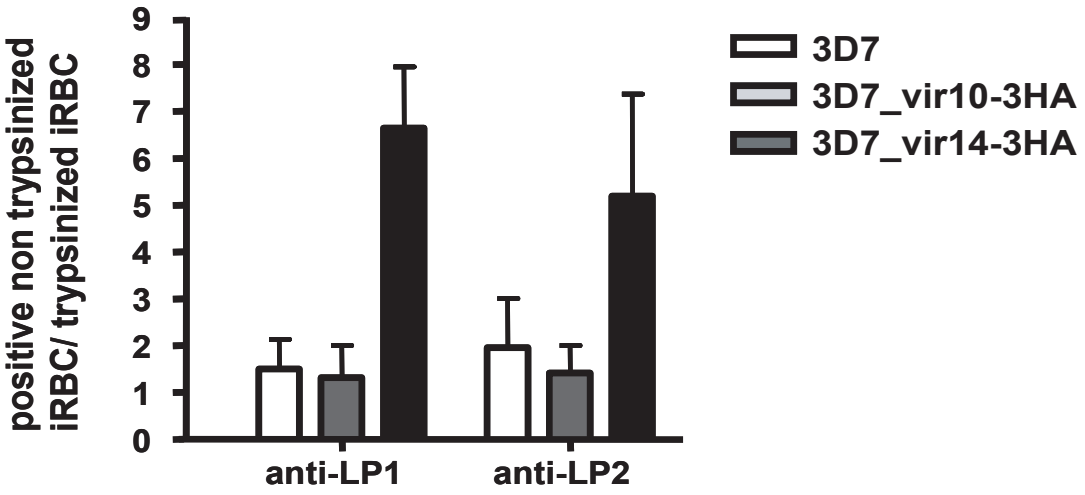

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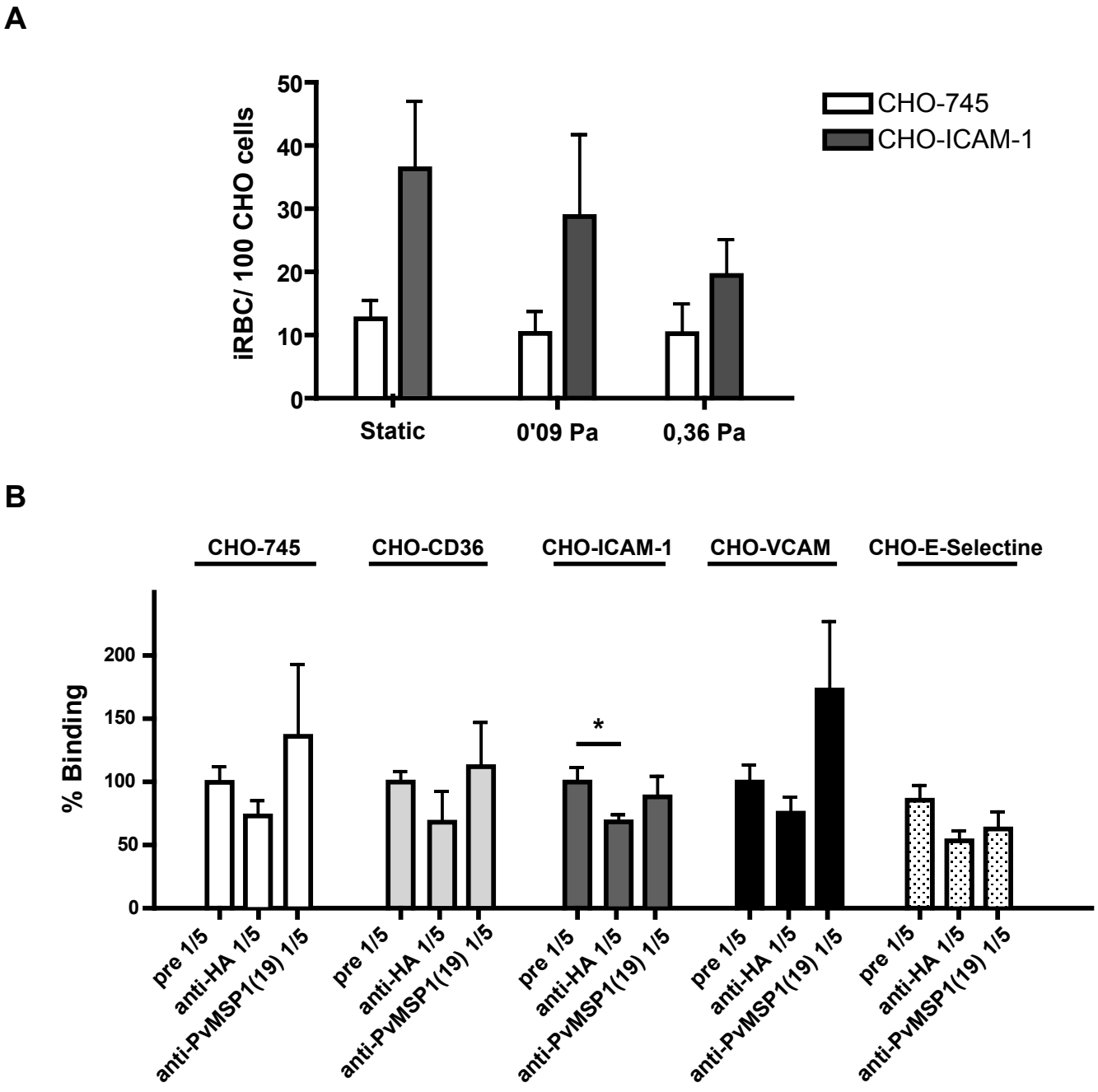

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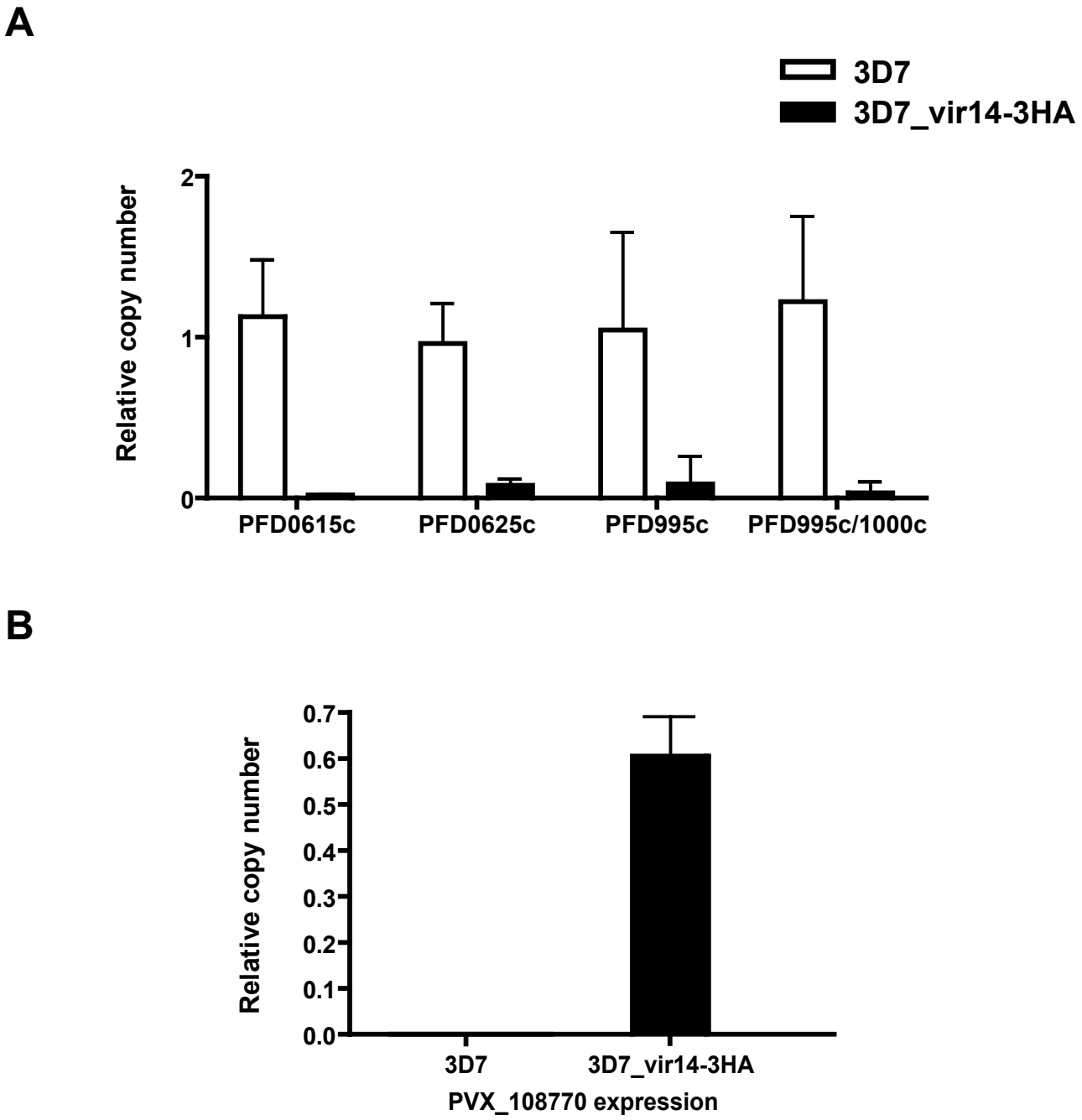

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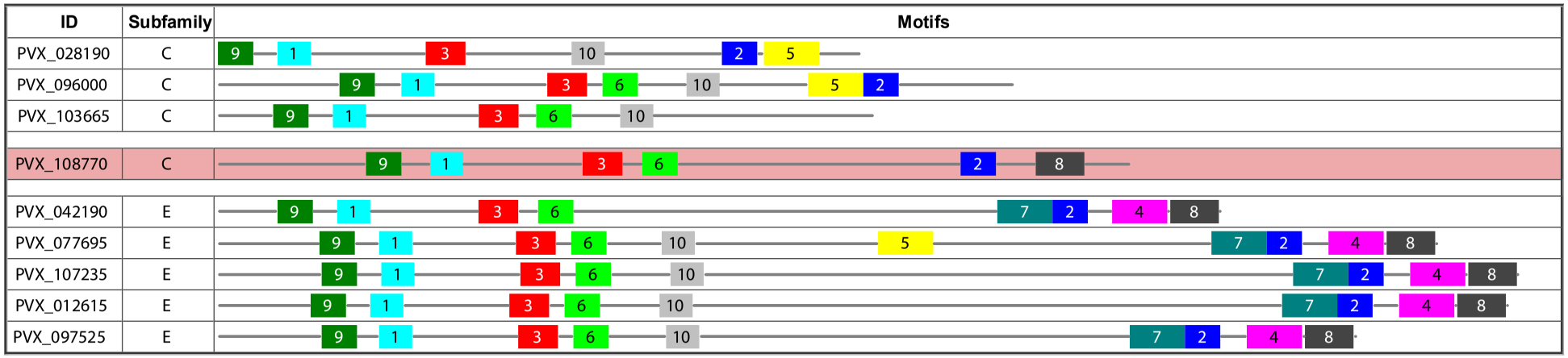

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BMC Genomics

RESEARCH ARTICLE Open Access

A new computational approach redefines the subtelomeric *vir* superfamily of *Plasmodium vivax*

Francisco Javier Lopez[1,3], Maria Bernabeu[1], Carmen Fernandez-Becerra[1] and Hernando A del Portillo[1,2,4*]

Abstract

Background: Subtelomeric multigene families of malaria parasites encode virulent determinants. The published genome sequence of *Plasmodium vivax* revealed the largest subtelomeric multigene family of human malaria parasites, the *vir* super-family, presently composed of 346 *vir* genes subdivided into 12 different subfamilies based on sequence homologies detected by BLAST.

Results: A novel computational approach was used to redefine *vir* genes. First, a protein-weighted graph was built based on BLAST alignments. This graph was processed to ensure that edge weights are not exclusively based on the BLAST score between the two corresponding proteins, but strongly dependant on their graph neighbours and their associations. Then the Markov Clustering Algorithm was applied to the protein graph. Next, the Homology Block concept was used to further validate this clustering approach. Finally, proteome-wide analysis was carried out to predict new VIR members. Results showed that (i) three previous subfamilies cannot longer be classified as *vir* genes; (ii) most previously unclustered *vir* genes were clustered into *vir* subfamilies; (iii) 39 hypothetical proteins were predicted as VIR proteins; (iv) many of these findings are supported by a number of structural and functional evidences, sub-cellular localization studies, gene expression analysis and chromosome localization (v) this approach can be used to study other multigene families in malaria.

Conclusions: This methodology, resource and new classification of *vir* genes will contribute to a new structural framing of this multigene family and other multigene families of malaria parasites, facilitating the design of experiments to understand their role in pathology, which in turn may help furthering vaccine development.

Keywords: Malaria, *Plasmodium vivax*, *vir* genes, VIR proteins, Subtelomeric multigene families, Sequence clustering, Similarity networks, Homology blocks

Background

Plasmodium vivax is the most widely distributed human malaria parasite, with an at-risk population of 2.5 billion people [1]. The widely held misperception of *P. vivax* as being relatively infrequent, benign, and easily treated explains its nearly complete neglect across the range of biological and clinical research. However, recent reports provide abundant evidence challenging this paradigm (reviewed in [2,3]).

Antigenic variation is a regular feature of all *Plasmodium species,* enabling parasites to evade the immune system [4]. Genes putatively responsible for antigenic variation in *P. vivax*, termed *vir* (*P. vivax* variant genes), were initially identified by analyzing a chromosome end from a *P. vivax* wild isolate [5]. Later, the publication of the *P. vivax* Salvador I strain genome sequence allowed the redefinition of the *vir* gene repertoire revealing a total of 346 *vir* genes, including 80 fragments and/or pseudogenes, 12 different subfamilies (A-L) and 84 "unclustered genes" which were not associated to any subfamily [6]. In addition, their gene structure revealed a complex organization including genes with different numbers of exons (1–5) and different sizes (156 to 2316 bp). Of interest, this multigene family shares sequence homology with other *Plasmodium* species and is included within the variant gene superfamily (*Plasmodium* interspersed repeats, *pir*) together with *kir* in *P. knowlesi*, and the *cir*/*yir*/*bir* family in *P. chabaudi*, *P. yoelii* and *P. berghei* [7,8].

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The function of the *vir* multigene family remains largely unknown. Analysis of the expressed *vir* repertoire in natural infections from individual parasites demonstrated that there is no allelic exclusion of *vir* genes and no clonal expression of VIR proteins at the surface of individually infected reticulocytes [9]. In addition, first-time infected *P. vivax* patients had naturally acquired antibodies capable of cross-reacting against different VIR proteins [9,10]. Moreover, only 160 deduced VIR proteins possess the PEXEL-like motif needed for exporting malarial proteins to the host cell surface [11]. Furthermore, subfamilies A and D share structural similarities, respectively, with the *P. falciparum surfin* and *Pfmc-2TM* multi-gene families [12]. This fact, together with the lack of PEXEL motifs in many of the VIR proteins, indicate that these proteins might have subcellular localizations other than the surface membrane of infected reticulocytes and different functions. This possibility, has been recently reported [13]. We thus reasoned that the original clustering of these 346 into a single superfamily might not be accurate and that some of these genes might belong to different multigene families.

In this work, a novel clustering procedure was applied to *vir* genes to re-analyze this subtelomeric multigene superfamily. Results presented here show that genes belonging to subfamilies A, D and H cannot longer be considered *vir* genes and that this computational approach facilitates grouping of unclustered genes, annotations of hypothetical proteins and studies of other multigene families. Procedures were implemented and integrated in web applications (http://bioinfold.fcrb.es/hb and http://bioinfold.fcrb.es/sequence_cluster).

Results

Contextual definitions

To facilitate the understanding of the developed method, we first introduce the definitions of several concepts: *E*-value threshold: the *E-value* parameter stands for the *Expect-value* threshold of the BLAST algorithm, which is used to calculate a similarity value for each two proteins [14].

The *Inflation* value is an input parameter of the Markov Clustering Algorithm [15]. It takes values in [1.1, 10.0] and determines the cluster granularity (the higher the *Inflation* value, the higher the granularity).

Homology Block concept is a key term regarding the search of conserved motifs. An homology block can be defined as a sequence profile determined from a multiple sequence alignment and modelled by a Hidden Markov Model (HMM).

Finally, note that we will name *unclustered* VIR proteins to those VIR proteins which were not previously associated to any subfamily, following the nomenclature by Carlton et al. [6], and *unclassified* VIR proteins to those predicted in this work that could not be associated to any multigene family.

Novel computational methods show VIR subfamilies cluster as independent graph components

The original annotation of *vir* genes was based on JIGSAW predictions, protein domains and sequence alignments [6]. Here, a new algorithm consisting of a pipeline having two recently reported pre-processing techniques [14,16] and the application of the Markov Clustering Algorithm [15] was implemented (Figure 1). In order to validate this clustering approach, we tested this algorithm using protein sequences from the RIFIN/STEVOR, Pfmc-2TM, fEMP1 and SURFIN subtelomeric multigene families of *P. falciparum* [19]. These multigene families have been demonstrated to have different sub-cellular localizations and functions [20-22]. We tested the clustering methodology by using distinct experimental set ups. As a starting point, we first determined the combination of *E*-value and *Inflation* (see "Methods") that allowed this methodology to correctly classify these different subtelomeric multigene families of *P. falciparum*. Results showed that with the *E*-value threshold set at 10^{-1} and *Inflation* value equal to 3, excepting for a single *rifin* gene (PFC0045w) which clustered with *stevor* genes, the procedure correctly clustered the different families (Figure 2A). Using these same values, all previously annotated VIR subfamilies excepting subfamilies D and H constitute a group of strongly related proteins (Figure 2B).

The *E*-value was next progressively restricted until each *P. falciparum* subtelomeric multigene family formed an independent graph component, *E*-value threshold set at 10^{-9} and *Inflation* value equal to 1.5 (Figure 2A). Of note, PFC0045w is still included within the *stevor* group and a single link remains between PFI0070w (*rifin*) and MAL13P1.7 (*stevor*). Using these conditions, five independent graph components were observed for VIR proteins (Figure 2B). One of them represents the VIR core, containing most of the VIR proteins, another one is exclusively formed by proteins which are not associated to any subfamily ("unclassified proteins"), and the three remaining components represent subfamilies A, D, and H (Figure 2B). These results suggest that genes from subfamilies A, D, H and the group of "unclassified proteins" do not belong to *vir* genes but rather represent novel *P. vivax* multigene families.

The VIR super-family and the new multigene families

To obtain a classification, as accurate as possible, of VIR proteins using this methodology, the combination of *E*-value and inflation was optimized for the VIR set (*E*-value 10^{-11} and *Inflation* = 1.3, see "Methods"). Using these values, a large cluster corresponding to the previously defined subfamilies B, C, E, G, I, J, K, remained inter-

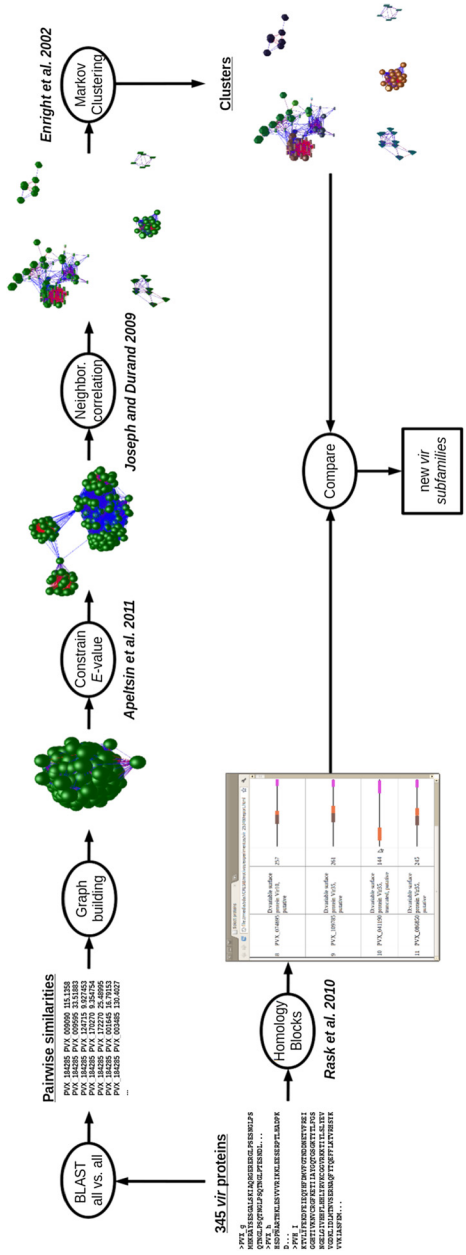

Figure 1 Pipeline procedure to reclassify *vir* genes. The procedure starts with the 345 VIR proteins annotated at PlasmoDB 7.2. Two graph pre-processing strategies [14,16] and the Markov Clustering Algorithm [15] are run to obtain sequence clusters. Then, the Homology Block concept [17] is used to further validate the clustering approach based on comparisons of conserved motifs. Graph figures were obtained using BioLayout [18].

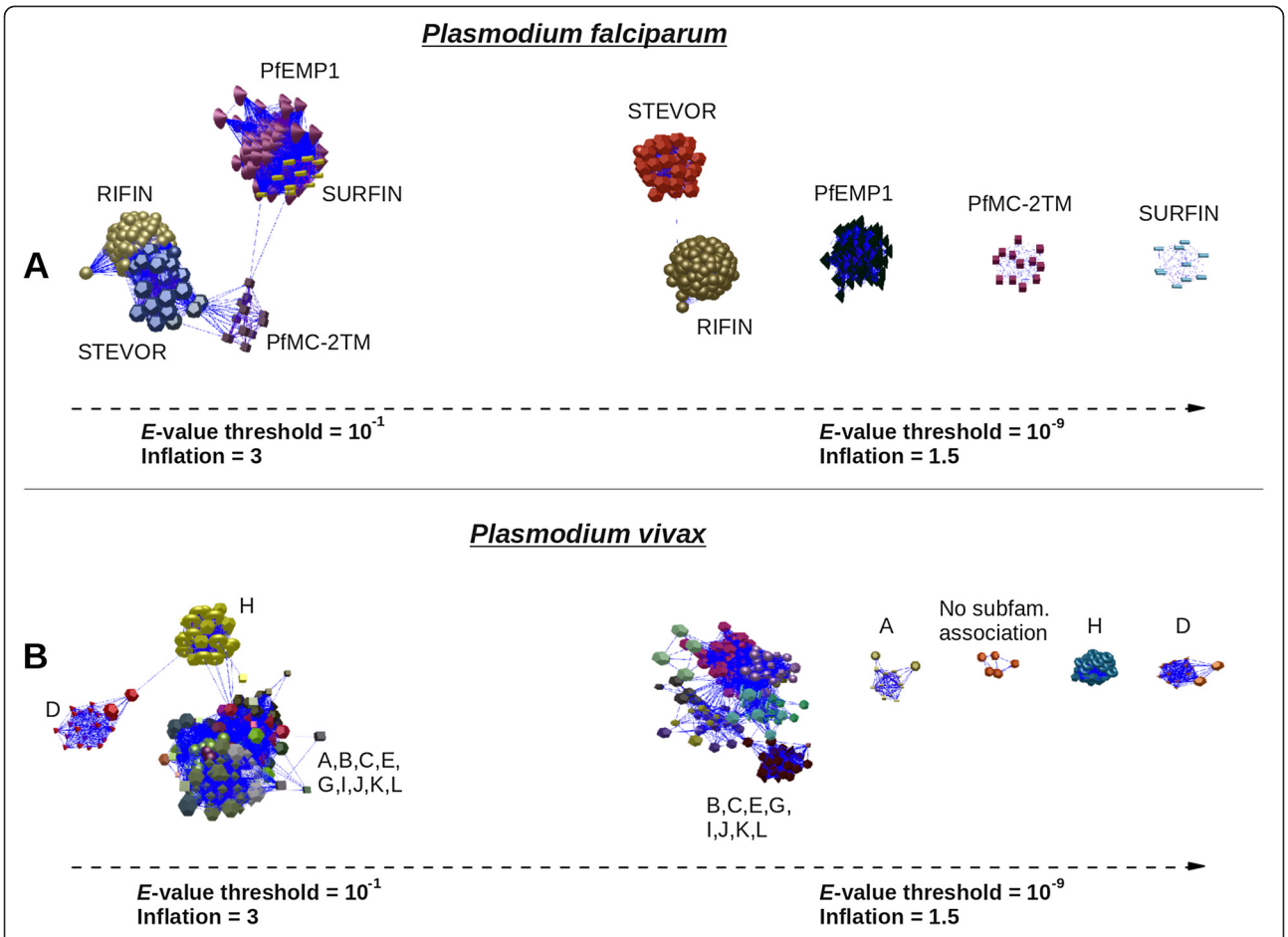

Figure 2 Comparative clusterization of subtelomeric multigene families from *P. falciparum* and *P. vivax*. Colors indicate cluster members. Different shapes indicate different (sub)families. **A**. Clustering result for the *P. falciparum* PfEMP1, PfMC-2TM, RIFIN, STEVOR and SURFIN subtelomeric families [19] at *E*-value = 10^{-1} and *Inflation* = 3 (left) and *E*-value = 10^{-9} and *Inflation* = 1.5 (right). **B**. Clustering result for the *P. vivax* VIR super-family [6] at *E*-value = 10^{-1} and *Inflation* = 3 (left) and *E*-value = 10^{-9} and *Inflation* = 1.5 (right). Graph figures were obtained using BioLayout [18].

connected (Figure 3, Additional file 1). Moreover, proteins of subfamily L appeared strongly related with proteins of subfamily E and were thus clustered all together as subfamily E. Also, all but 16 of the previous 84 unclustered *vir* genes appear now integrated within these subfamilies. It is unlikely that the remaining 16 singletons form a new subfamily since they are too divergent to group together and 12 are annotated as pseudogenes or truncated proteins. Yet, 19 new unclustered VIR proteins were observed (Additional file 1). In total, 295 VIR proteins belonging to ten subfamilies and 19 unclustered *vir* genes are included into this new classification (Additional file 1). As expected, independent graph components corresponding to subfamilies A, D and H were also observed under these *E*-value and inflation parameters (Figure 3, Additional file 2). To further support predictions from the graph components, we used heterologous transfections of *P. falciparum* to express three individual genes encoding VIR-C (PVX_108770), family D (PVX_102635) and family A (PVX_112645) proteins. As shown in Figure 3 and as recently published [13], members of these multigene families have different sub-cellular localizations suggesting different functions.

Proteome-wide analysis predicts 39 hypothetical proteins as belonging to variant proteins

To determine if this method could predict new VIR proteins, the algorithm was applied to the entire *P. vivax* proteome. 39 additional proteins appeared within the graph components of VIR core proteins, three with subfamily H and six with the independent graph component of "unclassified" proteins. Strikingly, all but one (PVX_123205, "CAF1 ribonuclease domain containing protein" contained in the non-clustered graph component), are annotated as "hypothetical proteins" (Additional file 1). In order to get additional information of the 47 hypothetical proteins (48 - PVX_123205), the genomic location of the genes was investigated (Additional file 3): (i) 21 are

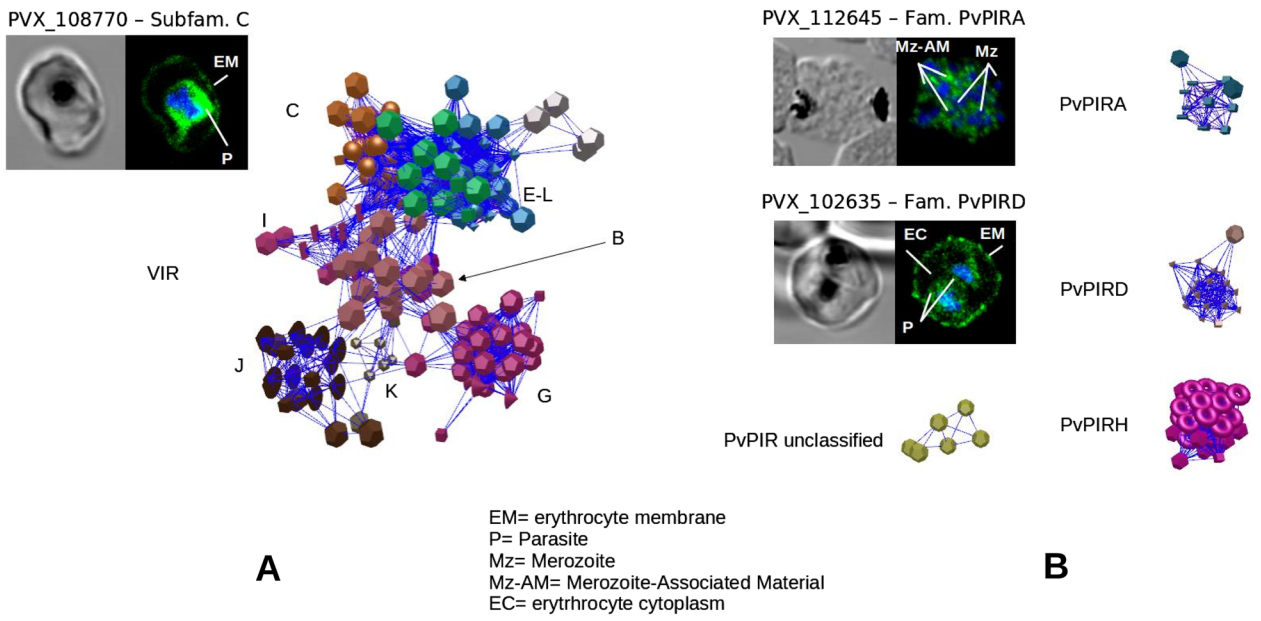

Figure 3 New clustering of the VIR superfamily and subcellular localization of VIR-C, PvPIRA, and PvPIRD. Clustering result for the *P. vivax* VIR super-family [6] at *E*-value = 10^{-11} and *Inflation* = 1.3. Colors indicate cluster members. Different shapes indicate different (sub)families. Graph figures were obtained using BioLayout [18]. Phase contrast and confocal immunofluorescence images of transgenic lines of *P. falciparum* 3D7 expressing VIR-C (PVX_108770), family PvPIRD (PVX_102635) and family PvPIRA (PVX_112645) proteins. Parasites were labelled with anti-HA (green) and DAPI for nuclear staining (blue).

located in subtelomeric regions. Out of the 21, 14 co-localize with members of their subfamilies in assembled chromosomes. Seven are subtelomeric but did not cluster with members of their own subfamilies due to the fact that most members of those subfamilies were not assembled. (ii) 3 genes are internally located, the three of them contained in the independent graph component of "unclassified" proteins (PVX_119620, PVX_092630 and PVX_123205). Of note, no hits were found between genes and PFL0030c (VAR2CSA) using PlasmoDB 7.2 sequences, NCBI BLAST 2.2.27 and E-value threshold = 0.01. (iii). The remaining 23 genes could not be assigned to any particular chromosomes since most chromosome ends from the genome of the *P. vivax* SalI strain remain unassembled [6].

Comparisons of conserved motifs among the newly defined VIR and non-VIR proteins reinforce this new classification

The Homology Block (HB) concept [17] was used to further validate this clustering algorithm based on comparisons of conserved motifs (see "Methods"). The prediction being that HBs from subfamilies A, D and H should have little or no intersections with the remaining subfamilies clustering as VIR proteins. First, we demonstrated that HBs capture previously defined VIR conserved motifs [6] (Additional file 4). Next, we aimed to determine whether there were cluster-specific HBs. Hence, for each cluster, we counted the number of HBs which were unique to that particular cluster and the number of those shared with one each other (Additional file 5). Noticeably, the proportion of specific/shared is either well-balanced or a considerably greater number of shared HBs appear in the VIR subfamilies. In contrast, an outstanding proportion of specific HBs were observed for families D and H (100% and 91% respectively). Note that subfamily A also formed an independent graph component when setting the BLAST *E*-value threshold at 10^{-9} (Figure 2). However, unlike subfamilies D and H, subfamily A shares 41% of HBs with the rest of VIR proteins. As expected, the analysis of the HB composition of proteins belonging to the same cluster revealed that the HB architecture is quite well conserved between members of the same subfamily. Moreover, this HB structure is also conserved in many of the hypothetical proteins predicted to be VIR members. Finally, InterproScan predictions were also obtained in order to provide a more general view of putative domains. It is worth noting that no matches were obtained by InterproScan for members of the newly defined subfamily H. (Additional file 6).

All together, these results validate the new classification of *vir* genes and exclude members from subfamilies A, D and H as VIR proteins. To avoid confoundings with their previous nomenclature as well as with the gene families (*Pv-fam-a-e* and *Pv-fam-g-i*) identified in the genome issue [6], we propose to term them PvPIRA, PvPIRD, and PvPIRH as they clearly fall into the PIR proteins super-family [8].

High expression levels of a subset of *vir* genes and most *pvpirH* genes in patients with symptomatic *P. vivax* infections

To illustrate the value of this new classification, we determined the expression pattern of *vir* genes and the newly defined *pvpirA, pvpirD* and *pvpirH* genes in parasites obtained from *P. vivax* patients [23]. This dataset contains the expression level of 5435 *P. vivax* genes in 10 blood samples of patients with typical symptoms of malaria. Interestingly, unlike members of the newly defined *pvpirA* and *pvpirD* genes, a subset of *vir* genes (38%) and most genes (91%) of the *pvpirH* family present high expression values in samples from all patients (Figure 4). Analysis of the set of *vir* genes expressed in all the samples (rows in red and dark red in Figure 4), showed no correlation with any one particular subfamily. These results thus illustrate the value of this new classification as they identified a subset of *vir* genes and *pvpirH* genes likely associated with clinical symptoms.

Discussion

The current definition and classification of *vir* genes was based on JIGSAW predictions, protein domains and sequence alignments [6]. Our approach also used homology-based BLAST analysis to build a protein graph; yet, the graph pre-processing strategies ensure that the weight of an edge is not exclusively based on the sequence similarity between the two proteins, but strongly dependant on the relations of their neighbours. In other words, the appearance of a protein within a group of nodes is a reliable indicator that there exists more than a simple sequence similarity relation between them. We found the application of the two pre-processing strategies to be essential for a good performance of the MCL algorithm over the VIR set. Using this methodology, our results corroborated the original classification of *vir* genes into different subfamilies. Moreover, results correlate well with OrthoMCL groups which also supports the good functioning of the procedure, but presents some significant differences mainly for subfamilies C, K, G and some of the groups of previously unclustered genes (Additional file 7). In addition, the whole clustering pipeline and results visualization allowed us to assess the strength and evolution of sequence similarities. Thus three of the subfamilies (A, D and H) formed independent graph components at an *E*-value and *Inflation* value where different multigene families of *P. falciparum* were observed as independent graph components. Hence, subfamilies A, D and H could be considered members of different families belonging to the PIR super-family and different data from subcellular and chromosomal locations and homology blocks fully supported this consideration. Accordingly, we propose to term these families PvPIRA, PvPIRD and PvPIRH to avoid confoundings with VIR proteins and with members of the *Pv-fam-a-e* and *Pv-fam-g-i* families described in the genome issue [6].

The Homology blocks concept reinforces the new classification of *vir* genes

To support this clustering approach, we applied the homology block (HB) concept originally coined by Smith and co-workers [24], and used to analyze the *P. falciparum* Erythrocyte Member Protein 1 (PfEMP1) as an iterative procedure for mining HBs from a set of PfEMP1 protein sequences [17]. The use of HBs facilitated a better classification and structural framing of *var* genes allowing recently the discovery of unique domain cassette-encoding *var* genes associated with severe disease in children [25]. Our results revealed that all of the HBs found in proteins of the PvPIRD proteins were family-specific. Likewise, all but two of the conserved motifs found in PvPIRH proteins were also family-specific. In contrast, motif-specific enrichment was not observed in proteins from the PvPIRA family which presented a balanced proportion of specific/shared HBs with VIR proteins. PvPIRA family still remains connected with VIR proteins at less stringent *E*-value and Inflation parameters partly explaining this result. Yet, it forms a completely independent graph component at E-value and inflation parameters where all known *P. falciparum* multigene families are independent. In addition, a member of this family has a different sub-cellular location as that of another member from the VIR family. We thereby consider PvPIRA an independent new subtelomeric family of *P. vivax*.

The clustering procedure facilitates the annotation of hypothetical proteins and evolutionary relatedness of malaria multigene families

Current annotation of the *P. vivax* proteome represents a great challenge as close to 60% of it remains annotated as hypothetical proteins. Remarkably, our approach predicted 39 hypothetical proteins as VIR proteins (Additional file 1). Moreover, data on location for those that could be assigned to assembled chromosomes revealed that they are located within subtelomeric regions. In addition, there were many occurrences of HBs related to VIR proteins reinforcing the predictions that these hypothetical proteins indeed represent VIR proteins (Additional file 6). Three of these proteins were associated with the "unclassified" group and are located in internal regions. VAR2CSA is an internal *var* gene directly involved in pregnancy-associated pathology in *P. falciparum* [26]. Whether any of these putative internal variant genes are related to pregnancy-associated pathology in *P. vivax* is presently unknown.

To determine if this clustering algorithm can facilitate annotation of proteins other than VIR and further our understanding of other malaria subtelomeric families, we ran the pipeline over the entire proteomes of *P. falciparum*,

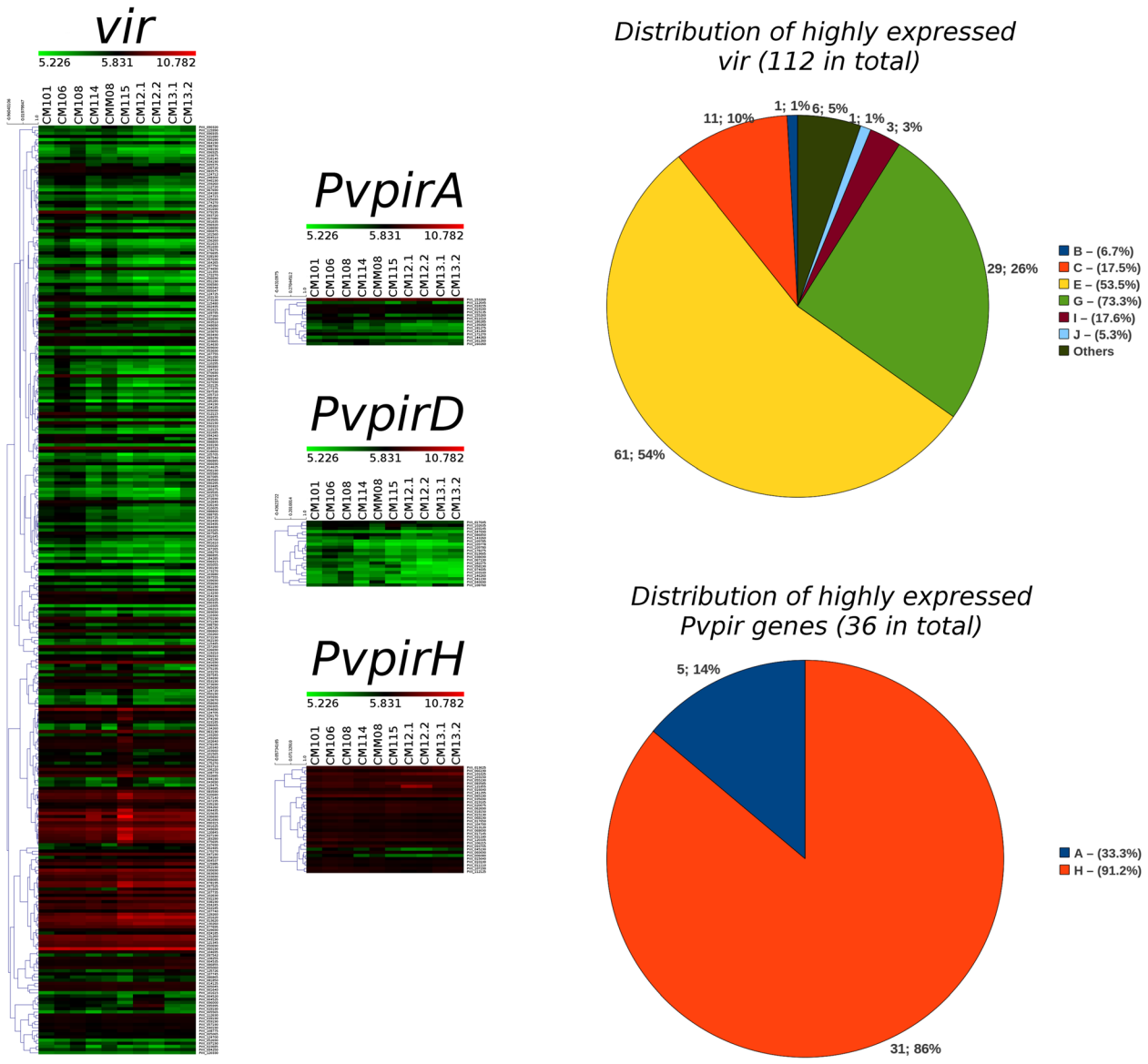

Figure 4 Gene expression profiles of *vir* and *pvpirA, pvpirD,* and *pvpirH* genes in symptomatic *P. vivax* patients. Left panel. Heatmaps drawn for the expression data of ten *P. vivax* patients [23]. Only *vir* and the newly defined *pvvirA, pvpirD and pvpirH* are shown. Color scale spans from 5.226 to 10.782 (upper bar), which represent the minimum and maximum expression values observed for this set of genes and samples (median value = 5.831). Dendrograms on the left of heatmaps show the result of average-linkage hierarchical algorithm. Right panel. Pie charts showing the distribution of expressed genes with expression values ≥ 5.831 for all samples. Percentages next to each (sub)family label indicate the proportion of genes within the corresponding (sub)family.

P. knowlesi, and the rodent malarias *P. yoelii, P. chabaudi* and *P. berghei* (Figure 5). Several inter-connected graph components belonging to different families of different species could be readily observed (Figure 5). For instance, the SURFIN family of *P. falciparum* shares relations with different families from all species suggesting a common origin. These results, however, need to be taken with caution as excepting for *P. falciparum*, remaining genomes are highly unassembled at chromosome ends where most of these families reside. Yet, as better coverage and assembling of these regions is achieved, this tool should facilitate the design of experiments to better understand the evolution and function of malaria subtelomeric families.

VIR proteins and pathology

The function of VIR proteins and other multigene families of this species remain largely unknown. Yet, recent evidences have demonstrated that a VIR protein belonging to

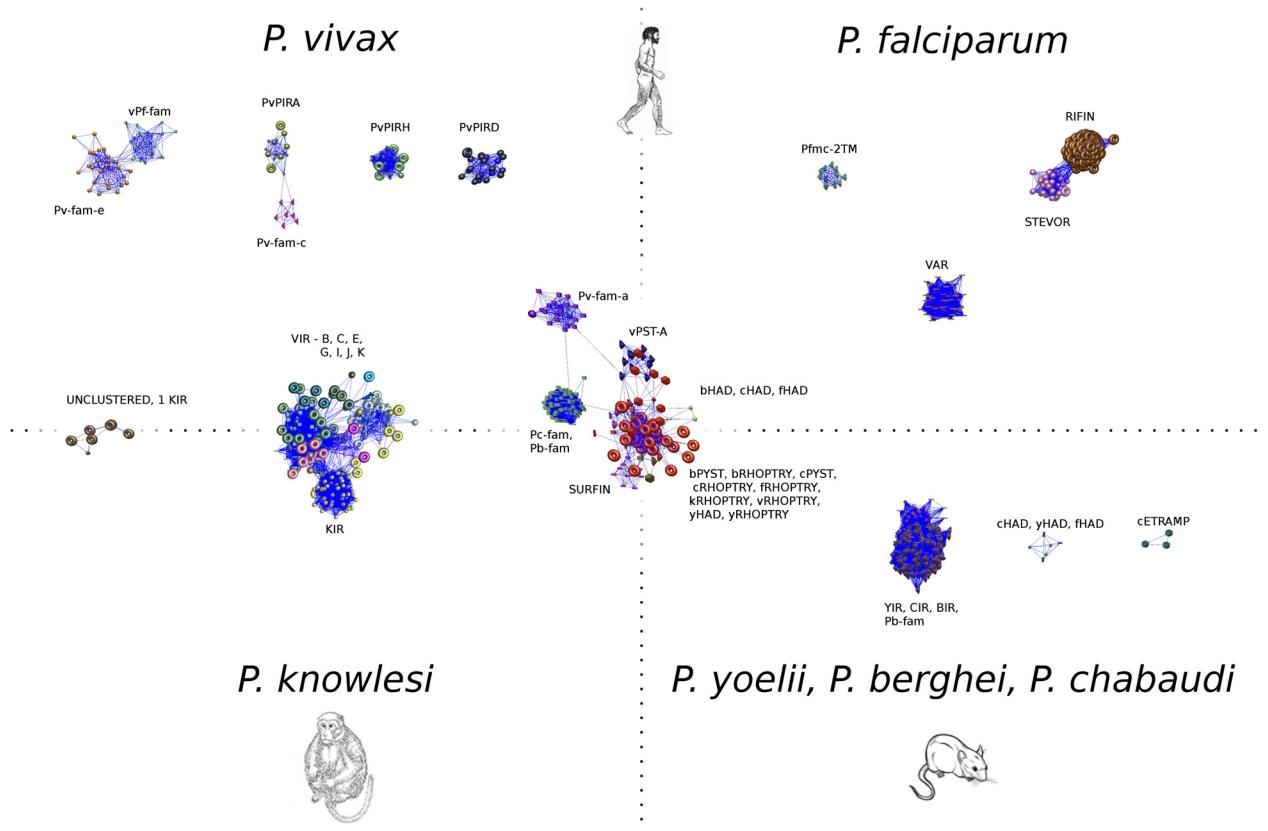

Figure 5 Protein similarity relations among several *Plasmodium* subtelomeric families. Results obtained when running the clustering procedure over the subtelomeric families of *P. vivax* (Pv or v): VIR, PvPIRA-D-H, Pv-fam, v-Pf-fam, vRHOPTRY, v-PST-A; *P. falciparum* (Pf or f): VAR, RIFIN, STEVOR, Pfmc-2TM, SURFIN, fRHOPTRY, fHAD; *P. knowlesi* (k): KIR, kRHOPTRY; *P. chabaudi* (Pc or c): CIR, Pc-fam, cRHOPTRY, cHAD, cPYIST, cETRAMP; *P. berghei* (Pb or b): BIR, Pb-fam, bRHOPTRY, bHAD, bPYIST, and *P. yoelii* (Py or y): YIR, yRHOPTRY, yHAD using an E-value 10^{-11} and Inflation of 1.3. The position of the clusters with respect to the axes is just a qualitative representation. Axes do not represent any metric. Graph figures were obtained using BioLayout [27].

subfamily C was exported and exposed at the surface of infected erythrocytes and that it mediated specific binding to the ICAM-1 endothelial receptor under flow physiological conditions [13]. This result, together with other evidence of in vitro cytoadherence of *P. vivax*-infected reticulocytes [28,29], indicates that this species, similar to *P. falciparum*, can cytoadhere. We thus computationally searched for other putative adhesins within members of VIR, PIRA, PIRD, and PIRH proteins using the MAAP predictor [30] (see "Methods"). Interestingly, only members representing subfamilies C and E as well as H proteins presented positive predictions. Thus, 39% (12/31) of H proteins present positive predictions, while only 7% (3/42) and 6% (5/88) of C and E VIR proteins, respectively, yielded a positive score. It is worth noting here that MAAP was trained with *P. falciparum* data, which implies that its performance may not be optimal when applied over *P. vivax* proteins. Yet, these data reinforce the view that variant proteins of *P. vivax* are involved in cytoadherence and pathology. In the absence of further experimental evidence this remains to be fully demonstrated.

Conclusions

A new computational approach was applied to revisit the original classification of *vir* genes, the largest subtelomeric multigene superfamily of human malaria parasites. Applying this pipeline, the *vir* gene super-family was redefined by excluding members of subfamilies A, D and H, by including unclusterd genes, and by facilitating the genome-wide annotation of 39 hypothetical proteins as new VIR proteins. In addition, analysis of gene expression data from febrile *P. vivax* patients illustrated the value of this new classification as it showed high expression levels of most genes belonging to the PvPIRH multigene family. Last, the clustering approach was extended to other subtelomeric multigene families of malaria parasites. It will thus improve the design of experiments to determine the role of subtelomeric multigene families in pathology, which in turn may help furthering vaccine development against malaria.

Methods

Data

Sequence information of the *P. vivax*, *P. falciparum*, *P. chabaudi*, *P. knowlesi* and *P. yoelii* predicted proteomes was obtained from PlasmoDB release 7.2 (May 2011) [31]. *P. berghei* sequences were obtained from PlasmoDB release 7.1 (Nov 2010). In addition, release A_25.0 of Pfam (March 2011) was downloaded from the HHpred ftp server [32] (ftp://toolkit.lmb.uni-muenchen.de/HHsearch/databases/).

Protein sequence clustering

The clustering procedure is based on a graph representation of the set of proteins, where nodes are the actual proteins and the weighted edges indicate the similarity relations between them. In our particular case, the weights of the edges are calculated as a function of the BLAST score obtained for each two proteins. A pipeline consisting of three different steps is followed to get the final set of clusters: 1) Estimating an appropriate BLAST *E*-value threshold, 2) Similarity value calculation and 3) Running the Markov Clustering Algorithm (MCL). The complete procedure was implemented and integrated in a web application (http://bioinfold.fcrb.es/sequence_cluster).

Estimating an appropriate BLAST E-value threshold

The *E-value* threshold stands for the *Expect-value* threshold of the BLAST algorithm, and describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. Sequence hits with an *E*-value greater than the threshold are discarded. Thus, an appropriate *E*-value threshold is essential to remove superfluous edges from the graph which introduce noise and disturb the clustering process. The heuristic reported by Apeltsin *et al.* [14] was followed. Briefly, it consists of an iterative procedure which starts running a BLAST all vs. all with a high *E*-value threshold (i.e. 1) [33]. An initial graph is thus built. Then, a parameter called *Nsv* by the authors is calculated as a function of the number of connected nodes and the number of edges in the graph:

$$N_{sv} = \frac{\#\ Edges}{\#\ Connected\_nodes}$$

Next, the *E*-value threshold is divided by 10 and the algorithm starts again by rebuilding the graph (unconnected nodes are removed). The procedure stops when the *Nsv* no longer decreases and begins to increase. The underlying reasoning is that those edges removed at the initial iterations connect proteins from different families, and therefore do not cause node removals. However, if the *Nsv* trend varies, is due to the removal of edges which link proteins within the same family. This, in turn, causes the isolation of nodes which are therefore deleted. Further details can be found in [14].

Calculating similarity values

Once the *E*-value threshold is set, the final weigths of the edges are calculated as a function of the similarity between each two of proteins. For each pair of sequences x and y, initial similarity values are calculated based on the High-scoring Segment Pairs (HSPs) returned by BLAST. The combination of non-overlapping HSPs which yields the highest score is obtained, and the corresponding bit score used as the initial similarity value, $S_{init}(x,y)$. Then, the strategy proposed by Joseph *et al.* [16] is applied to weight these similarity values. Let w_x be the vector of similarity values between x and the rest of proteins. For each pair of nodes x and y, the Neighbourhood Correlation score, NC_{xy}, is calculated as the Pearson Correlation Coefficient between w_x and w_y. Thus, the final similarity value between x and y and therefore the final weight of the edge than links x and y in the graph, is calculated as:

$$S(x,y) = S_{init}(x,y) \cdot NC_{xy}$$

Hence, it is important to emphasize that the strength of the association between two given proteins is not exclusively dependant on the similarity score, but also strongly conditioned on its neighbours and their associations. This step resulted to be of great importance to reducing noise and avoiding problems such as the domain chaining. Further details can be found in [16].

Markov clustering algorithm (MCL)

The Markov Clustering Algorithm (MCL) is a well-known procedure which has been extensively used for protein clustering [15]. Moreover, large scale projects such as the construction of the OrthoMCL [34] database made use of this algorithm for their purposes. It is a robust and efficient algorithm for graph clustering which uses an internal matrix representation. The underlying idea of the procedure is to simulate random walks within the graph, assuming that the number of longer paths between two arbitrary nodes in a given cluster is high. On the other hand, random walks on the graph will infrequently go from one cluster to another. Thus, the algorithm iteratively modifies the probabilities of random walks through the graph, by alternating two matrix operators called *expansion* and *inflation* until convergence. An important advantage of this algorithm is that it only requires to provide one parameter, the *Inflation*, which takes values in [1.1, 10.0] and determines the cluster granularity (the higher the *Inflation* value, the higher the granularity). Please, refer to the work reference in [15] for further details.

Selecting clustering parameters

Two are the main parameters that had to be set for family classification: *E*-value threshold and *Inflation* value. First, the heuristic reported by Apeltsin *et al.* was

followed as described above. The procedure estimated a minimum *E*-value threshold of 10^{-44}. Setting the *Inflation* value to 1.3, good clustering results were obtained which correlated well with the previous classification. Moreover, subfamily C was clearly divided in two different groups and two distinct subtypes of proteins could be unveiled among E members. Nevertheless, two facts evidenced that this *E*-value threshold was extremely restrictive. First, the HB architecture of the some subfamilies such as J or D, clearly indicated that highly similar proteins were being distributed in distinct clusters. Second, due to the highly restrictive threshold, up to 167 proteins lost all of their similarity associations with the rest of VIR members, and therefore were not included in the final clustering step. This meant that a lot of information was being missed and that the procedure was overestimating the *E*-value threshold. Recall here that the strategy is based on a heuristic and therefore may not provide the optimal solution. Thus, we looked for an *E*-value threshold as close as possible to the 10^{-44} which did not cause such a loss of data. It was observed that with the *E*-value threshold set at 10^{-11} just 16 proteins were discarded. Moreover, all of them were labelled as "Not clustered" by previous authors when the original VIR subfamilies were defined.

Once the *E*-value threshold was set, an appropriate *Inflation* value was estimated. A range of values were tested. Clustering results were visualized using BioLayout [18] and compared with the original classification, as well as with the HB architecture of the sequences. After manual inspection of the results, an *Inflation* value of 1.3 was chosen, since clusters were able to appropriately capture the structure of the graph, correlated well with the previous subfamilies and were in agreement with the HB architecture of the sequences.

Homology blocks

Mining homology blocks

An homology block represents a sequence pattern. Sequence patterns represented by Homology Blocks are usually observed after the alignment of multiple sequences which share a given motif. This motif (sequence pattern) is then modelled by a particular type of probabilistic models, called Hidden Markov Models (HMM). Thus, an homology block can be defined as a sequence profile determined from a multiple sequence alignment and modelled by a Hidden Markov Model (HMM). The procedure proposed by Rask *et al.* [17] was followed. The algorithm consists of an iterative process which repeats three main steps: 1) Selecting 100 promising *seeds* from the sequence database, 2) Building an HB for each seed and 3) Selecting the best HB and removing its occurrences. Since the original procedure is comprehensively described in the paper referenced in [17], we here describe just the three significant modifications of the original algorithm that were included in steps 1) and 2):

1) Selecting promising seeds. A seed can be defined as a promising short sequence extracted from the database. A score value is calculated for each seed which is, roughly, based on the number of homologies that the corresponding seed presents in the database [17]. After sorting the set of seeds according to their punctuation, it was observed that in many cases the score of the seed ranked at position 100, was the same than that of the seeds at position 101, 102 and so on. Therefore, it makes no sense to select only 100 seeds. Thus, some flexibility was allowed in the selection procedure, and all those seeds which score equals that of seed 100 were included in the selected set.

2) Saving HBs for posterior selection. Each time an HB is built from a certain seed, the original algorithm saves it for posterior selection in step 3. However, it was observed, that many low conserved motifs representing unsignificant sequence profiles were generated. In other words, many HBs with an empty logo appeared [27]. Hence, for each HB, the conservation level at each position was calculated [27]. Small sample correction was also incorporated. Those HBs which did not contain at least one significantly conserved position (conservation level > 0) were discarded.

3) Exhausting the seed list. If certain iteration returns no homology blocks, the next 200 seeds in the list are processed. The procedure is repeated until the seed list is empty. This ensures that all the seeds are processed and that no HB is lost.

Comparing HBs

The comparison between homology blocks was done by comparing their corresponding HMMs. Söding proposed a dynamic programming algorithm for aligning two given HMMs and developed the package HHpred [32]. The software HHsearch (included in HHpred 1.5.0) was used for comparing the set of HBs with the Pfam database. For each pair of HMMs, HHsearch returns the probability that both models overlap (range [0, 100]). Likewise, HHsearch was used for comparing the HBs with the previously defined MEME motifs. In this case, HMMs for the MEME motifs were built by using HMMER 3.0 [35].

Finally, a clustering algorithm was applied over the set of HBs to identify redundancies. The average-linkage hierarchical algorithm was used, setting distance threshold at 70. The distance measure was calculated as 100-*HHsearch_probability* (Additional file 4).

Transgenic lines generation and indirect immunofluorescence assays

P. falciparum culture, parasite transfection and indirect immunofluorescence assays were done as described in [13].

Adhesins prediction

Computational prediction of putative adhesins was carried out by applying MAAP [30]. The score threshold was set to 0.7 as suggested by the MAAP authors for the *P. vivax* proteome.

Additional files

Additional file 1: New VIR subfamilies. Excel sheet containing the new classification proposal of the VIR proteins. The list of subfamilies is shown along with their members. Rows in red indicate that the HB architecture of the corresponding protein does not clearly match with the rest of members in the subfamily.

Additional file 2: New subtelomeric PvPIRA, PvPIRD and PvPIRH families. Excel sheet containing the new subtelomeric families derived from previous VIR members. Rows in red indicate that the HB architecture of the corresponding protein does not clearly match with the rest of members in the subfa.

Additional file 3: Genome-wide distribution of newly predicted *vir* genes. Figure showing the chromosomal location of newly annotated hypothetical proteins as VIR proteins. For each chromosome, two rows of colored boxes are shown: one row illustrates the genomic location of the *vir* members (red) while the other illustrates the location of the putative *vir* (green) in that chromosome. Only genes annotated at specific chromosomes are shown.

Additional file 4: HB search results. Excel table with the list of Homology Blocks, their similarities with previously defined conserved motifs and PFAM annotations.

Additional file 5: Motif distribution across clusters. Two tables in an Excel data sheet showing the distribution of the conserved HBs across clusters: *i)* for each cluster, the number (and proportion) of HBs shared with other clusters is shown, as well as the number (and proportion) of cluster-specific HBs; *ii)* rows represent HBs, columns represent sequence clusters (subfamilies and families). The first row in the table (after the header) contains the size of each cluster. First column shows HB identifiers. Each cell in the table contains the number of sequences in a given cluster that contain the corresponding HB (and the proportion in brackets).

Additional file 6: Conserved motifs composition. Composition of clusters and the conserved motif structure of each of the proteins in the original VIR set, as well as the hypothetical proteins grouped with them. An illustration of the most representative homology blocks in each family is also included as well as InterproScan predictions for newly defined (sub)families.

Additional file 7: Comparison with OrthoMCL5. Results of the comparison between *vir*, *Pvpir* (sub)families and OrthoMCL5 groups.

Competing interests

All authors declare that they have no competing interests.

Authors' contributions

FJL suggested, implemented and performed computational analyses. MB and CFB suggested and performed biological experiments. HAP idealized and coordinated the study. FJL and HAP drafted the manuscript. All authors read and approved the final manuscript.

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Suplementary Figure 1

New classification proposal of the VIR proteins.

The list of subfamilies is shown along with their members. Rows in red indicate that the HB architecture of the corresponding protein does not clearly match with the rest of members in the subfamily.

In brackets, the new proposed annotation of hypothetical proteins.

MAAP (Malaria Adhesins and Adhesins-like Predictor)

Cluster 8 (Subfamily B)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
B	B	PVX_033190	357	variable surface protein Vir21, putative	No
B	B	PVX_180275	236	variable surface protein Vir26-like, truncated	No
B	B	PVX_164265	362	variable surface protein Vir21, putative	No
B	B	PVX_115480	276	variable surface protein Vir21, putative	No
B	B	PVX_110305	267	variable surface protein Vir21, putative	No
B	B	PVX_103680	361	variable surface protein Vir21, putative	No
B	B	PVX_096915	295	variable surface protein Vir21-related	No
B	B	PVX_088790	467	variable surface protein Vir21-like	No
B	B	PVX_086350	397	variable surface protein Vir21, putative	No
B	B	PVX_075195	302	variable surface protein Vir26, putative	No
B	B	PVX_062190	326	variable surface protein Vir21, truncated, putative	No
B	B	PVX_058690	299	variable surface protein Vir21, truncated, putative	No
B	B	PVX_025690	351	variable surface protein Vir21, putative	No
Not clustered	B	PVX_108775	62	variable surface protein Vir26, truncated, putative	No
Not clustered	B	PVX_059690	88	variable surface protein Vir26, putative	No

Cluster 2 (Subfamily C)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
C	C	PVX_043690	85	variable surface protein Vir32, truncated, putative	No
C	C	PVX_184285	348	variable surface protein Vir4, putative	No
C	C	PVX_175270	332	variable surface protein Vir14, truncated, putative	No
C	C	PVX_169270	346	variable surface protein Vir14, putative	No
C	C	PVX_149260	270	variable surface prtoein Vir14, truncated, putative	No
C	C	PVX_105700	340	variable surface protein Vir32/4, putative	No
C	C	PVX_096000	353	variable surface protein Vir4, putative	Yes
C	C	PVX_061190	342	variable surface protein Vir32, putative	No
C	C	PVX_051690	334	variable surface protein Vir32, putative	No
C	C	PVX_050190	340	variable surface protein Vir4, putative	No
C	C	PVX_030190	325	variable surface protein Vir14, truncated, putative	No
C	C	PVX_029190	335	variable surface protein Vir14, putative	No
C	C	PVX_027690	315	variable surface protein Vir16, putative	No
C	C	PVX_021680	355	variable surface protein Vir4, putative	No
C	C	PVX_016140	324	variable surface protein Vir4, putative	No
C	C	PVX_010610	315	variable surface protein Vir14, putative	No
C	C	PVX_006580	351	variable surface protein Vir14, putative	No
C	C	PVX_004520	363	variable surface protein Vir4, putative	No
C	C	PVX_127260	210	variable surface protein Vir16, putative, truncated	No
C	C	PVX_124710	313	variable surface protein Vir4-related	No
C	C	PVX_115475	229	variable surface protein Vir14, putative	No
C	C	PVX_113230	440	variable surface protein Vir14-related	No
C	C	PVX_108770	409	variable surface protein Vir 14, putative	No
C	C	PVX_104185	288	variable surface protein Vir16-related	No
C	C	PVX_103665	290	variable surface protein Vir4/32-like	Yes
C	C	PVX_102125	153	variable surface protein Vir32, truncated, putative	No
C	C	PVX_101615	361	variable surface protein Vir14-related	No
C	C	PVX_101560	302	variable surface protein Vir4-related	No
C	C	PVX_096945	314	variable surface protein Vir4-related	No
C	C	PVX_095995	315	variable surface protein Vir16/32-related	No
C	C	PVX_088800	358	variable surface protein Vir4-related	No
C	C	PVX_076695	325	variable surface protein Vir14/32-related	No
C	C	PVX_072690	324	variable surface protein Vir4/14-related	No
C	C	PVX_028190	284	variable surface protein Vir4, putative	Yes
C	C	PVX_026190	353	variable surface protein Vir14, putative	No
C	C	PVX_024690	343	variable surface protein Vir12-related	No
C	C	PVX_009090	337	variable surface protein Vir14-like	No
C	C	PVX_005580	297	variable surface protein Vir4, putative	No
C	C	PVX_004525	353	variable surface protein Vir16/32-related	No
C	C	PVX_004510	353	variable surface protein Vir4/14-related	No
C	C	PVX_003485	329	variable surface protein Vir4, putative	No
C	C	PVX_120330	418	variable surface protein Vir14-related	No
C	C	PVX_034190	403	variable surface protein Vir14-related	No
C	C	PVX_103660	360	variable surface protein Vir16/32-related	No
C	C	PVX_101600	372	variable surface protein Vir16/32-related	No

C	C	PVX_052690	279	variable surface protein Vir4-related, truncated	No
C	C	PVX_037190	404	variable surface protein Vir14/24-related	No
C	C	PVX_008085	418	variable surface protein Vir 14-related	No
Not clustered	C	PVX_044190	61	variable surface protein Vir32, putative	No
Not clustered	C	PVX_045690	140	variable surface protein Vir12-related	No
Not clustered	C	PVX_019670	172	variable surface protein Vir12-related	No
Not clustered	C	PVX_004535	330	variable surface protein Vir, putative	No
Not clustered	C	PVX_086875	292	variable surface protein Vir27-related	No
Unavailable	C	PVX_159260	541	variable surface protein Vir14, pseudogene, putative	No
Unavailable	C	PVX_066690	370	variable surface protein Vir16, pseudogene, putative	No
Unavailable	C	PVX_179275	240	variable surface protein Vir C1-29, pseudogene, putative	No
Unavailable	C	PVX_052190	388	variable surface protein Vir4, pseudogene, putative	No
Unavailable	C	PVX_046190	353	variable surface protein Vir32, putative; Truncated due to end of contig.	No
No Vir	C	PVX_124700	255	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	C	PVX_005065	198	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	C	PVX_090320	212	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	C	PVX_005575	138	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	C	PVX_094260	198	hypothetical protein (variable surface protein Vir, putative)	No

Cluster 1 (Subfamily E)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
C	E	PVX_101565	473	variable surface protein Vir24-related	No
C	E	PVX_124705	449	variable surface protein Vir12-related	No
E	E	PVX_107235	582	variable surface protein Vir12-related	Yes
E	E	PVX_106725	469	variable surface protein Vir12, putative	No
E	E	PVX_106220	481	variable surface protein Vir12, putative	No
E	E	PVX_103155	442	variable surface protein Vir12-related	No
E	E	PVX_097555	516	variable surface protein Vir12/16-related	No
E	E	PVX_093710	488	variable surface protein Vir24-related	No
E	E	PVX_090290	288	variable surface protein Vir12, putative	No
E	E	PVX_086860	447	variable surface protein Vir24-related	No
E	E	PVX_083575	510	variable surface protein Vir24-related	No
E	E	PVX_077695	545	variable surface protein Vir12-like	Yes
E	E	PVX_074690	427	variable surface protein Vir4-related	No
E	E	PVX_073690	433	variable surface protein Vir12-related	No
E	E	PVX_023185	476	variable surface protein Vir12-related	No
E	E	PVX_018660	493	variable surface protein Vir24-like	No
E	E	PVX_015635	529	variable surface protein Vir12-like	No
E	E	PVX_009595	392	variable surface protein Vir22, putative	No
E	E	PVX_007085	321	variable surface protein Vir24-related	No
E	E	PVX_003505	414	variable surface protein Vir24-related	No
E	E	PVX_001645	529	variable surface protein Vir12-related	No
E	E	PVX_001625	460	variable surface protein Vir12-related	No
E	E	PVX_001615	366	variable surface protein Vir12/24-related	No
E	E	PVX_172270	378	variable surface protein Vir22, truncated, putative	No
E	E	PVX_170270	332	variable surface protein Vir12, truncated, putative	No
E	E	PVX_158260	287	variable surface protein Vir12, truncated, putative	No
E	E	PVX_133260	268	variable surface protein Vir12, putative, truncated	No
E	E	PVX_124715	528	variable surface protein Vir5-related	No
E	E	PVX_120340	452	variable surface protein Vir12-related	No
E	E	PVX_119210	540	variant surface protein Vir22/5/24, putative	No
E	E	PVX_115485	302	variable surface protein Vir24-related	No
E	E	PVX_112630	650	variable surface protein Vir12, putative	No
E	E	PVX_110300	359	variable surface protein Vir 12/22/24-like	No
E	E	PVX_110295	553	variable surface protein Vir22-like	No
E	E	PVX_107755	657	variable surface protein Vir12/24-related	No
E	E	PVX_107745	351	variable surface protein Vir 12-like	No
E	E	PVX_104190	426	variable surface protein Vir12-like	No
E	E	PVX_101620	594	variable surface protein Vir16-related	No
E	E	PVX_097540	668	variable surface protein Vir24-related	No
E	E	PVX_097530	524	variable surface protein Vir22/12-related	No
E	E	PVX_097525	508	variable surface protein Vir 12, putative	Yes
E	E	PVX_096920	491	variable surface protein Vir12-related	No
E	E	PVX_094240	443	variable surface protein Vir12/24-related	No
E	E	PVX_093720	496	variable surface protein Vir24-like	No
E	E	PVX_090335	435	variable surface protein Vir12-like	No
E	E	PVX_090315	487	variable surface protein Vir12-like	No
E	E	PVX_090305	462	variable surface protein Vir12-related	No
E	E	PVX_088805	358	variable surface protein Vir22/24-like	No

E	E	PVX_081850	608	variable surface protein Vir22/23-related	No
E	E	PVX_078195	569	variable surface protein Vir12-related	Yes
E	E	PVX_076195	459	variable surface protein Vir12-related	No
E	E	PVX_075695	436	variable surface protein Vir12/22/24-related	No
E	E	PVX_042190	450	variable surface protein Vir12-related, truncated	Yes
E	E	PVX_022685	630	variable surface protein Vir24-related	No
E	E	PVX_022185	352	variable surface protein Vir12-related	No
E	E	PVX_017140	568	variable surface protein Vir12-like	No
E	E	PVX_013620	537	variable surface protein Vir12-like	No
E	E	PVX_005060	451	variable surface protein Vir12-like	No
E	E	PVX_003510	453	variable surface protein Vir24-related	No
E	E	PVX_003495	635	variable surface protein Vir22/24-related	No
E	E	PVX_002490	464	variable surface protein Vir12-related	No
E	E	PVX_002485	276	variable surface protein Vir12-related	No
E	E	PVX_002480	616	variable surface protein Vir12/24-related	No
L	E	PVX_102640	441	variable surface protein Vir12-related	No
L	E	PVX_083590	452	variable surface protein Vir12, putative	No
L	E	PVX_024685	419	variable surface protein Vir12-related	No
L	E	PVX_063190	483	variable surface protein Vir12-related	No
L	E	PVX_054190	396	variable surface protein Vir14-related, truncated	No
L	E	PVX_041690	379	variable surface protein Vir12-related, truncated	No
L	E	PVX_010105	421	variable surface protein Vir12-related	No
Not clustered	E	PVX_120845	587	variable surface protein Vir12-related	No
Not clustered	E	PVX_104180	386	variable surface protein Vir12, putative	No
Not clustered	E	PVX_020680	581	variable surface protein Vir12-like	No
Not clustered	E	PVX_183280	443	variable surface protein Vir12-related	No
Not clustered	E	PVX_157260	315	variable surface protein Vir12, truncated, putative	No
Not clustered	E	PVX_124720	545	variable surface protein Vir12-related	No
Not clustered	E	PVX_115985	574	variable surface protein Vir, putative	No
Not clustered	E	PVX_094245	584	variable surface protein Vir12-like	No
Not clustered	E	PVX_088785	314	variable surfave protein Vir12, putative	No
Not clustered	E	PVX_088780	446	variable surface protein Vir24-related	No
Not clustered	E	PVX_083580	575	variable surface protein Vir24-related	No
Not clustered	E	PVX_241290	771	variable surface protein Vir12-like	No
Not clustered	E	PVX_112720	505	variable surface protein Vir12-related	No
Not clustered	E	PVX_097545	451	variable surface protein Vir12-related	No
Not clustered	E	PVX_134260	104	variable surface protein Vir12, putative, truncated	No
Not clustered	E	PVX_124712	400	variable surface protein Vir24-related	No
Unavailable	E	PVX_005045	445	variable surface protein Vir12-related	No
Unavailable	E	PVX_096910	506	variable surface protein Vir23-related	No
No Vir	E	PVX_094250	525	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_086865	576	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_004537	466	hypothetical protein ((variable surface protein Vir, putative)	No
No Vir	E	PVX_097542	560	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_125726	364	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_107740	304	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_090295	542	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_065690	394	hypothetical protein (variable surface protein Vir, putative)	No

No Vir	E	PVX_049690	366	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_037690	67	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_036690	98	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_034690	404	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_027190	200	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_020170	333	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_005055	534	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_004495	449	hypothetical protein, conserved (variable surface protein Vir, putative)	No
No Vir	E	PVX_003490	612	hypothetical protein, conserved (variable surface protein Vir, putative)	No
No Vir	E	PVX_001635	634	hypothetical protein, conserved (variable surface protein Vir, putative)	No
No Vir	E	PVX_038190	58	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_090310	449	hypothetical protein, conserved (variable surface protein Vir, putative)	No
No Vir	E	PVX_061690	273	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_107735	362	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_124725	220	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_074190	323	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_001640	227	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	E	PVX_086895	553	hypothetical protein, conserved (variable surface protein Vir, putative)	No

Cluster 3 (Subfamily G)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
G	G	PVX_103670	459	variable surface protein Vir6-like	No
G	G	PVX_059190	438	variable surface protein Vir6, putative	No
G	G	PVX_050690	405	variable surface protein Vir6, putative	No
G	G	PVX_047190	325	variable surface protein Vir28-like	No
G	G	PVX_035190	410	variable surface protein Vir6, truncated, putative	No
G	G	PVX_014630	464	variable surface protein Vir6, putative	No
G	G	PVX_071190	437	variable surface protein Vir28-related	No
G	G	PVX_026690	460	variable surface protein Vir6, putative	No
G	G	PVX_007585	447	variable surface protein Vir28, putative	No
G	G	PVX_007080	451	variable surface protein Vir 28, putative	No
Not clustered	G	PVX_033690	105	variable surface protein Vir6, truncated, putative	No
Not clustered	G	PVX_121345	449	variable surface protein Vir6, putative	No
Not clustered	G	PVX_079195	427	variable surface protein Vir6-like	No
Not clustered	G	PVX_073190	492	variable surface protein Vir6-like	No
Not clustered	G	PVX_063690	456	variable surface protein Vir6-like	No
Not clustered	G	PVX_039190	438	variable surface protein Vir6, putative	No
Not clustered	G	PVX_031190	319	variable surface protein Vir6, putative	No
Not clustered	G	PVX_030690	186	variable surface protein Vir6, putative	No
Not clustered	G	PVX_029690	399	variable surface protein Vir6, putative	No
Not clustered	G	PVX_028690	464	variable surface protein Vir6, putative	No
Not clustered	G	PVX_024185	427	variable surface protein Vir6-like	No
Not clustered	G	PVX_048690	408	variable surface protein Vir7-like	No
Not clustered	G	PVX_112115	320	variable surface protein Vir6, putative	No
Not clustered	G	PVX_070190	182	variable surface protein Vir28, truncated, putative	No
Not clustered	G	PVX_106210	434	variable surface protein Vir6, putative	No
Not clustered	G	PVX_104695	435	variable surface protein Vir6-like	No
Not clustered	G	PVX_057190	118	variable surface protein Vir6, truncated, putative	No
Not clustered	G	PVX_056190	407	variable surface protein Vir6-related	No
Not clustered	G	PVX_032190	497	variable surface protein Vir28-related	No
Not clustered	G	PVX_018655	515	variable surface protein Vir28-like	No
Not clustered	G	PVX_012115	506	variable surface protein Vir 28/6-related	No
Not clustered	G	PVX_040190	266	variable surface protein Vir6, truncated, putative	No
Unavailable	G	PVX_014125	523	variable surface protein Vir6-like, pseudogene	No
Unavailable	G	PVX_174270	475	variable surface protein Vir6-like, pseudogene	No
No Vir	G	PVX_108255	442	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	G	PVX_060190	84	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	G	PVX_102645	92	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	G	PVX_102130	456	hypothetical protein (variable surface protein Vir, putative)	No

Cluster 7 (Subfamily I)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
I	I	PVX_072190	362	variable surface protein Vir7, putative	No
I	I	PVX_064190	364	variable surface protein Vir7, putative	No
I	I	PVX_014625	375	variable surface protein Vir7, putative	No
I	I	PVX_023685	292	variable surface protein Vir7, putative	No
I	I	PVX_002495	324	variable surface protein Vir7-like	No
I	I	PVX_177275	328	variable surface protein Vir7, putative	No
I	I	PVX_150260	361	variable surface protein Vir27, truncated, putative	No
I	I	PVX_102630	345	variable surface protein Vir27, putative	No
I	I	PVX_069190	343	variable surface protein Vir27-like, truncated	No
I	I	PVX_064690	363	variable surface protein Vir27, putative	No
I	I	PVX_053190	344	variable surface protein Vir27, putative	No
Not clustered	I	PVX_054690	51	variable surface protein Vir29, truncated, putative	No
Not clustered	I	PVX_032690	156	variable surface protein Vir7, putative	No
Not clustered	I	PVX_048190	117	variable surface protein Vir27, truncated, putative	No
Not clustered	I	PVX_039690	66	variable surface protein Vir27, truncated, putative	No
Not clustered	I	PVX_055690	192	variable surface protein Vir27, truncated, putative	No
Unavailable	I	PVX_248300	400	variable surface protein Vir29, pseudogene, putative	No

Cluster 6 (Subfamily J)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
J	J	PVX_010605	286	variable surface protein Vir15, putative	No
J	J	PVX_167265	278	variable surface protein Vir15, truncated, putative	No
J	J	PVX_109795	301	variable surface protein Vir33, putative	No
J	J	PVX_107750	301	variable surface protein Vir2/15-like	No
J	J	PVX_105710	245	variable surface protein Vir1/9, putative	No
J	J	PVX_096935	304	variable surface protein Vir9-related	No
J	J	PVX_096930	248	variable surface protein Vir1-related, truncated	No
J	J	PVX_070690	260	variable surface protein Vir33, truncated, putative	No
J	J	PVX_067690	266	variable surface protein Vir1-like	No
J	J	PVX_057690	301	variable surface protein Vir33, putative	No
J	J	PVX_000020	234	variable surface protein Vir9-like	No
Not clustered	J	PVX_121355	222	variable surface protein Vir15-like	No
Not clustered	J	PVX_106720	220	variable surface protein Vir33 (truncated), putative	No
Not clustered	J	PVX_186290	133	variable surface protein Vir15-related, truncated	No
Not clustered	J	PVX_096005	367	variable surface protein Vir15-related	No
Unavailable	J	PVX_185285	345	variable surface protein Vir2, truncated, putative	No
Unavailable	J	PVX_168270	343	variable surface protein Vir15, pseudogene, putative	No
Unavailable	J	PVX_096925	289	variable surface protein Vir15-like	No
No Vir	J	PVX_105705	191	hypothetical protein (variable surface protein Vir, putative)	Yes

Cluster 11 (Subfamily K)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
K	K	PVX_056690	190	variable surface protein Vir30, truncated, putative	No
K	K	PVX_145260	305	variable surface protein Vir30, putative	No
K	K	PVX_053690	309	variable surface protein Vir30, putative	No
K	K	PVX_042690	337	variable surface protein Vir30, putative	No
K	K	PVX_031690	268	variable surface protein Vir30, putative	No
Not clustered	K	PVX_001610	338	variable surface protein Vir25-related	No
Unavailable	K	PVX_163265	345	variable surface protein Vir30, pseudogene, putative	No
No Vir	K	PVX_005565	209	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	K	PVX_086885	266	hypothetical protein (variable surface protein Vir, putative)	No
No Vir	K	PVX_115990	271	hypothetical protein (variable surface protein Vir, putative)	No

Cluster 10 (Subfamily: Not clustered)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
Not clustered		PVX_086855	721	variable surface protein Vir24-like	No
Not clustered		PVX_011615	351	variable surface protein vir23, putative	No
Not clustered		PVX_173270	346	variable surface protein Vir23, putative	No
Not clustered		PVX_086880	268	variable surface protein Vir23-like	No
Not clustered		PVX_069690	327	variable surface protein Vir23, putative	No
Not clustered		PVX_093725	392	variable surface protein Vir24-related	No
Not clustered		PVX_051190	286	variable surface protein Vir23, truncated, putative	No
Not clustered		PVX_096940	292	variable surface protein Vir24-related	No
Unavailable		PVX_005047	527	variable surface protein Vir22/23-related	No
Unavailable		PVX_103675	462	variable surface protein Vir23, pseudogene, putative	No

Cluster 13 (Subfamily: Not clustered)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
Not clustered		PVX_009600	259	variable surface protein Vir5-related	No
Not clustered		PVX_021685	426	variable surface protein Vir5, putative	No
Not clustered		PVX_101570	386	variable surface protein Vir5-related	No
Not clustered		PVX_093715	499	variable surface protein Vir5-like	No
Unavailable		PVX_156260	213	variable surface proteinVir5, pseudogene, putative; truncated	No

Cluster 14 (Subfamily: Not clustered)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
Not clustered		PVX_043190	319	variable surface protein Vir12, truncated, putative	No
Not clustered		PVX_131260	278	variable surface protein Vir12, truncated, putative	No
Not clustered		PVX_130260	108	variable surface protein Vir12, truncated, putative	No
Not clustered		PVX_129260	59	variable surface protein Vir12, putative	No

Suplementary Figure 2

Description: new subtelomeric families derived from previous *vir* members. Rows in red indicate that the HB architecture of the corresponding protein does not clearly match with the rest of members in the family.

MAAP (Malaria Adhesins and Adhesins-like Predictor)

Cluster 9 (Family PvPIRA)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
A	PvpirA	PVX_011610	362	variable surface protein Vir17, putative	No
A	PvpirA	PVX_161260	209	variable surface protein Vir3, truncated, putative	No
A	PvpirA	PVX_160260	166	variable surface protein Vir3, truncated, putative	No
A	PvpirA	PVX_141260	385	variable surface protein Vir17, putative	No
A	PvpirA	PVX_112645	321	variable surface protein Vir17-like	No
A	PvpirA	PVX_019160	394	variable surface protein Vir17, putative	No
A	PvpirA	PVX_018155	393	variable surface protein Vir17, putative	No
A	PvpirA	PVX_015135	383	variable surface protein Vir17, truncated, putative	No
Not clustered	PvpirA	PVX_153260	67	variable surface protein Vir17, truncated, putative	No
Not clustered	PvpirA	PVX_144260	430	variable surface protein Vir17, putative, pseudogene	No
Unavailable	PvpirA	PVX_186285	306	variable surface protein Vir3, truncated, pseudogene	No
Unavailable	PvpirA	PVX_181275	485	variable surface protein Vir17, pseudogene, putative	No
Unavailable	PvpirA	PVX_171270	253	variable surface protein Vir17, pseudogene, putative	No
Unavailable	PvpirA	PVX_155260	369	variable surface rptoein Vir3, pseudogene, putative; No 5' found.	No
Unavailable	PvpirA	PVX_139260	362	variable surface protein Vir3, truncated,putative	No

Cluster 5 (Family PvPIRD)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
D	PvpirD	PVX_103160	254	variable surface protein Vir10-like	No
D	PvpirD	PVX_178275	267	variable surface protein Vir11, putative	No
D	PvpirD	PVX_146260	239	variable surface protein Vir11, putative	No
D	PvpirD	PVX_143260	248	variable surface protein Vir35, putative	No
D	PvpirD	PVX_109785	261	variable surface protein Vir35, putative	No
D	PvpirD	PVX_109780	266	variable surface protein Vir35, putative	No
D	PvpirD	PVX_108760	261	variable surface protein Vir35, putative	No
D	PvpirD	PVX_103145	168	variable surface protein Vir10, putative	No
D	PvpirD	PVX_102635	198	variable surface protein Vir10-related	No
D	PvpirD	PVX_086850	245	variable surface protein Vir35, putative	No
D	PvpirD	PVX_074695	257	variable surface protein Vir10, putative	No
D	PvpirD	PVX_067190	235	variable surface protein Vir35, putative	No
D	PvpirD	PVX_047690	267	variable surface protein Vir10, truncated, putative	No
D	PvpirD	PVX_040690	166	variable surface protein Vir35, putative	No

D	PvpirD	PVX_038690	199	variable surface protein Vir35-like	No
D	PvpirD	PVX_019665	282	variable surface protein Vir11, putative	No
D	PvpirD	PVX_017645	230	variable surface protein Vir35, putative	No
D	PvpirD	PVX_182275	151	variable surface protein Vir11, truncated, putative	No
D	PvpirD	PVX_041190	144	variable surface protein Vir35, truncated, putative	No
Not clustered	PvpirD	PVX_058190	123	variable surface protein Vir10/35, truncated, putative	No
Unavailable	PvpirD	PVX_109778	240	variable surface protein Vir, putative	No

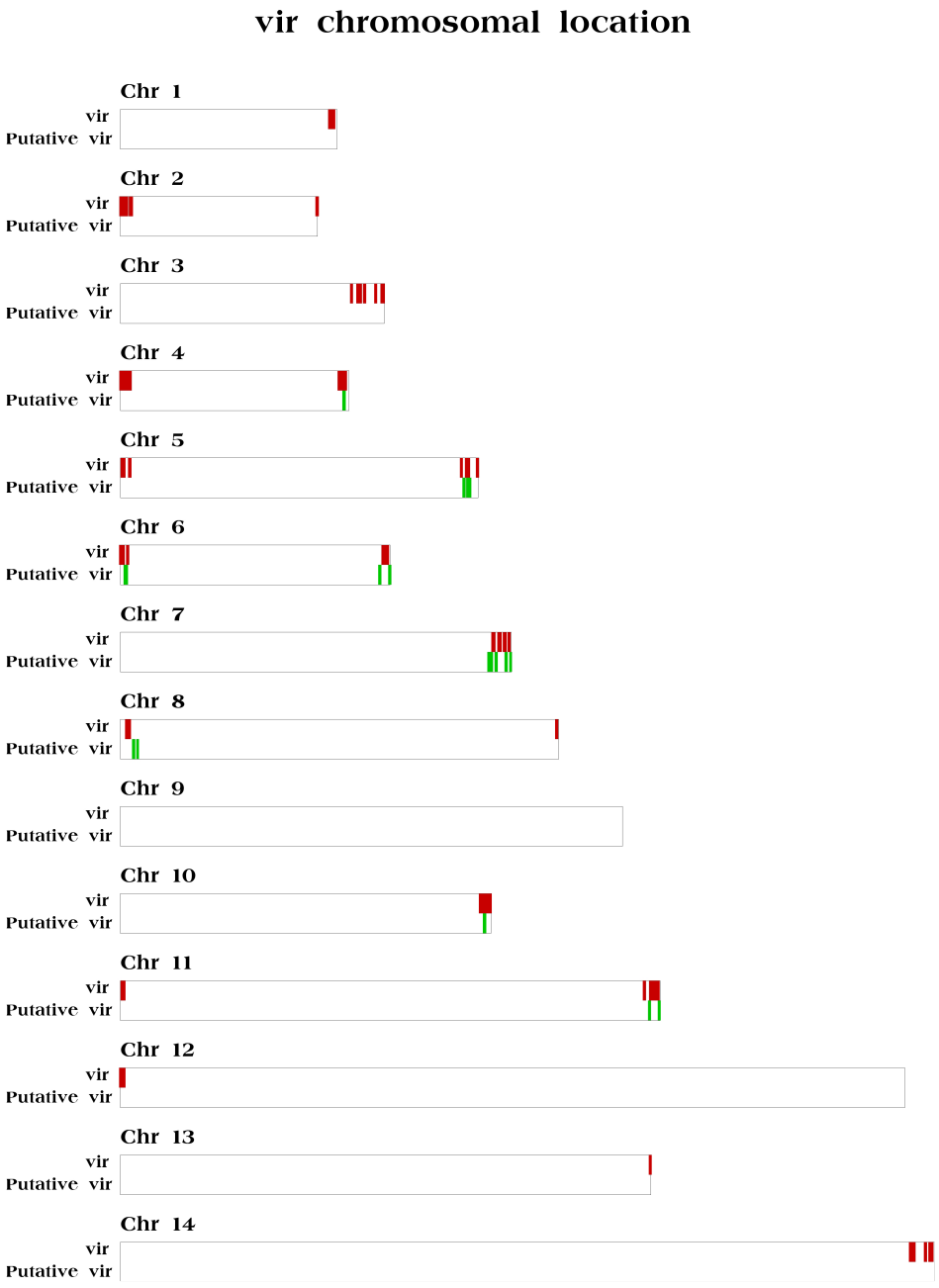
Cluster 4 (Family PvPIRH)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
H	PvpirH	PVX_017145	478	variable surface protein Vir18, putative	No
H	PvpirH	PVX_241295	489	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_112125	423	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_104700	475	variable surface protein Vir18, putative	No
H	PvpirH	PVX_103150	488	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_101625	527	variable surface protein Vir18-like	No
H	PvpirH	PVX_083585	548	variale surface protein Vir18-related	No
H	PvpirH	PVX_068690	529	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_068190	511	variable surface protein Vir18, putative	No
H	PvpirH	PVX_065190	442	variable surface protein Vir18, truncated, putative	No
H	PvpirH	PVX_062690	462	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_055190	378	variable surface protein Vir18, truncated, putative	No
H	PvpirH	PVX_021180	467	variable surface protein Vir18, putative	No
H	PvpirH	PVX_020675	495	variable surface protein Vir18, putative	No
H	PvpirH	PVX_019165	503	variable surface protein Vir18, putative	No
H	PvpirH	PVX_018150	510	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_017650	502	variable surface protein Vir18, putative	Yes
H	PvpirH	PVX_016640	483	variable surface protein Vir18-like	Yes
H	PvpirH	PVX_013120	475	variable surface protein Vir18, putative	No
H	PvpirH	PVX_011110	475	variable surface protein Vir18-like	No
H	PvpirH	PVX_010100	478	variable surface protein Vir18, putative	Yes
Not clustered	PvpirH	PVX_121855	402	variable surface protein Vir18, putative	No
Not clustered	PvpirH	PVX_066190	463	variable surface protein Vir18-like	No
Not clustered	PvpirH	PVX_060690	656	variable surface protein Vir18-related	Yes
Not clustered	PvpirH	PVX_015130	459	variable surface protein Vir18-like	No
Not clustered	PvpirH	PVX_006080	577	variable surface protein Vir18-related	No
Not clustered	PvpirH	PVX_093705	318	variable surface protein Vir18, putative	No
Not clustered	PvpirH	PVX_045190	130	variable surface protein Vir18-related	No
Not clustered	PvpirH	PVX_015640	427	variable surface protein Vir18-related	No
Not clustered	PvpirH	PVX_120335	485	variable surface protein Vir18-related	Yes
Unavailable	PvpirH	PVX_013625	381	variable surface protein Vir18, putative	Yes
No Vir	PvpirH	PVX_107230	416	hypothetical protein	No
No Vir	PvpirH	PVX_106215	328	hypothetical protein	No
No Vir	PvpirH	PVX_035690	298	hypothetical protein	No

Cluster 12 (Unclassified)

Previous subfamily	Proposed subfamily	ID	Length (aa)	Annotation	MAAP
Not clustered		PVX_096970	491	variable surface protein Vir8-related	No
Not clustered		PVX_120840	324	variable surface protein Vir8-related	No
Not clustered		PVX_096985	387	variable surface protein Vir, putative	No
Not clustered		PVX_096980	396	variable surface protein Vir, putative	No
Not clustered		PVX_086890	271	variable surface protein Vir8-like	No
Not clustered		PVX_176270	339	variable surface protein Vir8	No
No Vir		PVX_123205	2024	CAF1 ribonuclease domain containing protein	No
No Vir		PVX_092630	2712	hypothetical protein, conserved	No
No Vir		PVX_096975	556	hypothetical protein	No
No Vir		PVX_119620	1384	hypothetical protein, conserved	No
No Vir		PVX_096965	285	hypothetical protein	No
No Vir		PVX_183275	391	hypothetical protein	No

Supplementary Figure 3



Description: For each chromosome, two rows of colored boxes are shown: one row illustrates the genomic location of the vir members (red) while the other illustrates the location of the putative vir (green) in that chromosome. Only genes annotated at specific chromosomes are shown.

Supplementary Figure 5

Distribution of the conserved HBs accross clusters.

For each cluster, the number (and proportion) of HBs shared with other clusters is shown, as well as the number (and proportion) of cluster-specific HBs.

Cluster	Exclusive	Shared
Cluster 1 (Subfamamily E)	63 (53%)	56 (47%)
Cluster 2 (Subfamily C)	36 (46%)	42 (54%)
Cluster 3 (Not clustered)	8 (24%)	25 (76%)
Cluster 4 (Family PvPIRH)	20 (91%)	2 (9%)
Cluster 5 (Family PvPIRD)	8 (100%)	0 (0%)
Cluster 6 (Subfamily J)	2 (8%)	24 (92%)
Cluster 7 (Subfamamily I)	15 (44%)	19 (56%)
Cluster 8 (Subfamily B)	15 (56%)	12 (44%)
Cluster 9 (Family PvPIRA)	16 (59%)	11 (41%)
Cluster 10 (Not clustered)	10 (29%)	24 (71%)
Cluster 11 (Subfamily K)	6 (43%)	8 (57%)
Cluster 12 (Subfam. Not clustered)	1 (14%)	6 (86%)
Cluster 13 (Subfam. Not clustered)	2 (29%)	5 (71%)
Cluster 14 (Subfam. Not clustered)	0 (0%)	13 (100%)

Suplementary Figure 4, 5, 6 and 7

Due to the extensive length of supplementary figures 4, 5, 6 and 7, these figures are found in the electronic version of this thesis and in the website of the article.

Introduction

Hypothesis and objectives

Results

- Article 1
- Article 2
- **Unpublished results**

Discussion

Conclusions

Annex

- Summarized Catalan version
- Apicoplast's RNA Binding Protein Research Article
- Other contributions

Bibliography

Plasmodium vivax subtelomeric variant proteins and cytoadherence to the human spleen

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Abstract

The lack of a continuous *in vitro* culture system for blood stages of *Plasmodium vivax* has severely limited our understanding of the molecular basis of pathology in this species. Noticeably, recent data have challenged the dogma that *P. vivax*-infected reticulocytes do not cytoadhere in the deep vasculature of internal organs. Here, we show that a transgenic line of *P. falciparum* expressing a VIR protein but not another transgenic line expressing a Pv-FAM-D protein, mediated adherence to human spleen fibroblasts. Spleen-adherence specificity was shown as neither transgenic line bound to human lung fibroblasts. To extrapolate these results to natural infections, adhesion experiments using *P. vivax*-infected reticulocytes obtained from human patients were performed on human spleen fibroblasts. Results demonstrated adherence, albeit variable, among different isolates. Moreover, this adherence was inhibited by an anti-VIR antibody. These data reinforce the fact that cytoadherence is not exclusive of *P. falciparum* and challenges the dogma that the spleen is the evolutionary driven force for cytoadhesion of infected red blood cells in other organs for avoidance of spleen- clearance.

Introduction

Plasmodium vivax is geographically the most widespread human malarial parasite [1], accounting for 25-40% of the world malaria burden and it is estimated that causes from 132 to 391 million clinical cases each year [2]. Despite its historical perceiving as a benign infection, recent clinical data is challenging this accepted view. Clinical vivax malaria cases can lead to severe symptoms such as severe anemia, respiratory distress and spleen rupture [3-6] and recently, severe and fatal disease has been associated to *P.vivax* and confirmed by PCR [6].

One of the key features of *P. falciparum* pathogenesis is parasites' mature stages sequestration in the capillaries of the inner organs that can produce organic failure and fatal malaria [7, 8]. In consequence, by cytoadhering to endothelial receptors, *P. falciparum* rigid mature stages avoid spleen filtration and clearance [9]. On the other hand, because *P. vivax* mature stages are detected in peripheral circulation, it is widely believed that it does not cytoadhere to the deep vasculature of internal organs. Contrary to this view, recent articles have described *in vitro* cytoadhesion of *P. vivax* to brain and lung endothelial cells [10], placenta cryosections [10, 11] and binding to placenta glycosaminoglycans such as CSA [11]. Moreover, it has been demonstrated the scattered presence of the parasite in intervillous space of 3 placentas sections [12] and a large presence of this parasite in the spleen of a splenectomized patient [13].

The *P. falciparum var* subtelomeric gene family encoding the collectively known Erythrocyte Membrane Protein 1 (PfEMP1) is the main ligand that mediates cytoadhesion in this species [14-16]. On the other hand, in *P. vivax,* a multigene family termed *vir* (*P. vivax* variant genes) was described in chromosomal subtelomeric ends of the parasite [17]. Initial studies revealed that, unlike *P. falciparum var* genes [18, 19], *vir* genes and VIR proteins were not expressed under allelic exclusion nor clonally, respectively, questioning their role in the strict sense of antigenic variation [20]. The complete annotation of the *P. vivax* Sal1 strain genome described 346 *vir* genes subgrouped in 12 different subfamilies [21]. However, a new computational approach has excluded previous subfamilies A, D and H as members of the *vir* multigene family that now includes 295 members [22]. In the absence of a *P. vivax* long term *in vitro* culture system

for blood stages, we constructed *P. falciparum* transgenic lines expressing three different VIR-tagged proteins to study the biology of these proteins and gain insights into its function [23]. Of them, VIR14 pertaining to subfamily C was expressed at the surface of infected red blood cells (iRBC) and specifically cytoadhered under static and flow conditions to CHO cells expressing the endothelial receptor ICAM-1.

Based on a reticulocyte-prone nonlethal rodent malaria model [24], our group postulated a daring hypothesis suggesting that *P. vivax* triggers a remodeling of the spleen by the reorganization of barrier cells of fibroblastic origin. It was proposed that infected reticulocytes specifically cytoadhere to these cells through VIR proteins or other ligand(s) yet to be identified [25]. In doing so, *P. vivax* infected reticulocytes are protected of the spleen macrophage clearance. In addition, the merozoite-reticulocyte encounter will be facilitated, as spleen erythropoiesis is induced by infections [26, 27]. Recently, our group described the presence of a barrier of fibroblastic origin in the spleen of mice infected with the reticulocyte-prone non-lethal *P. yoelii* 17X strain. Notably, reduction of macrophage clearance was described, altogether with reduction of parasite motility and directionality observed by intravital microscopy. Moreover, parasite accumulation associated to this barrier was observed [28].

The demonstration of *P. vivax* cytoadherence to the human spleen is a rather difficult objective due to fact that biopsies of this organ are ethically undoable as well as to the low cases of *P. vivax* spleen rupture before antimalarial treatment. Nevertheless, these studies remain of fundamental value to understand the pathophysiology of *P. vivax.* In here, we describe the use of *P. falciparum* transgenic strains expressing proteins of *P. vivax* subtelomeric multigene families to assess the binding of VIR proteins to spleen fibroblast and to decipher the role of ICAM-1 in this adhesion. Moreover, adhesion of *P. vivax* Brazilian wild isolates to spleen fibroblast will also be presented.

Results

VIR14 mediates adhesion to human spleen fibroblasts

To assess the adhesion of VIR proteins to human spleen fibroblasts, we performed cytoadhesion assays by using the *P. falciparum* transgenic line that expresses VIR14 (PVX_108770)(3D7_vir14-3HA). This transgenic strain presented cytoadhesion under static conditions to CHO cells expressing CD36, ICAM-1, VCAM and E-Selectine and specifically to CHO- ICAM-1 cells under physiological flow conditions [23]. To perform cytoadhesion assays we got a commercial cell line of human spleen fibroblast (Hs 697.Sp) from a patient who previously had a granulomatous lymph node and Hodgkin's disease without spleen involvement. Significant cytoadhesion of transgenic line 3D7_vir14-3HA was found under both flow and static conditions compared to the parental strain 3D7 (Figure 1A). Remarkably, adhesion to Hs 697.Sp was five times higher than adhesion to CHO-ICAM- 1 cells (Figure 1A and C). To avoid confounding, we generated a human spleen fibroblast cell line (1010T) by culturing for three weeks the cells obtained from a homogenized healthy spleen from a donor. The transgenic strain 3D7_vir14-3HA also presented a significant adhesion to 1010T fibroblasts compared to the 3D7 parental strain (Figure 1B and C). To determine the specificity of adhesion to spleen fibroblasts and knowing that static and flow conditions gave not statistically significant different results, static adhesion experiments were done using commercial lung fibroblasts (WI-38). No significant adhesion of the transgenic line 3D7_vir14-3HA was found to the WI-38 lung fibroblasts (Figure 1B and C).

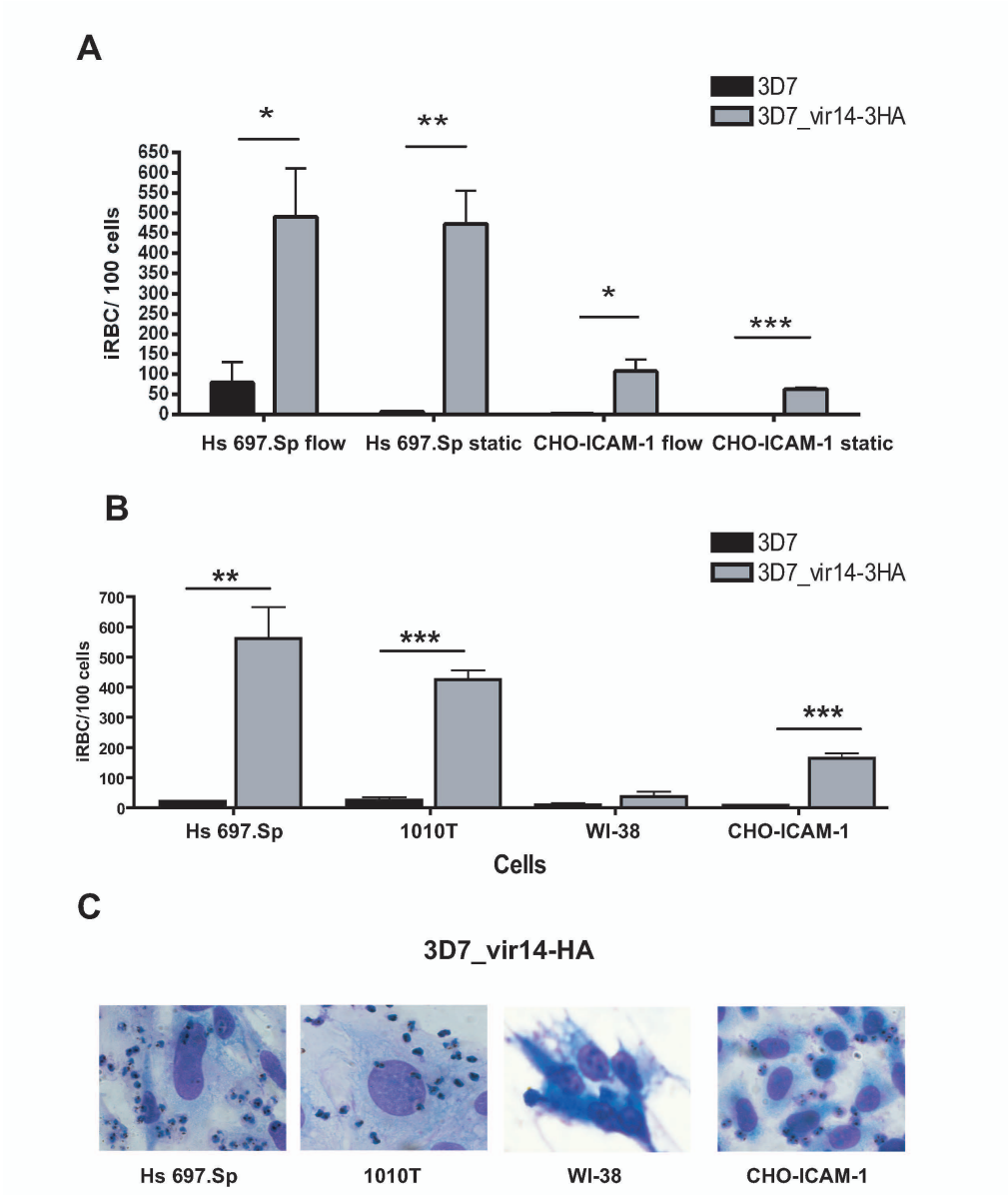

Figure 1. The transgenic strain 3D7_vir14-3HA mediates adhesion to human spleen fibroblasts

Cytoadherence was expressed as iRBC per 100 cells. Transgenic lines that significantly adhered to the cells compared with 3D7 parental strain, are marked with an asterisk (t-Student test) (*P<0,05; **P < 0.01; ***P < 0.005). Results are shown as the mean of the binding of 3 to 5 experiments ± standard error.

A. Cytoadherence of 3D7 (black bars) and the *P. falciparum* transgenic line 3D7_vir14-3HA (grey bars) to CHO-ICAM-1 cells and to human spleen fibroblasts Hs 697.Sp, under static and flow conditions at a wall shear stress of 0.09 Pa.

B. Cytoadherence of 3D7 (black bars) and 3D7_vir14-3HA (grey bars) to CHO-ICAM-1 cells, lung fibroblasts WI-38 and spleen fibroblasts Hs 697.Sp and 1010T.

C. Stained images of cytoadhesion of transgenic line 3D7_vir14-3HA to human spleen fibroblasts Hs 697.Sp (left) and 1010T (center left), lung fibroblasts WI-38 (center right) and CHO-ICAM-1 cells (right).

Finally, global transcriptional analysis of highly synchronous cultures was done to exclude the possibility that the observed adhesion could be mediated by the overexpression of a certain PfEMP1 variant triggered by the expression of VIR14 (Table 1). Only exported proteins of unknown function and the Knob Associated Histidine Protein (KAHRP) were differentially expressed in transgenic line 3D7_vir14-3HA. Due to an unknown mechanism, overexpression of KAHRP is always triggered in our *P. falciparum* transgenic lines that express *P. vivax* proteins. Despite no global expression analysis was done in the non-adherent cell line that also expresses a VIR protein in the membrane (3D7_vir10-3HA) [23], the presence of knobs was suggested due to the possibility to concentrate mature stages by the use of gelatin containing media (data not shown), compared to the inability to concentrate the long term cultivated 3D7 strain [55]. As a consequence, these data point to a role of VIR14 in mediating adherence to human spleen fibroblasts.

Gene	Name	3D7	3D7_vir14-3HA
PFB0105c	Plasmodium exported protein (PHISTc)	3.09 ± 0.48	9.00 ± 1.43
PFB0075c	Plasmodium exported protein (hyp9)	3.16 ± 0.1	6.92 ± 0.26
PFB0080c	Plasmodium exported protein (PHISTb)	3.09 ± 2.65	6.04 ± 0.05
PFB0100c	Knob associated histidine-rich protein (KAHRP)	4.50 ± 0.47	10.27 ± 0.21

Table 1. Global transcriptional analysis of highly synchronous cultures. Upregulated genes in the transgenic strain 3D7_vir14-3HA compared to 3D7 parental strain. Mean values ± standard deviation of two biological replicates are shown.

Role of ICAM-1 in the adhesion to spleen fibroblasts

To determine the role of ICAM-1 in the adhesion of the transgenic line expressing VIR14 to CHO-ICAM-1 cells and spleen fibroblast, we performed adhesion inhibition assays using the monoclonal antibody anti-ICAM-1 15.2. This antibody successfully blocks the adhesion of infected *P. falciparum* parasites to ICAM-1 [29]. Transgenic strain 3D7_vir14-3HA had an adhesion to CHO-ICAM-1 cells 30% lower in the presence of the inhibitor antibody, and there was a 40% inhibition in the case of the spleen fibroblast Hs 697.Sp. Despite the inhibition was statistically significant, none complete inhibition of the adhesion was observed in neither CHO-ICAM-1 nor Hs 697.Sp cells (Figure 2A). On the other hand, no inhibition of the adhesion was found in the case of the 1010T spleen fibroblasts.

Because the results obtained in inhibition adhesion assays were unclear, we wanted to determine the expression of ICAM-1 in the cells used in adhesion experiments. Immunofluorescence assays were carried out using CHO-ICAM-1 cell line as a positive control of expression and CHO-745 cells as a negative control. Both spleen fibroblasts cell lines showed a much fainter stain than CHO-ICAM-1 cells. On the other hand, WI-38 lung fibroblasts presented an even slightly fainter stain than spleen fibroblasts. Similar results were obtained in FACS analysis (data not shown). Despite the significant inhibition of the adhesion to CHO-ICAM-1 and Hs 697.Sp fibroblasts, those experiments exclude a main role of ICAM-1 in mediating the adhesion to the 3D7_vir14-3HA strain.

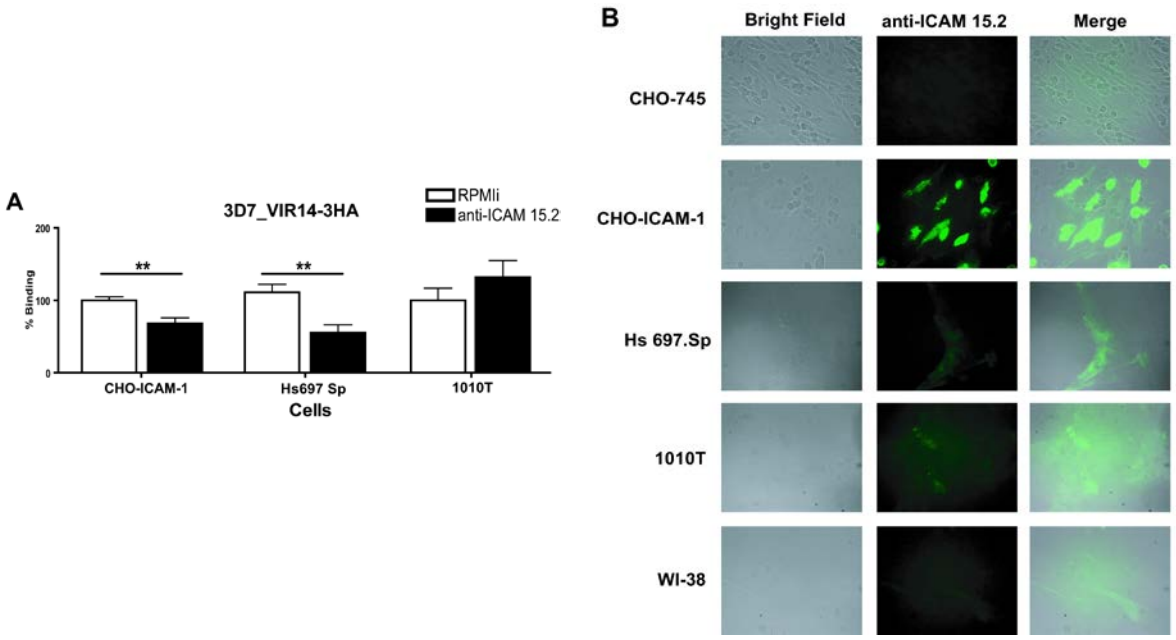

Figure 2. Role of ICAM-1 in the adhesion to spleen fibroblasts

A. Adhesion-inhibition assay with the anti-ICAM 15.2 monoclonal antibody. Adhesion of *P. falciparum* transgenic line 3D7_vir14-3HA in the presence (black bars) or absence (white bars) of anti-ICAM 15.2 to CHO-ICAM-1 cells and to human spleen fibroblasts (Hs 697.Sp, 1010T) under static conditions. Results are shown as % of binding. For these assays, binding of iRBCs to cells pretreated with incomplete RPMI media were considered to have a 100% of cytoadhesion. Statistically significant differences in cytoadhesion are shown with an asterisk (**P < 0.01) (t-Student test). Mean ± standard error of three independent experiments is shown.

B. Indirect immunofluorescence assay. Fixed cells were stained with anti-ICAM 15.2 monoclonal antibody (green). The first column represents DIC and the third column the merge of the two images.

Adhesion of *P. vivax*- infected reticulocytes to spleen fibroblasts

To assess whether wild isolates present adhesion to spleen fibroblast, we performed adhesion assays using peripheral blood of *P. vivax* patients that came to the Tropical Medicine Foundation Dr. Heitor Vieira Dourado (FMT-HVD) in Manaus (Brazilian Amazon). The patients were diagnosed by an expert microscopist through the examination of thick blood smears, suffered from non-severe malaria and were in age between 20-40 years old. As the majority of samples that we received presented early blood stages, these samples were matured for 16h in McCoy's 5A medium in the presence

of 20% human serum at 37°C in low O_2 conditions. Depending on the initial stage of the parasite in the peripheral blood, infected-reticulocytes used in these experiments ranged from 28h to 46h stages of the asexual blood cycle. As expected, huge variability in adhesion levels to human spleen fibroblast Hs 697.Sp was observed depending on the wild isolate used (Figure 3A). A group of samples had a low binding to spleen fibroblasts (Pv61, Pv65, Pv67 and Pv74). There was also a group with a medium adhesion to Hs 697.Sp cells (Pv59, Pv63, Pv64, and Pv70). Noticeably, this group had adhesion levels just slightly lower than the transgenic strain 3D7_vir14-3HA (note that due the low parasitaemias associated to *P. vivax,* 20 times less parasite was used in these adhesion assays). Last, one strain presented a very strong adhesion to human spleen fibroblasts (Pv69) (Figure 3A). Surprisingly, the isolates with a medium and high fibroblast adhesion rate bound in a concentrated pattern to only a subpopulation of Hs 697.Sp cells (data not shown). Of interest, the binding observed seems independent of the stage of the parasite, as early trophozoites citoadhered in patient Pv69, late trophozoites in patient Pv59 and mature schizonts in the case of patient Pv64 (Figure 3B).

Finally, to decipher the role of VIR proteins as possible ligands and ICAM-1 as a plausible receptor, Hs 697.Sp adhesion-inhibition experiments were done using the monoclonal anti-ICAM-1 15.2 antibody and guinea pig sera raised against anti-VIR conserved motifs (anti-LP1 and anti-LP2) [23]. Noticeably, no inhibition of adhesion was shown in the inhibition assays done with the anti-ICAM-1 15.2 monoclonal antibody (Figure 3C). In addition, the nine wild isolates tested did not present binding to CHO-ICAM-1 cells (data not shown). In contrast, statistically significant inhibition of the adhesion was found when the anti-LP2 antibody was used. Of interest, this antiserum was raised against C- and N-terminal conserved motifs of the VIR proteins. This inhibition was observed indistinctly in the isolates with low, medium and high adhesion to Hs 697.Sp cells. Finally, no inhibition of the adhesion was observed in the case of the anti-LP1 antiserum against central core conserved motifs (Figure 3C).

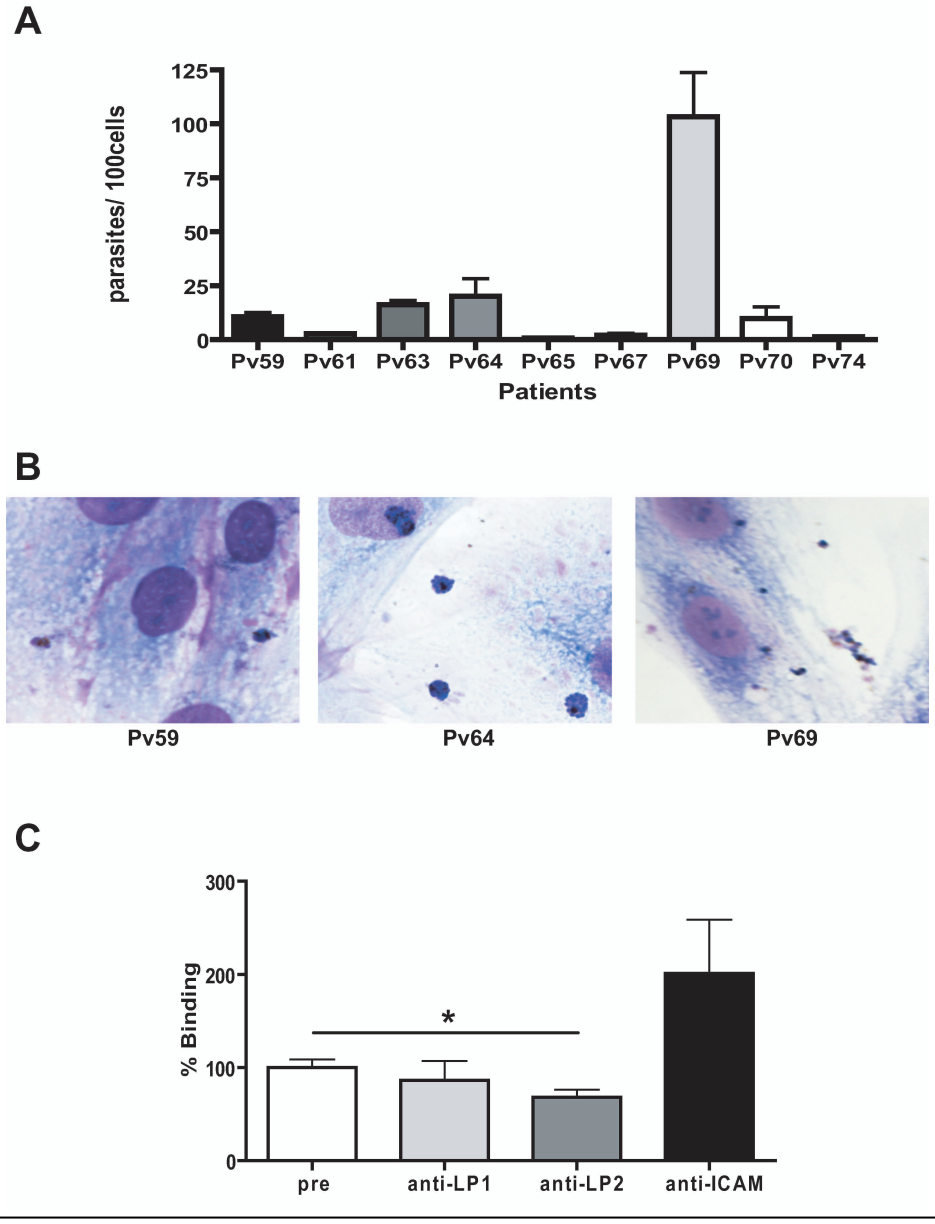

Figure 3. *P. vivax* isolates cytoadhere to human spleen fibroblasts (Hs 697.Sp) through VIR proteins.

A. Cytoadhesion of *P. vivax* wild isolates from infected patients (Pv59-74) to human spleen fibroblasts Hs697.Sp. Cytoadherence was expressed as parasites per 100 cells. Results are shown as mean of the binding of duplicates ± standard error.

B. Stained image of the cytoadhesion of patients Pv59, Pv64 and Pv69 to human spleen fibroblasts Hs 697.Sp.

C. Inhibition-adhesion assays to Hs 697.Sp done with the anti-VIR antisera (anti-LP1 and anti-LP2) and the anti-ICAM 1.2 monoclonal antibody. Results are shown as % of binding. For these assays, cells pre-treated with incomplete RPMI media (anti-ICAM 15.2) or parasites pre-treated with pre-immune sera (anti-LPs) were considered to have a 100% of cytoadhesion. Statistically significant differences in cytoadhesion are shown with an asterisk (*$P < 0.05$) (t-Student test). Mean ± standard error of duplicates is shown

Role in cytoadhesion of other variant multigene families

To decipher if other variant multigene families could be implicated in adhesion, we generated a *P. falciparum* transgenic line that expressed a member of the *pv-fam-d* multigene family (PVX_101580). This gene presents a signal peptide and two transmembrane domains thus revealing a possible export of this protein. The *P. vivax* gene was expressed into plasmid pARL1a- with a triple hemagglutinine tag (3xHA) and under the control of the *P. falciparum* CRT promoter and its expression in the *P. falciparum* transgenic line was validated by RT-PCR using a forward primer recognizing the *pv-fam-d* gene and a reverse primer that recognizes the tag (Figure 4A). Indirect immunofluorescence assays were done to determine the subcellular localization of the protein. As expected, Pv-FAM-D was found in the parasite membrane already in early stages of the asexual blood cycle. Co-localization assays using primers raised against Skeleton Binding Protein 1 (anti-SBP1) and the conserved cytosolic domain of PfEMP1 (anti-ATS) confirmed its membrane localization (Figure 4B and C) but apparently in a more external "layer" than the cytoplasmic tail of PfEMP1 (Figure 4C). In addition, a membrane surface expression of the protein was found in live immunofluorescence assays (Figure 4D). Later, cytoadhesion assays to human fibroblasts and CHO cells expressing endothelial receptors were carried on. No relevant adhesion to human endothelial receptors expressed in CHO and to human spleen fibroblast was found (neither Hs 697.Sp nor 1010T cells) (Figure 4E and F). A similar or even lower adhesion than the 3D7 parental strain was found, while the 3D7_vir14-3HA strain displayed a cytoadhesion higher than 300 iRBC/cell (Figure 4E and F). Last, a role in adhesion to lung fibroblast was excluded as no adhesion of the 3D7_Pv-fam-D-3HA strain was found to WI-38 fibroblasts (Figure 4F).

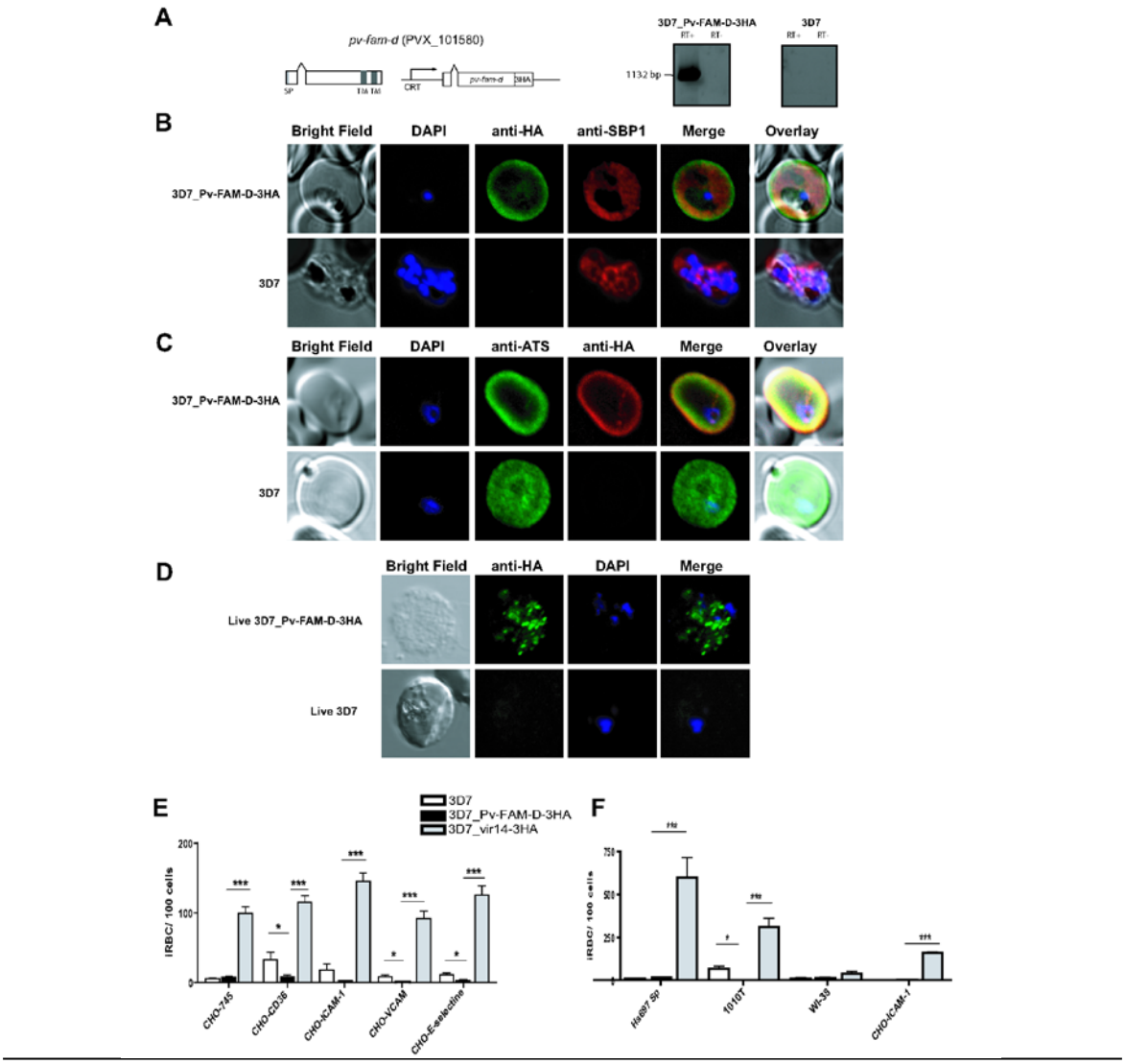

Figure 4. A PV-FAM-D member is expressed in the surface of the *P. falciparum* transgenic line and it is not implicated into adhesion to endothelial receptors nor human spleen and lung fibroblasts.

A. General structure of the *pv-fam-d* gene expressed *in P. falciparum*. SP: Signal Peptide and TM: Transmembrane domain (left). Diagram of the expression cassette of the transgenic line (left center). RT- PCR expression of the *pv-fam-D* gene in the transgenic *P. falciparum* line (3D7_Pv-fam-D-3HA) (right center) and in 3D7 (right). RT+ indicates cDNA treated with reverse transcriptase and RT- not treated.

B. Co-immunofluorescence images of transgenic strain 3D7_Pv-fam-D-3HA (upper row) and 3D7 (lower row) labeled with anti-HA (green), anti-SBP-1 (red) and DAPI (blue) for nuclear staining. The first column represents DIC, the fifth the merge of the fluorescent staining and the last the overlay of all five images.

C. Co-immunofluorescence images of transgenic strain 3D7_Pv-fam-D-3HA (upper row) and 3D7 (lower row) labeled with anti-HA (red), anti-ATS (green) and DAPI (blue) for nuclear staining. The first column represents DIC, the fifth the merge of the fluorescent staining and the last the overlay of all five images.

D. Live immunofluorescence assay. The anti-HA (green) antibody recognized the 3D7_Pv-fam-D-3HA transgenic line and did not recognize the 3D7 parental strain (lower row). The first column represents DIC, the third DAPI for nuclear staining and the last the overlay of all fluorescence images.

E and F. Cytoadhesion assays of the 3D7_Pv-fam-D-3HA transgenic strain. Cytoadherence was expressed as iRBC per 100 cells. Transgenic lines that have significant adhesion differences to a specific receptor, compared with 3D7 parental strain, are marked with an asterisk (t-Student-test) (*<0.05; ***P < 0.005). Results are shown as the mean of the binding of 3–5 experiments ± standard error. E. Cytoadherence of 3D7 and transgenic lines 3D7_vir14-3HA and 3D7_Pv-fam-D-3HA to CHO cells expressing human endothelial receptors in static conditions. F. Cytoadherence of 3D7 and transgenic lines 3D7_vir14-3HA and 3D7_Pv-fam-D-3HA to human fibroblasts (Hs 697.Sp, 1010T and Wi-38) and CHO-ICAM-1 cells.

Discussion

Despite it is widely believed that there is no cytoadherence in *Plasmodium vivax*, recent data is challenging this dogma [10-13]. Here, we demonstrate that both *P. vivax* infected-reticulocytes and the *P. falciparum* transgenic line 3D7_vir14-3HA present cytoadhesion to fibroblasts derived from the human spleen. Our results clearly demonstrated that at least some VIR proteins specifically mediate this cytoadherence. Moreover, a principal role of ICAM-1 in this adhesion was ruled out. Finally, the generation of a new transgenic line expressing a member of the *pv-fam-d* multigene family that localizes at the surface of the infected erythrocyte, excluded a role of this protein in cytoadhesion to neither endothelial receptors nor spleen fibroblasts and confirmed the specificity of adhesion of VIR14.

The Balb/*c P. yoelii* rodent malaria model revealed a remodeling of the spleen upon the infection with the reticulocyte-prone non-lethal strain 17X and the formation of a spleen blood barrier of fibroblastic origin where infected RBCs adhered [28]. Noticeably, we have shown that the *P. falciparum* transgenic line expressing VIR14 presented an adhesion fifty times higher than the 3D7 parental strain to commercial human spleen fibroblasts Hs 697.Sp and only slightly lower levels of adhesion to human spleen fibroblasts from a different origin (1010T). As the Hs 697.Sp line was derived from a donor who had a granulomatous lymph node and Hodgkin's disease, to minimize confounding effects we generated a spleen fibroblasts cell line (1010T) from a donor with an intact and healthy spleen. Spleen-adherence specificity was shown as no adhesion of the transgenic line 3D7_vir14-3HA was found to lung derived fibroblasts (WI-38). To

extrapolate these results, we also demonstrated adhesion of *P. vivax*-infected reticulocytes to spleen fibroblasts. Albeit variability between patients, similar binding rates as those observed in the 3D7_vir14-3HA strain were found in some patients. In addition, these results complement unpublished results of the group that also showed similar levels of adhesion of *P. vivax* wild isolates to human spleen cryosections (Mireia Ferrer PhD Thesis, 2012). Both results strongly complement previous results reported in the literature that demonstrated intact *P. vivax*-infected reticulocytes presence in the spleen of an untreated patient who suffered abdominal trauma and splenectomy [13]. We thus believe that active cytoadhesion as opposed to mechanical trapping is the most plausible explanation for the elevated number of parasites found in that spleen. In addition, spleen rupture is a *P. vivax* non-rare derived complication and has been described several times despite it has been probably under- reported [5, 30-34] and could be in part, the consequence of parasite accumulation.

The significant adhesion of the transgenic strain 3D7_vir14-3HA makes difficult to argue against a role of at least, some VIR proteins in this adhesion. The presence of VIR14 in the surface of the RBC, together with the absence of overexpression of any member of the PfEMP1 family seem to point VIR14 as the main responsible of active binding to human spleen fibroblasts. Interestingly, a member of the *P. falciparum* PHISTc family was upregulated in the transgenic line expressing VIR14. Of interest, the only sub-cellular localization in *Plasmodium* of a member of this multigene family has been shown in *P. cynomolgi* using antibodies raised against the Pv-FAM-B *P. vivax* protein. This study revealed the presence of the Pv-FAM-B (PHISTc) orthologous protein in the CVC and hypothesized that these structures might play a role in the implantation of VIR proteins into the membrane of the reticulocyte [35]. Because this variant family seems to display a role in traffic export, we rule out a direct intervention in mediating the adherence. On the other hand, there is no doubt that the overexpression of KAHRP may contribute in the generation of a scaffold that increases the binding of VIR14. However, a high presence of Knobs was also presented in the non-adherent transgenic line 3D7_vir10-3HA (data not shown), thus arguing against a direct contribution of KAHRP in the adhesion. But without doubt, the main arguments in defense of the role of VIR proteins in mediating (at least in part) spleen fibroblast adherence are the similar

binding rates obtained in both *P. vivax*-infected reticulocytes and the *P. falciparum* transgenic line together with the partial inhibition of the *P.* vivax-infected reticulocytes binding by the anti-LP2 sera. In addition, similar inhibition-adhesion results to those obtained in this thesis were found in adhesion assays of *P. vivax* to human spleen cryosections (Mireia Ferrer PhD thesis).

Differences in expression of variant proteins have been shown in *P. falciparum*, *P. knowlesi* and in other *Plasmodium* species depending on spleen presence [9, 36-41]. Noticeably, expression of VIR14 in a *P. vivax* Sal1 adapted strain was upregulated upon reinfection in *Aotus* monkeys with an intact spleen, after several passages in splenectomized animals (unpublished data) thus pointing to a role in mediating spleen clearance. For this reason, we can conclude that the spleen dependent expression of VIR14, together with the functional demonstration of the adhesion of VIR proteins in both transgenic lines and wild isolates, reinforces the role of at least a subset of these proteins as parasite spleen ligands. On the other hand, the expression of Pv-FAM-D at the surface of the iRBC in *P.falciparum* and its lack of adhesion to both fibroblasts and endothelial receptors reinforces the role of VIR14 as a *P. vivax* ligand. Remarkably, this member of the *pv-fam-d* subfamily was also overexpressed upon reinfection in monkeys with an intact spleen (unpublished data). Despite Pv-FAM-D function still remains unknown; the upregulation might reveal a spleen related function. Contrary to what happens in *P. falciparum, P. vivax* infection increases the deformability of the infected reticulocyte [42]. Because this protein presents two different transmembrane domains, it is tempting to speculate that Pv-FAM-D family members might play a role in increasing the infected-reticulocyte deformability. Further studies to reveal the function of this multigene family need to be done.

Unlike VIR proteins, the role of ICAM-1 in spleen adhesion appears much less clear. Despite immunofluorescence assays demonstrated that all fibroblasts express the receptor, all fibroblasts presented a much fainter stain than CHO-ICAM-1 cells. This low expression of the receptor argues against a principal role of ICAM-1 in spleen adhesion. In addition, non-adherent WI-38 lung fibroblasts presented similar expression levels than spleen fibroblasts. For this reason, despite ICAM-1 may be one factor in spleen homing, another receptor might play a much important role in this adhesion.

Indeed, a collaborative role of receptors is not an unusual event in *P. falciparum* infections and it has been demonstrated that ICAM-1 synergizes with CD36 to mediate firm iRBC binding [43]. The existence of an unknown receptor is supported by the fact that the transgenic strain 3D7_vir14-3HA showed a surprising adhesion for all the CHO cells expressing endothelial receptors under static conditions, including the CHO-745 that does not express any human endothelial receptor. In addition, this transgenic strain was unable to bind to human ICAM-1 purified protein (data not shown in collaboration with Professor Alister Craig). Last, when flow was applied in the CHO cells adhesion experiment, the binding levels to all cells types fell, except for ICAM-1 [23]. The most plausible explanation to this phenomenon is that ICAM-1 strength and binding avidity increases with flow in *P. falciparum* [43, 44]. Another argument against a main role if ICAM-1 in this adhesion, is the contradictory differences obtained in the inhibition adhesion assay. The monoclonal antibody anti-ICAM-1 15.2 presented a moderate but significant adhesion-inhibition of the line expressing the VIR14 to CHO-ICAM-1 cells and to Hs 697.Sp fibroblasts. In the contrary, this antibody failed in both inhibition assays of 3D7_vir14-3HA and *P. vivax* infected-reticulocytes to 1010T and to Hs 697.Sp fibroblasts, respectively. In *P. falciparum,* mutations in human receptors, such as ICAM-1 and CD36, and globin genes have been associated with different adhesion levels and susceptibility to severe *falciparum* malaria [45-47]. It could be feasible that the lower adhesion to 1010T cells as well as the absence of inhibition in this cell line may be due to the fact that the ICAM-1 polymorphism expressed by these cells is not favoring the adhesion to VIR14. Collectively, these data suggest that a secondary role of ICAM-1 in spleen adhesion. Under this scenario, the exact identity of the main receptor remains to be further investigated.

In summary, the data presented here clearly demonstrates that *P. vivax* presents cytoadhesion to the spleen and reveals that at least, some VIR proteins are involved in this adherence. Because, the role ICAM-1 in this adhesion remains unclear, further studies are needed to reveal the identity of this receptor. Finally, there is no doubt that the study of the evolutionary advantages of this adhesion and its implication in disease pathology could be a potential target to prevent severe symptoms produced by this neglected disease.

Experimental procedures

Ethics statement

All the studies involving human patients' *P. vivax* infected blood has received the oral informed consent of their donors. The procedures were approved by the Ethics Committee Board of the FMT-HVD (CAAE 03050512.70000.0005)

CHO cells and fibroblasts culture

CHO cells and 1010T fibroblasts were cultivated in complete RPMI medium (supplemented with 10% FBS serum) and Hs 697.Sp and WI-38 fibroblasts were culture in complete Dulbecco's Modified Eagle Medium (DMEM) (supplemented with 10% FBS serum). Cell trypsinization was done when the cells reached a 90% confluence.

***P. falciparum* static and flow cytoadhesion assays**

Static binding assays were performed as described previously [48]. Briefly, $5x10^4$ CHO cells (CHO-746, CHO-CD36, CHO-ICAM-1, CHO-VCAM and CHO-E-Selectine kindly donated by Professor Artur Scherf) [49] were seeded in 24 wells plates in coverslips (Nunc) and left to attach for 2 days. Half of the content of a 75cm2 flask of Hs 697.Sp, 1010T and WI-38 cell lines was used to seed 12 wells. In the case of Hs 697.Sp cell line, the cells were seeded 5 days before performing the experiment. Mature stages of cultured *P. falciparum* strains were enriched using a 70% Percoll solution and parasites were quantified using both a Neubauer chamber and a Giemsa stained smear. In the adhesion experiment, cells were washed previously in binding medium (RPMI pH 6,8 supplemented with 10% AB plasma) and 1 x 10^6 *P. falciparum* mature stages in 0,5 ml of binding medium were added to each well. Each experiment was run in triplicate. Cells plus iRBCs were incubated for 1 hour at 37°C in binding medium in a 5% CO_2 incubator. Unbound cells were washed by dipping twice coverslips in binding medium and exposed to a 30 minutes gravity wash in a 45^o angle with coverslips upside down. To avoid cell clumping Adhesion experiments with the WI-38 cell line were done in binding medium at a pH of 7.2, in this adhesion experiment the same conditions were used in parallel for CHO-ICAM-1 and Hs 697.Sp cells. For flow cytoadhesion assays, coverslips seeded with either CHO or Hs 697.Sp cell lines were mounted in a Cell Adhesion Flow Chamber. The system was connected to a precise infusion/withdrawal pump (model KDS120, IBIDI) to control the flow of the iRBCs suspension through the perfusion chamber.

$1x10^7$ iRBC were flowed over for a total of 30 minutes, and then binding buffer was flowed over for 10 minutes to remove unbound cells. The flow rate yielded a wall shear stress of 0.09 Pa, which mimics wall shear stresses in the microvasculature. Static and flow coverslips were fixed in methanol and stained with 10% Giemsa for 15 minutes. Adhesions were quantified in an optical light microscope.

***P. vivax* static cytoadhesion assays**

CHO-ICAM-1 and Hs 697.Sp cells were subcultered as described in the previous section. Blood from patients with confirmed *P. vivax* infection was collected in EDTA tubes and White Blood Cells were removed using a Plasmodipur filter (EuroProxima). Samples containing early stage parasites were matured for 16h in McCoy's 5A Medium supplemented with 20% of human AB+ serum and growth in low O_2 conditions (2%). Mature infected erythrocytes were enriched using a 40% Percoll gradient as described in [10] and quantified using both a Neubauer chamber and a slide stained with Panotico kit. Adhesions were performed using $5x10^4$ parasites per coverslip covered with cells grown to 60-80% confluence. Adhesions were done in duplicate. Cells plus iRBCs were incubated for 1 h at 37°C in a 5% CO_2 incubator in binding medium and unbound cells were washed as described in the previous section. Coverslips were fixed in methanol and stain using the Panotico kit.

***P. falciparum* and *P. vivax* Inhibition assays**

Inhibition adhesion assays to ICAM-1 were done by pre-incubating the CHO-ICAM-1, the Hs697 Sp and the 1010T cells for one hour in a 5% CO_2 incubator in incomplete RPMI containing the monoclonal mouse Anti-ICAM-1 antibody 15.2 (Abcam) at a 0,01mg/ml final concentration. Inhibition adhesion assays to VIR proteins were done incubating *P. vivax* mature stages enriched isolates in the presence of a 1/5 dilution of both anti-LP1 and anti-LP2 anti-sera [23] in incomplete RPMI for one hour at room temperature in constant agitation and then, parasites were washed in binding medium (pH 6,8) before the adhesion protocols described above were done.

Statistical analysis.

The statistical significance of adhesion to different cells types at various conditions was determined using the t-Student test. Experiments in *P. falciparum* transgenic strains were performed at least in three different experiments. Calculations were performed using Prism software (version 4; GraphPad Software). Differences were considered significant at *<0.05;

**<0,01 ***P < 0.005.

***P. falciparum* culture, plasmid constructs and parasite transfection**

P. falciparum parasites were cultured with B+ human erythrocytes (3% haematocrit) in RPMI media (Sigma) supplemented with 10% AB+ human plasma using standard methods [50]. *Pv-fam-D* gene (PVX_101580) was amplified from *P. vivax* Sal1 gDNA using primers F-PvfamD: GGTACCATGAAAATGAAAAAAAATAAG; R-PvfamD: ctgcagATTTCTTGGTCTTTTTTTTG and was cloned in the KpnI-PstI cloning sites of modified transfection vector pARL1a-3HA [23]. The plasmid pARL1a-PvfamD-3HA was transfected into 3D7 parasites by electroporating ring-stage parasites (> 5% parasitaemia) with 100 µg of purified plasmid DNA (Qiagen) as previously described [51] using 0.310 kV and 950 F electroporation conditions. 6h after transfection, 2,5nM WR99210 was added to the culture media. Culture was maintained in drug selection and 20 to 30 days after drug application parasites appear.

RNA preparation and RT-PCR

Trophozoite stage parasites were harvested at a parasitaemia higher than 5%, treated with 0.15% saponine and lysed in TRIzol reagent (Invitrogen). RNA was extracted using manufacturer's instruction. To minimize the risk of DNA contamination, RNA was DNase treated (Invitrogen). First-strand cDNA synthesis was performed as described in [23]. For RT- PCR, a specific forward primer of the *pv-fam-D* gene and a specific reverse primer of the triple HA tag were used.

***P. falciparum* indirect immunofluorescence assays**

Cultured *P. falciparum* 3D7_Pv-fam-D-3HA transgenic line presenting mixed stages was washed in PBS and then fixed with 4% EM grade paraformaldehyde and 0.075% EM grade glutaraldehyde in PBS [52]. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 hour at room temperature in 3% PBS-Bovine Serum Albumin (PBS-BSA). Samples were incubated overnight with primary antibody [rabbit anti-HA (1:50, Molecular Probes), rat anti-HA (1:50, Roche), mouse anti-ATS (1:50) or rabbit anti-SBP1 (1:750)] diluted in 3% PBS-BSA followed by 1 h incubation with secondary antibody [anti-mouse or anti-rat IgG conjugated with Alexa Fluor 488 and anti-rabbit IgG conjugated with Alexa Fluor 594 (1:100, Molecular Probes)] diluted in 3% PBS-BSA. Nuclei were stained in the secondary antibody incubation with 4,6-diaminido-2-phenylindole (DAPI, 2 mg/ml diluted in 3% PBS-BSA). Samples were mounted in Vectashield (Vector Labs) and confocal microscopy was performed using a laser scanning

confocal microscope (TCS-SP5; Leica Microsystems), at microscopy scientific and technical services of Universitat de Barcelona. Images were processed using ImageJ image browser software.

***P. falciparum* live immunofluorescence assays**

Cultured *P. falciparum* transgenic lines were washed in incomplete RPMI and blocked for 1 hour at room temperature in 3% RPMI-BSA. Samples were incubated for 1h with rabbit anti-HA [1:50, Molecular Probes] diluted in 3% RPMI-BSA. Followed by 1 h incubation with secondary antibody anti-rabbit IgG conjugated with Alexa Fluor 488 (1:100, Molecular Probes) diluted in 3% RPMI-BSA. Nuclei were stained in the secondary antibody incubation with DAPI (2 mg/ml). Samples were mounted in Vectashield (Vector Labs) and confocal microscopy was performed using a laser scanning confocal microscope (TCS-SP5; Leica Microsystems) and images were processes using ImageJ image browser software.

CHO cells and fibroblasts immunofluorescence assays

Cells subcultured in coverslips for 2 days were fixed for 10 minutes in 4% EM grade paraformaldehyde. Dried coverslips were blocked for 1 hour at room temperature in 3% PBS-BSA. Coverslips were incubated for 1hour with a mouse monoclonal anti-ICAM-1 antibody 15.2 [1:25, Abcam] diluted in 3% PBS-BSA. Followed by 1 hour incubation with a secondary antibody anti-mouse IgG conjugated with Alexa Fluor 488 [1:100, Molecular Probes] diluted in 3% PBS- BSA. Samples were mounted in Vectashield (Vector Labs) and microscopy was performed using an epifluorescence microscope (DMI 6000B; Leica Microsystems).

Global transcriptional analysis

Parasites were highly synchronized by a 70% Percoll gradient in cultures with mature stages, followed by a 5% sorbitol synchronization 4 to 5 hours later when merozoites egress had occurred. RNA was extracted using the RNeasy Mini Kit (QIAGEN) and RNA purification was assessed using a bioanalyzer an Agillent 2100 Bioanalyzer. A GeneChip Plasmodium/Anopheles Genome Array (Affymetrix) was used and two biological replicates were hybridized at the IDIBAPS genomic unit. Expression levels were normalized by using RMA (Robust Multi-array Average) [53]. Then, a low intensity filter was applied which removed all of those probes that yielded very low intensities (<5) for all samples. Thus, 2899 probes (222 genes) were selected for downstream analysis. Finally, differentially expressed genes were identified by running SAM (Significance Analysis of Microarrays) [54].

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Discussion

The main contribution of this thesis was the functional study of subtelomeric variant genes of *Plasmodium vivax* and the demonstration that some can mediate cytoadherence to endothelial receptors and to human spleen fibroblasts. In the absence of a continuous *in vitro* culture system for blood stages of *P. vivax*, we have used heterologous transfections in *P. falciparum* as previous reports had demonstrated the feasibility of this approach for functional analyses of *P. vivax* genes (Sa *et al.*, 2006, O'Neil *et al.*, 2007, Auliff *et al.*, 2010). Our results demonstrated that a variant VIR protein pertaining to the highly polymorphic subfamily C can mediate cytoadherence to the ICAM-1 endothelial receptor (Bernabeu *et al.*, 2012b) and to human spleen fibroblasts under flow physiological conditions. Specificity of such adherence was verified as this transgenic line failed to bind to human lung fibroblasts. Furthermore, the lack of adhesion of the transgenic line expressing a member of the *pv-fam-d* family, to both lung and spleen fibroblasts also reinforce the specificity of this adhesion. Noticeably, we showed that *P. vivax*-infected reticulocytes adhere to human spleen fibroblasts, albeit variably, challenging the dogma that adherence is the driving force to avoid spleen clearance in malaria. Last, a new computational approach redefined VIR proteins, and excluded subfamilies A and D from the *vir* multigene family raising the possibility that VIR proteins might still be related to antigenic variation in its strict sense.

Vivax malaria: a neglected infection

Historically, *P. vivax* has been considered a benign infection and even today, the view of vivax as a generally harmless disease prevails. Presumably this assumption is established, in part, due to the low and self-limiting parasitaemias present in peripheral blood, when compared to *P. falciparum* (Baird, 2013). However, recent data from vivax endemic countries demonstrates that *P. vivax* is non-rarely associated with severe illness and fatal outcomes (Baird, 2013). Hospital and village studies in areas where the two species coexist affirm that severe morbidity and mortality appear only slightly weighted toward falciparum malaria (Genton *et al.*, 2008, Tjitra *et al.*, 2008, Andrade *et al.*, 2010, Kochar *et al.*, 2010b, Manning

et al., 2011, Kaushik *et al.*, 2012, Mahgoub *et al.*, 2012). This suggests *P. vivax* infections carries similar risks of associated severe illness and death than *P. falciparum* (5-15% risk of death in severe cases) in populations which have been exposed to both parasites. These recent epidemiological data do not appear a consequence of an increase in the severity of the disease, as similar mortality rates occurred in neurosyphilis patients treated with *P. vivax* at the beginning of the last century (Baird, 2013).

But it is not only clinical data asking for more attention to be given to this parasite. Remarkably, *P. vivax* is recolonizing in geographic areas that had successfully eradicated these parasites during the last century. For the last few years, continuous transmission of this parasite has been reported in limited geographical areas in Greece. The fact that favorable conditions continue to exist in several parts of Greece and in other countries in the southern borders of Europe, suggests there should be implementation of specific awareness actions (Andriopoulos *et al.*, 2013, Kousoulis *et al.*, 2013). On the other hand, it has been considered that *P. vivax* is absent in West, Central and Tropical Africa, where the presence of a Duffy negative population reaches frequencies of almost 95-100%. This is based upon the apparent dependence on the Duffy antigen for *P. vivax* invasion (Miller *et al.*, 1976, Horuk *et al.*, 1993). However, several recent lines of evidence indicate that *P. vivax* is circulating in known Duffy negative regions in Africa (Rubio *et al.*, 1999, Muhlberger *et al.*, 2004, Ryan *et al.*, 2006, Culleton *et al.*, 2009, Dhorda *et al.*, 2011). Indeed, as part of a collaborative study, we have demonstrated a 30% prevalence of vivax infections in all malaria cases in three different regions of Mali (Bernabeu *et al.*, 2012a). Even though we could not determine the Duffy phenotype of the infected patients examined, the population in these areas presents a Duffy negative frequency from 90 to 98%. In this line, recent reports have described the presence of *P. vivax* in the blood of Duffy negative individuals (Cavasini *et al.*, 2007, Rosenberg, 2007, Menard *et al.*, 2010, Mendes *et al.*, 2011, Wurtz *et al.*, 2011, Woldearegai *et al.*, 2013). The most probable explanation suggests the presence of a new phenotype of parasites which uses a less efficient invasion mechanism (Carlton *et al.*, 2008).

Taken all together, the data reviewed above clearly indicate that the historical view of vivax malaria as a benign disease is no longer tenable. In addition, it has to be taken into account that present malaria control tools mainly directed to *P. falciparum,* will not suffice for *P. vivax.* Insecticide treated bed nets appear of little usage given the fact that vectors that

transmit *P. vivax* are mostly outdoor biting (Mueller *et al.*, 2009). Moreover, chloroquine and primaquine have been in continuous use since 1952, despite the fact that chloroquine resistance is now widespread and primaquine produces mild and severe hemolytic anaemia in patients with a deficiency in the Glucose-6-Phospate dehydrogenase enzyme (G6PD). In this scenario, relapses of the disease are difficult to control due to the fact that primaquine is the only available drug that targets *P. vivax* hypnozoites (Abeyaratne *et al.*, 1968, Baird *et al.*, 2004, Cappellini *et al.*, 2008, Burgoine *et al.*, 2010, Howes *et al.*, 2012). Furthermore, the presence of gametocytes in peripheral blood before the appearance of clinical symptoms, together with the high presence of asymptomatic patients may serve as a reservoir to successfully transmit the disease (Mueller *et al.*, 2009). Last, the vast majority of funds dedicated to malaria research are still assigned to *P. falciparum.* Under this scenario it is tempting to speculate that *P. vivax* will be the last human malarial species to be eliminated. Thus, there is a clear need to increase our knowledge of the biology and pathophysiology of this parasite to develop species-specific tools.

Multigene variant families: a way to escape the hosts' immune system

Pathogenic unicellular organisms maintain chronic infections to guarantee survival and disease transmission. To accomplish this, mammalian pathogens must face the continuous and complex pressure of adapting to different hosts' immune systems. In addition, microorganism's survival also depends on their capacity to adapt to any intrinsic host differences such as immune pressure or host genetics. The race against these common rivals has contributed to the development of surprisingly similar mechanisms in different pathogens from distant evolutionary lineages (Deitsch *et al.*, 2009). These strategies rely on the presence of large, multicopy repertoires of genes, in which each individual sequence encodes an antigenically distinct surface protein to overcome and evade the mammal hosts' immune system. By systematically altering the proteins displayed in the surface, using a strategy known as antigenic variation, the host is confronted with a continually changing population that is difficult or impossible to eliminate.

The recent availability of extensive genome sequences has allowed the identification of several hypervariable multigene families. In bacteria, these are typified by the subtelomeric

vsp and *vlp* genes in *Borrelia hermsii* (Kitten *et al.*, 1990), the *pil* genes in *Neiserria* species (Merz *et al.*, 2000, Plant *et al.*, 2003), and the family of *tpr* genes in *Treponema spp* (Gray *et al.*, 2006). In the fungus kingdom, classic examples of such repertoires can be found in *Pneumocystis carinii* (*msg, msc,* and *prt* gene families) (Keely *et al.*, 2005), *Candida albicans* (*als* genes) (Hoyer, 2001) and *Candida glabrata* (*epa* genes) (Filler, 2006). Protozoan parasites also present large families of antigen-encoding sequences. The prototypical example is the *Trypanosoma brucei vsg* family which has undergone a massive expansion (Pays *et al.*, 2004, Taylor *et al.*, 2006). Other protozoan species such as *Giardia* (Prucca *et al.*, 2009) and *Babesia* (Allred *et al.*, 2000) present smaller but similar hypervariable gene families.

Plasmodium is not an exception in the protozoan phylum, and has also developed and expanded multigene families to generate antigenic variation. In *P. falciparum,* variant multigene families are represented by the *var* multigene family (Baruch *et al.*, 1995, Smith *et al.*, 1995, Su *et al.*, 1995) and the members of the 2TM superfamily (*rif, stevor* and *Pfmc-2tm*) (Sam-Yellowe *et al.*, 2004, Petter *et al.*, 2007, Niang *et al.*, 2009, Khattab *et al.*, 2011, Bachmann *et al.*, 2012). On the other hand, some of the multigene families in *P. vivax*, *P. knowlesi*, and the rodent malarias (*P. berghei*, P. *chabaudi,* and *P. yoelii*) are integrated in the PIR superfamily (Janssen *et al.*, 2002, Janssen *et al.*, 2004, Cunningham *et al.*, 2010). Within most species (*P. falciparum, P. vivax, P. berghei* and *P. chabaudi*) hypervariable families are mostly located at subtelomeric regions, where repeat structures and nuclear localization facilitate recombination and chromosomal exchange for generation of antigenic diversity (Carlton *et al.*, 2008, Cunningham *et al.*, 2010). In addition to this complexity, members of distinct multigene families of unknown function, *Pb-fam, Pk-fam* and *Pv-fam,* are also present in *P. berghei, P. knowlesi* and *P. vivax,* respectively (Hall *et al.*, 2005, Carlton *et al.*, 2008, Pain *et al.*, 2008).

***P. vivax m*ultigene families: different localizations, functions and new classification**

Subcellular localization and computational approaches were key methodologies used in this thesis to uncover insights into gene function; these procedures have played a major role in functional analysis of multigene families in *P. falciparum.* For instance, studies on RIFIN have

clearly demonstrated the importance of these procedures, since distinct subcellular localizations of members of A and B-RIFIN groups have suggested that they might play different roles (Petter *et al.*, 2007, Joannin *et al.*, 2008, Bachmann *et al.*, 2009, Joannin *et al.*, 2011). As the *vir* multigene family displays an extreme diversity, it was speculated that the VIR members could also present different subcellular localizations and functions (Carlton *et al.*, 2008, Fernandez-Becerra *et al.*, 2009). In addition to that, the variability in the presence of exports motifs within the family and the fact that some subfamilies share structural similarities with distinct *P. falciparum* multigene families also point into the multiple function hypothesis (Carlton *et al.*, 2008). Here, this thesis confirms that the transgenic strains of *P. falciparum* expressing VIR proteins from subfamilies A, C and D present different localizations in the infected Red Blood Cell (iRBC) (Bernabeu *et al.*, 2012b). Nevertheless, our new computational approach postulated that the differences in protein architecture and subcellular localization were due to the classification of members of subfamily A and D into new and distinct multigene families (Lopez *et al.*, 2013). Now, questions therefore arise as to whether or not the newly described VIR core (subfamilies B, C, E, G, I, J and K) present different subcellular localizations and functions. The application of our new classification approach on the multigene families of *Plasmodium* species, revealed that the new VIR family still remains the most divergent (Lopez *et al.*, 2013). In line with these results, immunofluorescence analysis in *P. vivax*- infected reticulocytes clearly showed distinct subcellular localizations dependent upon the isolate and the stage of the parasite (Bernabeu *et al.*, 2012b). Therefore, the presence of multiple functions over the new *vir* family must still be considered. However, the fact that the new VIR family could present a unique subcellular localization and function cannot be dismissed.

Different subcellular localizations were presumed originally on the *vir* multigene family, due to predictions that suggested that members from subfamily A and D shared structural characteristics between the *P. falciparum surf* and the *pfmc-2TM* families, respectively (Merino *et al.*, 2006, Carlton *et al.*, 2008). Based on confocal images of the transgenic line 3D7-vir10_3HA, we discarded a similar role of subfamily D (PvPIRD) and the *pfmc-2TM* multigene family, as no localization of VIR10 was found in the Maurer's clefts (Sam-Yellowe *et al.*, 2004, Bernabeu *et al.*, 2012b). On the other hand, we suggested that subfamily A could have similar functions to the *surf* family, as we found similar subcellular localizations of

VIR17 and SURFIN 4.1 (Mphande *et al.*, 2008, Bernabeu *et al.*, 2012b). However, a recent study that revisits the subcellular localization of this SURFIN member has shown that this protein is exported to the Maurer's clefts. As they demonstrate that the TM domain, together with the N-terminal region and the C-cytoplasmic tail are essential for the correct export of this protein to the host cytoplasm, it is tempting to speculate that lack of TM regions in the entire subfamily A (PvPIRA) points to an internal localization of these proteins and a different function than the SURFIN members (Zhu *et al.*, 2013). Indeed, Zhu and collaborators suggested a homology between the *surf* and the *pvstp* family (Zhu *et al.*, 2013). In this direction, we also showed a clear relationship between *surf* and *pvstp*, as depicted in the scheme that represents the protein similarity among *Plasmodium* variable families (Lopez *et al.*, 2013).

In the absence of a *P. vivax* culture, we chose heterologous transfection to make functional analysis of *P. vivax* genes. Question remain as to which extend, *P. falciparum* is able to recognize *P. vivax* export and transport motifs. Successful heterologous transfections of *P. vivax* proteins targeted to different organelles have been reported before. First, a study of our group revealed a correct subcellular localization of the PvCRT-o in the digestive vacuole (Sa *et al.*, 2006). Later, this strategy has been successfully used in addressing interactions between the PvDHFRT-TS and new antifolates (O'Neil *et al.*, 2007, Auliff *et al.*, 2010). Finally, in this thesis we successfully targeted the PvACP to the apicoplast of the transgenic line 3D7_ACP-GFP to demonstrate a correct subcellular localization of *P. vivax* proteins whose expression is driven by the *pfcrt* promoter of the pARL1- plasmid (Bernabeu *et al.*, 2012b). However, none of these reports have demonstrated heterologous export of *P. vivax* proteins to the *P. falciparum* iRBC cytoplasm. Because non-falciparum malarias present a surprisingly small number or PEXEL positive proteins, it is believed that PEXEL- negative exported proteins (PNEPs) play a more prominent role in these species (Sargeant *et al.*, 2006). In particular, VIR proteins can be considered PNEPs, as most of them do not present a hydrophobic signal sequence and only half of the VIR repertoire presents the pentameric PEXEL-like export motif (Carlton *et al.*, 2008). Remarkably, recent findings in *P. falciparum* traffic machinery argue against a possible misrecognition of *P. vivax* export signals: it has been demonstrated that PEXEL and PNEPs proteins are exported by a common export mechanism and the members that mediate this export pathway to the cytoplasm of the host

(Plasmepsin V and proteins from the PTEX translocon), are conserved among the *Plasmodium* genus (Gruring *et al.*, 2012). Having considered this, despite the variability in export signals in Plasmodium species (Pick *et al.*, 2011), we can conclude that *P. falciparum* may be exporting correctly subfamilies C and D (PvPIRD).

Subcellular localization assays must also be interpreted within the context of the PIR superfamily. This multigene family was defined in terms of similarity in; gene copy number, genome location, expression pattern and intra-family sequence conservation (Janssen *et al.*, 2002, Janssen *et al.*, 2004). However, if we once again pay attention into the classification of *Plasmodium* variant families made by our new approach we can observed that while BIR/CIR/YIR members are represented in a single dense cluster, the VIR cluster still appears spread with long edges that separate its members. Therefore reflecting the extreme diversity of the *P. vivax* hypervariable family (Lopez *et al.*, 2013). Moreover, discrepancies were found in sub-cellular localization assays among members of the PIR multigene family. While YIR proteins were detected in the surface of the iRBC by FACS analysis (Cunningham *et al.*, 2005), the export of BIR proteins into the host cytosol without expression in the membrane, was reported in two different publications (Sijwali *et al.*, 2010, Pasini *et al.*, 2013). For these reasons we conclude that despite malaria rodent models appearing attractive because they provide an *in vivo* model of the disease with quicker transfections, the extrapolation of *bir/cir/yir* multigene families to infer VIR subcellular location and function should be approached with caution, as they may not represent the whole complexity of the *P. vivax* family.

Cytoadhesion in *P. vivax*

As part of the variability strategy, most multigene families exhibit the unique function of creating a thick coat to avoid immune pressure, such as *vsp* genes in *Giardia lamblia* (Prucca et al., 2009) or the *vsg* genes in *Trypanosoma brucei* (Pays *et al.*, 2004, Taylor *et al.*, 2006). In contrast, other hypervariable families have evolved to generate alternative functions that increase microorganism fitness, such as cytoadhesion. Multigene families with cytoadhesive properties range from bacterial *Neisseria* species (Merz *et al.*, 2000, Plant *et al.*, 2003) to the

well-studied PfEMP1 protein encoded by the *var* multigene family in *Plasmodium falciparum* (Baruch *et al.*, 1995, Smith *et al.*, 1995, Su *et al.*, 1995).

One of the main debates in the study of *P. vivax* pathophysiology is focused on the cytoadhesive properties of *P. vivax.* Apparently, the plausible lack of cytoadhesion in the inner organs seems to render the parasite vulnerable in front of the spleen defensive response. If so, how is this parasite able to escape from spleen clearance? In this thesis we try to unveil the molecular mechanisms that mediate such escape. The well-developed cytoadhesive molecular machinery in *P. falciparum* mediates the parasite sequestration in the capillaries and the sinuses of the inner organs, to scape spleen-clearance of rigid mature stages and only early asexual stages are found in the peripheral blood of *P. falciparum* non-severe patients (Miller *et al.*, 2002). Because all sexual stages are found in peripheral blood of *P. vivax* patients, it is believed that sequestration does not occur in this species. However, analysis of the literature revealed that, in spite of having different parasite circulating blood-stages, it is mostly young forms that circulate (Field *et al.,* 1963).This evidences have also been proven by our group in an extensive and carefully examination of peripheral blood smears of *P. vivax* infected patients from the Brazilian amazon that also has demonstrated partial depletion of mature stages (Thomson et al., unpublished results).

In the last few years, many studies are providing evidences that challenge the dogma that *P. vivax* do not present cytoadherence. Carvalho and collaborators showed the first evidences of *P. vivax* cytoadhesion and demonstrate binding of infected-reticulocytes to lung endothelial cells, *Saimiri* brain endothelial cells and placenta cryosections. This adhesion presented a 10-fold lower frequency than *P. falciparum* but similar strength under flow conditions (Carvalho *et al.*, 2010). Later, Chotivanich and collaborators showed cytoadhesion to glycosaminoglycan's receptors (Chotivanich *et al.*, 2012) and Machado-Siqueira and Mayor showed the presence of *P. vivax* infected-reticulocytes in the spleen and the placenta, respectively (Machado Siqueira *et al.*, 2012, Mayor *et al.*, 2012). However, all these studies disagree with the fact that little evidence of parasite accumulation has been reported in organ autopsies of *P. vivax* infected patients (Billings and Post, 1915, Bruestsch, 1932, Clark and Tomlinson, 1949) (Anstey *et al.*, 2009, Lacerda *et al.*, 2012).

Another question arises about this parasite pathophysiology: How does low-grade and self-limiting parasitaemias cause such severe disease in *P. vivax*? Two different explanations have been proposed. Anstey and collaborators suggest that the low parasite biomass is due to the fact that *P. vivax's* strong predilection to invade reticulocytes limits its multiplication rate. For this reason, this school of thought hypothesizes that severe disease is a consequence of the greater inflammatory response produced by this species. Therefore, their main argument to ignore cytoadhesion events is the absence of evidence from *in vivo* assays and autopsies (Anstey *et al.*, 2012). On the other hand, del Portillo (del Portillo *et al.*, 2004) and Baird (Baird, 2013) hypothesize that vivax malaria may be primarily an infection of hematopoietic tissues rich in reticulocytes, such as the bone marrow and the spleen. In particular, our group hypothesized that changes in the spleen tissue architecture occur in response to *P. vivax* infection, generating a closed circulation that facilitates merozoite-reticulocyte encounter and avoids macrophage clearance. This closed circulation would be a consequence of the formation of a blood spleen barrier of fibroblastic origin (Weiss, 1990) and the adhesion would be mediated at least in part by the VIR proteins (del Portillo *et al.*, 2004, Fernandez-Becerra *et al.*, 2009).

Role of the spleen in vivax malaria

Analysis of the infection in splenectomized hosts has demonstrated that the spleen does play an important role in modulating expression among all *Plasmodium* variant multigene families. The first evidence of this modulation was reported in *P. knowlesi* by Barnwell and collaborators in which they demonstrated that no parasite agglutination occurred with immune sera of chronically infected monkeys, after several passages in splenectomized monkeys (Barnwell *et al.*, 1983, Howard *et al.*, 1983). For this reason, they hypothesized that the SICAvar antigen expression in the surface of the infected red blood cells is driven by the presence of the spleen. This phenomenon has been elegantly described in *P. falciparum* in the case of a splenectomized patient that presented a large parasitaemia with the presence of mature stages in peripheral blood. Those parasites did not expressed multigene families, such as *var, stevor,* and *rif-A,* and did not mediate binding to endothelial receptors (Bachmann *et al.*, 2009). In conclusion, both articles hypothesize that the expression of

malaria surface variant proteins that convert the parasite into a target of the immune system, is not necessary when the spleen is absent. Hence, they postulate that the spleen is the driver for the malaria evolutionary process that favors cytoadhesion in other organs.

To understand the role of the spleen in the expression of *P. vivax* variable families, a study utilizing a similar approach to the Barnwell experiment in 1983 was untaken by our group in *Aotus* monkeys. Three independent algorithms revealed, in a global transcriptional analysis, that VIR14 (PVX_108770) was clearly upregulated upon infection in monkeys with an intact spleen after several passages in splenectomized monkeys (unpublished data). The transgenic line that expressed this surface protein mediated cytoadherence to spleen fibroblasts of distinct origin and the specificity of this adhesion was determined by the absence of binding to lung fibroblasts. In addition, these results were reinforced by cytoadhesion of *P. vivax* infected-reticulocytes to one of the spleen fibroblasts, albeit at different levels (unpublished manuscript of this thesis). All together, these results clearly demonstrated that some VIR proteins at least partially mediate the adhesion to the spleen. In contrast, the role of ICAM-1 in such adhesion appears not as clear, as has been amply discussed in the third manuscript of this thesis. The only fact that seems certain is that this receptor does not play a principal role and may synergize with and unknown principal receptor to mediate *P. vivax* adhesion (Figure 10). Apart from our study, contradictory results have been shown in recent reports on the role of ICAM-1 in the adhesion to *P. vivax* (Carvalho *et al.*, 2010, Chotivanich *et al.*, 2012). Last, a secondary role of this receptor is in consistence with the *P. vivax* adhesion evidences in infected patients autopsies. As ICAM-1 is highly and widely expressed, if this receptor displayed a key role in *P. vivax* cytoadhesion, a greater presence of this parasite in organ autopsies would be expected (Billings and Post, 1915, Bruestsch, 1932, Clark and Tomlinson, 1949).

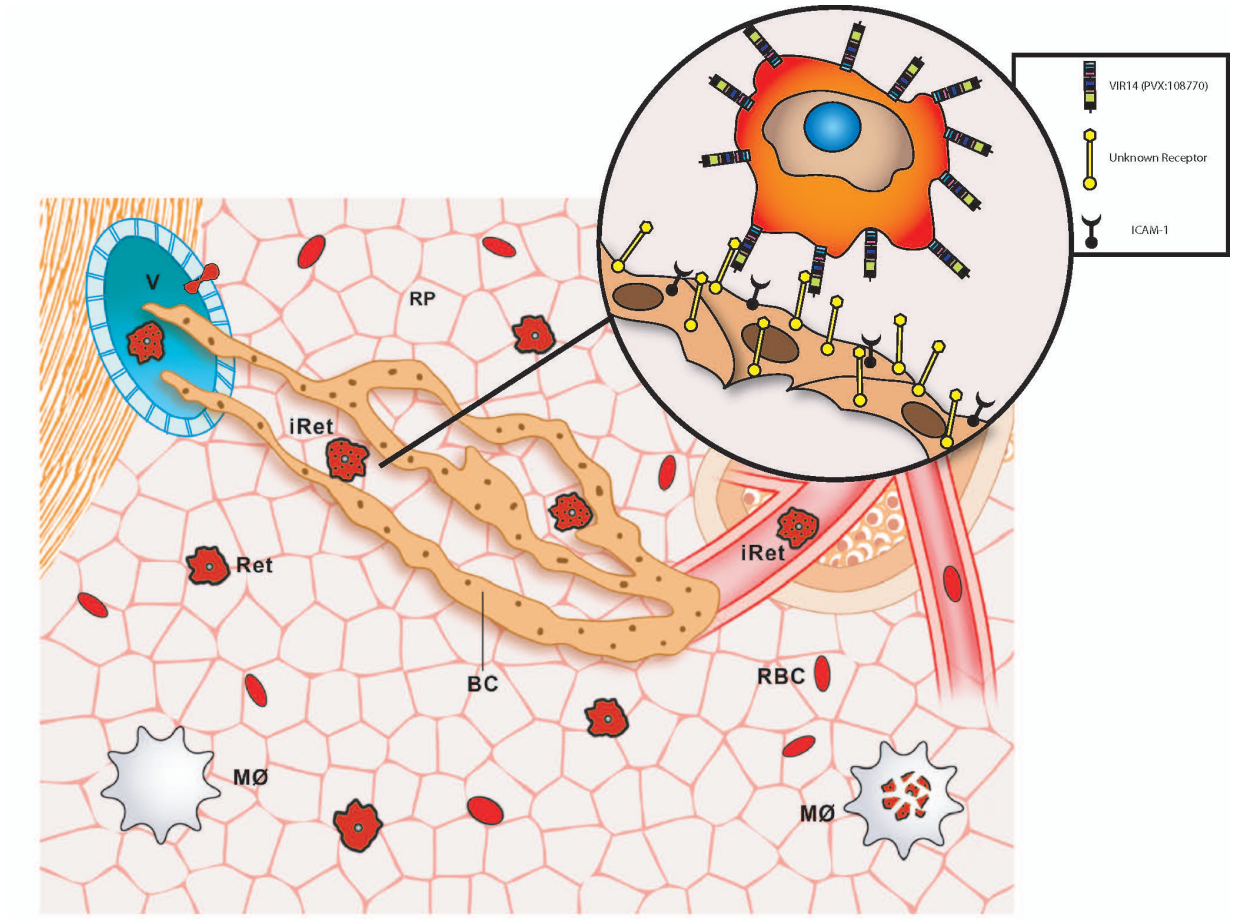

Figure 10. Model of the *P. vivax* spleen-clearance evasion mechanism mediated by VIR adhesion to spleen fibroblasts. Source: modified from (Martin-Jaular et al., 2011) by Lluís Gasulla and Maria Bernabeu. Upon infection with *P. vivax* the parasite induces the formation of a spleen barrier of fibroblastic origin (BC, barrer cells). VIR14 (PVX_108770) expressed in the surface of the infected-Reticulocytes (iRet) will mediate the adherance to a spleen fibroblast unknown receptor. MØ: Macrophages; V: Venules.

Taking into account our hypothesis, is not a chance that it is the first time that cytoadhesion to a cell of fibroblastic origin is reported in malaria. In murine species, the presence of contractile fibroblasts cells located in filtration beds of the red pulp has been previously described (Weiss, 1991). In addition, the studies performed by our group in the *P. yoelii* reticulocyte-prone strain 17X revealed that upon infection, the "open" circulation of the spleen suddenly changed to a "closed" circulation due to the reorganization of a syncytial layer of fibroblasts that formed a physical barrier. Because *P. yoelii* 17X was found to be associated to this barrier, it was speculated that fibroblasts expressed putative receptors for this species (Martin-Jaular *et al.*, 2011). Even though the presence of barrier cells in the spleen has been verified in humans in other pathologies (Bowdler, 2002), their presence in the spleen in malaria infected patients remains controversial. The further identification of

the specific receptor of spleen fibroblasts that mediated cytoadhesion in this thesis, could help in the future identification of barrier cells in prospective immunhistochemical analysis in spleens from malaria patients. Taking this data into account, despite the fact that the presence of barrier cells in the spleen of vivax patients has not yet been identified, the adhesion of this species in this organ seems not arguable. First, for the enormous accumulation of intact parasites in the spleen of a malaria patient (Machado Siqueira *et al.*, 2012). Last, for the results observed in cytoadhesion assays of *P. vivax* infected reticulocytes to human spleen cryosections (Mireia Ferrer thesis) as well as to human spleen fibroblasts, as shown in this thesis.

Finally, it is important to determine if the adhesion of the spleen could be related with pathology in *P. vivax.* Of note, rupture or infarct of the spleen, a relatively rare and seriously threatening complication, is not uncommon in severe vivax malaria (Anstey *et al.*, 2012, Lacerda *et al.*, 2012). On the other hand, anaemia, a common manifestation of severe vivax disease in both children and adults, could also be related to spleen cytoadherence (Del Portillo *et al.*, 2012). Finally, a role of the spleen in thrombocytopenia, another frequent vivax severe disease manifestation, has also been described in mice infected with *P. chabaudi* (Watier *et al.*, 1992). The identification of other VIRS that mediate spleen cytoadhesion, together with the homology block (HB) analysis made by our group (Lopez *et al.*, 2013) might enable the identification of domain cassettes associated with spleen adhesion and pathology on *P. vivax.* Remarkably, the HB concept was developed originally to analyze the *P. falciparum* PfEMP1 multigene family and has allowed the possibility to identify domain cassettes associated with adhesion (Rask *et al.*, 2010). For this reason, if spleen cytoadhesion events are related to vivax disease pathology, adhesion domain cassettes represent potential targets for intervention to prevent or treat clinical episodes.

Future perspectives

- *P. vivax* continuous *in vitro* culture for blood stages

The present understanding of *P. falciparum* biology and pathophysiology that we have today has been mostly achieved due to the availability of a reliable continuous *in vitro* culture for

this species. The first assay to establish a *P. vivax in vitro* culture was done a hundred years ago (Bass and Johns, *et al.,* 1912) and after that, several attempts have been carried out, especially in the last years. The most crucial step that has to be addressed is the availability of a reliable source of reticulocytes. Besides that, a secondary but still important step is the establishment of an optimal culture medium that will cover the requirements of the parasite. Until now, most reports have shown a good development and maturation of the parasite only during the first schizogonic cycle (Noulin *et al.*, 2012) followed by a fast decrease in parasite densities (Panichakul *et al.*, 2007, Udomsangpetch *et al.*, 2007). Without doubt, the establishment of a long term *P. vivax* continuous *in vitro* culture will represent the greatest breakthrough in the research on this parasite. Once this has been achieved, the development of a reliable transfection system is also essential to elucidate *P. vivax* gene function.

- Functional analysis of *P. vivax* genes

Apart from VIR proteins, we wanted to determine the role that new muligene families exert in *P. vivax* expressing a member of the multigene family Pv-FAM-D in *P. falciparum*. The protein was exported to the host surface of the iRBC but the transgenic line did not present cytoadherence to neither endothelial receptors nor spleen fibroblasts. Because its transcription is also spleen dependent (unpublished results), it is tempting to speculate that the protein might be involved in changes in the rheological properties of the iRBC. It is obvious that a wide range of possibilities is now open regarding research on functional gene discovery in VIR proteins and new multigene families, including PvPIRA, PvPIRD, and PvPIRH. Taking this into account, the relations established between vivax multigene families and other multigene families in *Plasmodium* species in our computational approach could be extremely useful as a startpoint to get insights into the function of these proteins.

- Future model of spleen adhesion

The spleen is a very complex organ that presents different compartments, a complex 3D structure and different blood flows are observed when the blood circulates through it (Bowdler, 2002). The main limitation of the adhesion assays that have been done in either *P. vivax* or *P. falciparum* assays is that we have underestimated the complexity of this organ. A major step forward to understand the role of the spleen in falciparum malaria has been

achieved by perfusion of human spleens *ex vivo* with parasites demonstrating the retention of ring stages in the slow compartment of the red pulp (Safeukui *et al.*, 2008). However, similar experiments in *P. vivax* appear non-viable due to the fact that the high amount of parasites that are required cannot be obtained from *P. vivax* patients. Taking advantage of the great advents in nanotechnology and microfluidics, we are developing a spleen on a chip that reproduces the minimal functional unit of the spleen (in collaboration with Professor Josep Samitier). This chip will reproduce the compartmentalization of this organ and the different blood flows that are observed through it. At the same time, the small scale of the model will allow the study of adhesion in multiple samples of vivax endemic areas.

- Antigenic variation

As said before, surface antigenic variation was a mechanism employed by pathogenic microorganisms to maintain chronic infections under continuous immune pressures. Three requirements are essential for antigenic variation in its strictest sense: i) The presence of a family of homologous genes encoding antigenically different surface molecules. ii) Reversible mechanisms that guarantees the mutually exclusive expression of only one antigen at a time. iii) Mechanisms of reversible to switch the expression of these proteins in individual cells. *P. falciparum* PfEMP1 antigenic variation has been well established and accomplishes the three requirements (Guizetti *et al.*, 2013). On the contrary, expression of VIR proteins is neither clonal nor mutually exclusive (Fernandez-Becerra *et al.*, 2005). The PIR multigene family does not shed any more light on understanding *vir* antigenic variation mechanisms despite similar transcription patterns, as well as expression of just a small number of variant proteins in a single cell have been foun in *P. vivax* (Fernandez-Becerra *et al.*, 2005, Bozdech *et al.*, 2008), *P. yoelii* (Cunningham *et al.*, 2009) and *P. chabaudi* (Lawton *et al.*, 2012). Finally in *P. falciparum*, contrary to what happens in the *var* multigene family, different RIFINs could be detected at the iRBC per parasite (Cabral *et al.*, 2009) and high switching rates can be detected for the *stevor* and *pfmc-2TM* families (Lavazec *et al.*, 2007). Under this confuse scenario, different hypothesis could be formulated. The first one takes suggests that just a subset of proteins (subfamilies and/or groups) may play a role in antigenic variation. The second one postulates that the expression of many variants at levels which are below the threshold for induction of a strong immune response could help to escape the immune system. In any case, further research needs to be conducted in this field to understand

antigenic variation of *Plasmodium* variant multigene families and the mechanisms that control their expression.

- *P. vivax* multigene families and antigen discovery

Another aspect that needs further research is the immunogenicity of *P. vivax* variant proteins. Present data suggest that despite their inner variability (Merino *et al.*, 2006, Gupta *et al.*, 2012), VIR proteins are capable of eliciting cross-reacting antibodies already in first time infected patients (Fernandez-Becerra *et al.*, 2005). Despite the fact that the scientific community has remained skeptical about vaccines against variant surface proteins in *P. falciparum,* some present research is currently oriented towards this strategy and efforts are focused in finding conserved motifs that mediate cerebral malaria (Avril *et al.*, 2012, Claessens *et al.*, 2012, Lavstsen *et al.*, 2012, Bengtsson *et al.*, 2013) or pregnancy associated malaria (Hviid, 2010). Instead of interrupting transmission or a complete eradication of the infection, there is no doubt that such vaccines could be extremely useful complementary tools to reduce the burden of malaria. For all these reasons, further research in *P. vivax* is needed to understand the role that variant determinants play in immunogenicity and protection against disease pathology.

Concluding remarks

In conclusion, in this thesis we have studied the neglected parasite *P. vivax,* with a focus on subtelomeric multigene families, in particular the *vir* variant family. The lack of a long term *P. vivax in vitro* culture impaired the use of homologous reverse genetics approaches to understand the role of such family during infections. Yet, expressions *in trans* of some of these proteins in *P. falciparum* have shed light on *P. vivax* multigene organization and subcellular localization. Noticeably, it has demonstrated that VIR proteins play an important role in cytoadhesion to the human spleen. Adhesion of *P. vivax*-infected reticulocytes to the human spleen further indicated that the this organ plays a yet to be fully defined role in the life cycle of *P. vivax* and that VIR proteins mediate, at least partially, this host-parasite interaction. Further understanding of this multigene family and the roles that it may play in *P. vivax* pathology will undoubtedly provide new targets for disease intervention.

Conclusions

The key conclusions of this thesis can be summarized as follows:

- VIR proteins from subfamilies A, C and D present different subcellular localizations when expressed in *P. falciparum* indicating that they have different functions.
- Polyclonal antibodies against conserved globular domains from VIR proteins indicate that VIR proteins also present different subcellular localizations in natural infections.
- A new computational approach redefined VIR proteins and excluded subfamilies A, D and H from being members of the VIR protein repertoire.
- VIR14 from subfamily C mediates adhesion to CHO cells expressing ICAM-1 and to spleen fibroblasts under physiological flow conditions.
- Members from multigene family Pv-FAM-D do not present cytoadhesive properties, thus highlighting the specificity of adhesion of VIR14.
- *P. vivax* infected reticulocytes from patients from the Brazilian Amazon cytoadhere to spleen fibroblast. This adhesion may help the parasite to encounter new reticulocytes and avoid spleen clearance.
- As determined by partial though specific inhibition of adhesion, a subset of VIR proteins mediate adhesion to spleen fibroblasts as demonstrated in *P. vivax* wild isolates. The adhesion assays done in the *P. falciparum* transgenic lines demonstrate that at least, subfamily C might be specifically involved in mediating this adhesion.
- In the absence of a long term *P. vivax in vitro* culture, the heterologous expression of *P. vivax* in *P. falciparum* may be extremely helpful in the study of the function of vivax proteins and their implications in pathology.
- All together, these data challenge the dogmas that there is no cytoadhesion of *P. vivax*-infected reticulocytes and that adherence is the driving force to avoid spleen clearance in malaria.

Versió en català

Introducció

Malària: conceptes generals

La malària és una malaltia infecciosa causada per protozous del gènere *Plasmodium.* Aquests paràsits han evolucionat conjuntament amb l'home durant milers d'anys i fins i tot la seva incidència ha fet canviar el curs de la història. Les primeres escriptures que descriuen aquesta malaltia daten de l'any 2700 a. de C. a la Xina (Cox, 2002) i es creu que figures rellevants com Alexandre el Gran o Genghis Khan van morir a causa d'aquesta malaltia. Les seves presentacions clíniques són diverses: des d'individus immunitzats que presenten infeccions asimptomàtiques fins a manifestacions severes que inclouen febre, anèmia, distrès respiratori i coma, podent arribar a produir la mort del pacient.

Tots els paràsits del gènere *Plasmodium* són transmesos a través de la picada de mosquits femelles del gènere *Anopheles.* En humans, la infecció és deguda a quatre espècies diferents: *P. falciparum, P. vivax, P. ovale* i *P. malariae*. A més a més, recentment s'ha descobert que *P. knowlesi* és capaç d'infectar humans però, afortunadament, encara es tracta només d'una zoonosi localitzada a l'illa de Borneo, a Malàisia (Singh *et al.*, 2004). D'aquestes cinc espècies, pràcticament només *P. falciparum* i *P. vivax* produeixen casos clínics d'aquesta malaltia.

Actualment, s'estima que la meitat de la població mundial està en risc de patir aquesta infecció. L'Organització Mundial de la Salut estima que el 2010 es van produir uns 219 milions de casos clínics de malària, dels quals 660.000 van acabar amb la mort del pacient (WHO 2012). Malgrat que a l'any 2000 es va calcular que amb les noves mesures de control i prevenció de la malaltia s'havien reduït en un 25% el nombre de morts, la infecció encara continua sent un greu problema de salut pública. La majoria d'estratègies de control es divideixen en intervencions contra el vector o el paràsit. Les mesures contra el vector estan centrades bàsicament en l'ús de mosquiteres

impregnades amb insecticides o el ruixament de les cases amb aquests químics. Les mesures contra el paràsit estan majoritàriament centrades en la quimioteràpia per a tractar les infeccions diagnosticades. Al llarg de la història s'ha creat un gran nombre de fàrmacs a mesura que el paràsit ha anat desenvolupant resistències contra ells (Clyde *et al.*, 1957, Cowman *et al.*, 1994, Trape, 2001, Sa *et al.*, 2009, Meng *et al.*, 2010, Briolant *et al.*, 2011) i avui en dia, el tractament més efectiu contra la malaltia està basat en l'ús de teràpies combinades amb artemisina (ACTs) (Egan *et al.*, 2007). Malauradament, en els darrers anys s'ha observat una menor sensibilitat als ACTs en els paràsits circulants en alguns països del Sud-est Asiàtic (Noedl *et al.*, 2008, Petersen *et al.*, 2011), fet que revela la importància de programes de recerca dirigits cap al descobriment de nous fàrmacs. En paral·lel, gran part dels esforços dedicats a la prevenció de la malària estan centrats en la generació d'una vacuna efectiva. Actualment, la vacuna RTS,S es troba en fase III de desenvolupament i ha demostrat una eficàcia del 50% en nens de 5 a 17 mesos (Agnandji *et al.*, 2011) i del 30% en infants de 6 a 12 setmanes (Agnandji *et al.*, 2012).

Cicle de vida del paràsit

Com s'ha esmentat anteriorment, el paràsit presenta dos tipus d'hostes diferents. En els humans es dóna el cicle de vida asexual del paràsit i en els mosquits, el sexual. La picada del mosquit diposita centenars d'esporozoïts en el teixit subcutani que entraran al sistema circulatori i viatjaran fins al fetge. Allà, es produirà la invasió dels hepatòcits, que donarà lloc a la replicació nuclear del paràsit i la generació de milers de merozoïts (Sturm *et al.*, 2006). Aquests, tornaran al torrent sanguini on es produirà la fase sanguínia de la infecció, que és la responsable dels principals símptomes clínics. Els merozoïts lliures infectaran eritròcits i maduraran subseqüentment fins convertir-se en anells, trofozoïts i esquizonts en un cicle que dura 48 hores. Finalment, la ruptura de l'esquizont generarà aproximadament 20 nous merozoïts que infectaran altres eritròcits (Gruring *et al.*, 2011, Gruring *et al.*, 2012), perpetuant així el cicle del paràsit. Eventualment, alguns estadis sanguinis poden arribar a diferenciar-se en gametòcits masculins i femenins que seran ingerits pels mosquits en una nova picada, perpetuant així el cicle de vida del paràsit.

P. vivax, una malària no benigna però desatesa

Històricament s'ha cregut que la gran majoria de morts derivades de la malària eren degudes a *P. falciparum,* principalment al continent africà. No obstant, els últims estudis epidemiològics han demostrat que *P. vivax* també és capaç de generar casos clínics de la malaltia que poden acabar en mort (Anstey *et al.*, 2012, Baird, 2013). Actualment, es calcula que 2.850 milions de persones tenen risc de patir aquesta infecció en 95 països arreu del món (Guerra *et al.*, 2010). A més a més, es calcula que un 25-40% dels casos de malària fora de l'Àfrica són deguts a aquest paràsit, que produeix entre 132 i 391 milions de casos clínics cada any (Price *et al.*, 2009). A diferència de *P. falciparum,* que presenta una gran incidència al continent africà, els casos de *P. vivax* es presenten principalment a Sud-Amèrica i al Sud i Sud-Est asiàtic.

La cloroquina, és encara avui en dia la teràpia més utilitzada contra *P. vivax,* malgrat que ja fa més de 20 anys que s'observen resistències contra aquest fàrmac arreu del món (Rieckmann *et al.*, 1989, Baird *et al.*, 1996, Fryauff *et al.*, 1998, Soto *et al.*, 2001, Phan *et al.*, 2002, Sumawinata *et al.*, 2003, Kurcer *et al.*, 2006, Taylor *et al.*, 2006, de Santana Filho *et al.*, 2007, Ratcliff *et al.*, 2007, Guthmann *et al.*, 2008, Karunajeewa *et al.*, 2008, Teka *et al.*, 2008, Lee *et al.*, 2009, Sutanto *et al.*, 2009). La cloroquina s'utilitza sovint en combinació amb la primaquina, un fàrmac que elimina els hipnozoïts, que són uns estadis latents exclusius de *P. vivax* que es troben al fetge. Aquest fàrmac però, pot causar casos greus d'hemòlisi aguda en persones deficients en l'enzim Glucosa-6-fosfat deshidrogenasa (G6PD), una deficiència força estesa a tot el món (Abeyaratne *et al.*, 1968, Baird *et al.*, 2004, Cappellini *et al.*, 2008, Burgoine *et al.*, 2010, Howes *et al.*, 2012).

La recerca en *P. vivax* ha estat històricament desatesa. En primer lloc, per la falsa creença que aquest paràsit no dóna lloc a casos severs de la malaltia. En segon lloc, per la impossibilitat de cultivar *P. vivax* a llarg termini, ja que aquest paràsit només infecta reticulòcits, les formes immadures dels eritròcits. Per últim, les baixes parasitèmies associades a aquesta malaltia fan difícil una òptima utilització de mostres de camp en recerca. Les grans diferències que existeixen en el cicle d'ambdós paràsits fan que la

majoria d'eines efectives avui en dia per prevenir i/o tractar *P. falciparum* no siguin eficients enfront *P. vivax* (Mueller *et al.*, 2009). Per aquesta raó, la recerca en aquest últim ha de ser fortament impulsada, si algun dia volem aconseguir l'eradicació d'aquesta malaltia.

Bases moleculars de la patologia de la malària

- ***P. falciparum***

L'adhesió i acumulació d'eritròcits infectats als teixits de l'hoste és un dels principals factors que contribueixen a la patologia ocasionada per *P. falciparum.* Els trofozoïts i esquizonts madurs són capaços d'adherir-se a i) cèl·lules endotelials (Newbold *et al.*, 1999), ii) eritròcits no infectats (formació de rosetes) (Udomsangpetch *et al.*, 1989), iii) altres eritròcits infectats (formació d'agregats o "clumping") (Pain *et al.*, 2001a) o iv) cèl·lules del teixit placentari (Walter *et al.*, 1982). Es creu que la citoadhesió és un mecanisme originat pel paràsit per evitar el pas per la melsa i el conseqüent filtratge i destrucció. Degut a aquest fenomen, la majoria de pacients infectats per *P. falciparum* no presenten formes madures en circulació perifèrica, mentre que els paràsits aïllats de pacients sense melsa sí que ho fan (Demar *et al.*, 2004, Bachmann *et al.*, 2009).

Mitjançant l'adhesió a capil·lars del cervell, pulmons o placenta, *P. falciparum* produeix l'obstrucció del flux sanguini, cosa que pot arribar a provocar danys als teixits, fallida de l'òrgan i, fins i tot, la mort (Miller *et al.*, 2002). L'adhesió de les formes madures es dóna gràcies a la unió a receptors humans com CD36 (Barnwell *et al.*, 1989), ICAM-1 (Intercellular Adhesion Molecule-1) (Berendt *et al.*, 1989), CSA (Chondroitin Sulphate A) (Rogerson *et al.*, 1995), CR1 (Complement Receptor 1) (Rowe *et al.*, 1997) o gC1qR (Biswas *et al.*, 2007). Els principals lligands parasitaris involucrats en aquesta adhesió són les proteïnes de membrana PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) (Baruch *et al.*, 1996). Aquestes proteïnes estan codificades per la família multigènica *var* (Su *et al.*, 1995), composta per 60 gens diferents (Gardner *et al.*, 2002) localitzats a les regions subtelòmeriques dels cromosomes de *P. falciparum.* Cada paràsit només expressa una única variant de PfEMP1 a la superfície de la membrana de l'eritròcit infectat. Per evitar ser reconegut pel sistema immunològic de l'hoste, *P. falciparum* té

la capacitat de canviar la proteïna expressada a la superfície en un procés conegut com a variació antigènica (Scherf *et al.*, 1998). A més de la família multigènica *var,* el paràsit també presenta altres famílies multigèniques de funció encara desconeguda com *rifin* (Petter *et al.*, 2007)*, stevor* (Niang *et al.*, 2009)*, pfmc-2tm* (Sam-Yellowe *et al.*, 2004) i *surf* (Winter *et al.*, 2005).

- ***P. vivax***

Clàssicament s'ha pensat que els reticulòcits infectats per *P. vivax* no presentaven citoadhesió als capil·lars dels òrgans interns, ja que no és estrany trobar formes madures del paràsit en sang perifèrica. No obstant, els últims estudis contradiuen aquest dogma històric. Mitjançant estudis *in vitro* utilitzant mostres de pacients de Brasil, Carvalho i els seus col·laboradors van demostrar adhesió de *P. vivax* a cèl·lules endotelials de pulmó i cervell, a talls de placenta (Carvalho *et al.*, 2010) i a cèl·lules CHO que expressen el receptor ICAM-1. En la mateixa línia, Chotivanich i col·laboradors van demostrar adhesió de paràsits de Tailàndia a talls de placenta i al receptor CSA (Chotivanich *et al.*, 2012). Finalment, s'ha demostrat la presència de paràsit en placentes de dones infectades (Mayor *et al.*, 2012) i una gran acumulació de *P. vivax* a la melsa d'un pacient que va patir una esplenectomia abans de rebre tractament antimalàric (Machado Siqueira *et al.*, 2012). Malgrat tots aquests estudis, els lligands implicats en l'adhesió de *P. vivax* encara no són del tot coneguts.

L'any 2001, el nostre grup va descobrir l'existència de la família multigènica *vir* (*P. vivax* variant proteins) a les regions subtelòmeriques dels cromosomes de *P. vivax.* En aquell moment es va creure que aquesta família multigènica estava formada per 600-1.000 proteïnes i que les proteïnes VIR es trobaven a la superfície del reticulòcit infectat (del Portillo *et al.*, 2001). Posteriorment, gràcies a la seqüenciació del genoma de *P. vivax* es va determinar que aquesta família gènica esta formada per 346 proteïnes i 80 pseudogens dividits en 12 subfamílies (A-L) (Carlton *et al.*, 2008). El fet que aquestes proteïnes presentin una gran diversitat estructural, va fer preveure que puguessin tenir altres localitzacions subcel·lulars a part de la membrana del reticulòcit infectat i exercir diferents funcions (Merino *et al.*, 2006, Carlton *et al.*, 2008). A diferència de la família *var,* gran part dels gens *vir* es troben expressats en

una infecció (Bozdech *et al.*, 2008, Westenberger *et al.*, 2010), no presenten expressió clonal ni exclusió al·lèlica i els pacients infectats per primera vegada ja presenten anticossos capaços de reaccionar contra proteïnes VIR de diferents subfamílies (Fernandez-Becerra *et al.*, 2005). Degut a aquestes últimes evidències i la seva localització subcel·lular, es va creure que la família multigènica *vir* podria tenir una funció d'evasió del sistema immune de manera alternativa a l'exclusió al·lèlica. Avui en dia la funció d'aquestes proteïnes resta desconeguda, encara que la seva variabilitat fa creure que podrien exercir diferents funcions. Finalment, *P. vivax* també presenta altres famílies multigèniques subtelomèriques de funció desconeguda anomenades *pv-fam-A-E i pv-fam-G-I* (Carlton *et al.*, 2008).

La família multigènica *vir* esta integrada dins la superfamília PIR juntament amb les famílies *kir (P. knowlesi), bir (P. berghei), cir (P. chabaudi) i yir (P. yoelii)* (Janssen *et al.*, 2002, Janssen *et al.*, 2004). L'existència d'aquesta superfamília està basada en la presència de múltiples gens per família, en la localització genòmica, en la conservació de l'estructura secundària i en patrons d'expressió similars al llarg del cicle. La funció d'aquestes altres famílies multigèniques també resta encara desconeguda (Pain *et al.*, 2008, Cunningham *et al.*, 2009, Cunningham *et al.*, 2010).

Paper de la melsa en malària

La melsa té un paper molt important en el control de la malària. A la vegada que s'encarrega del filtratge dels eritròcits infectats, també modula l'expressió de les proteïnes de les famílies multigèniques. Concretament, s'han demostrat diferències en l'expressió d'aquestes proteïnes variants en individus esplenectomitzats i infectats per *P. knowlesi, P. falciparum, P. fragile* i *P. chabaudi* (Barnwell *et al.*, 1983, David *et al.*, 1983, Hommel *et al.*, 1983, Handunnetti *et al.*, 1987, Gilks *et al.*, 1990, Bachmann *et al.*, 2009). A més a més, la melsa també juga un paper protector important gràcies a la resposta immune innata dels macròfags (Looareesuwan *et al.*, 1993, Chotivanich *et al.*, 2000).

En gran part dels pacients, la infecció per *P. vivax* provoca esplenomegàlia i indueix una reorganització de l'arquitectura de la melsa (Machado Siqueira *et al.*, 2012). Ja al

2004, el nostre grup va hipotetitzar que després de la infecció per aquest paràsit, els fibroblasts contràctils presents a la melsa es reorganitzen tancant la circulació d'aquest òrgan i formant una barrera física que permet l'eritropoesi i la maduració dels eritròcits. Concretament, amb anterioritat s'havia demostrat que el número de cèl·lules de barrera augmenta significativament en melses humanes i de ratolins patològiques (Weiss, 1990, Weiss, 1991) (Tablin et al., 2002) A més, també es va hipotetitzar que els paràsits tindrien la capacitat d'adherir-se a les cèl·lules de barrera mitjançant les proteïnes VIR. D'aquesta manera, aquests quedarien protegits dels macròfags presents en aquest òrgan, a la vegada que es facilitaria l'encontre merozoït-reticulòcit (del Portillo *et al.*, 2004, Fernandez-Becerra *et al.*, 2009).

Descobriment de la funció dels gens de *P. vivax*

La majoria dels avenços aconseguits en el coneixement de la biologia de *P. falciparum* s'han produït gràcies a la possibilitat de cultivar el paràsit (Trager *et al.*, 1976) i de poder modificar-lo mitjançant tècniques de genètica reversa (Rug *et al.*, 2013). En canvi, la recerca a nivell molecular en *P. vivax* es manté pràcticament aturada en comparació amb *P. falciparum*, degut a la impossibilitat de cultivar aquest paràsit a llarg termini. Per aquesta raó, en aquesta tesi utilitzarem com a alternativa la transfecció heteròloga en *P. falciparum* com a model per a estudiar les proteïnes de famílies multigèniques de *P. vivax.* Aquesta estratègia ja s'ha utilitzat amb èxit amb anterioritat. Fa uns anys el nostre grup va expressar el gen *pvcrt*-o (Chloroquine resistance transporter) en *P. falciparum* i va demostrar colocalització dels dos ortòlegs a la vacuola digestiva del paràsit. Addicionalment, aquesta línia transgènica presentava una resistència a la cloroquina 2,5 cops major que la soca parental (Sa *et al.*, 2009). A més, altres grups han utilitzat aquesta aproximació per estudiar la resistència de *P. vivax* a antifolats (O'Neil *et al.*, 2007, Auliff *et al.*, 2010).

Hipòtesi

La família multigènica *vir* és una de les més diverses dins del gènere *Plasmodium* (del Portillo *et al.*, 2001, Fernandez-Becerra *et al.*, 2005, Merino *et al.*, 2006, Carlton *et al.*, 2008). La nostra hipòtesi és que les proteïnes VIR podrien presentar diferents

localitzacions subcel·lulars, a part de la membrana de l'eritròcit infectat, i d'aquesta manera exercir diferents funcions. A més, hipotetitzem que algunes proteïnes VIR podrien exercir un paper important permetent l'adhesió del paràsit a la melsa, evitant-ne la destrucció per part d'aquest òrgan i facilitant la invasió de nous reticulòcits.

Objectius

El principal objectiu d'aquesta tesi és conèixer més a fons la família multigènica *vir* i entendre quin paper pot exercir en la patologia de la malaltia. En l'absència d'un cultiu a llarg termini de *P. vivax,* utilitzarem com a estratègia la transfecció heteròloga de *P. falciparum*. Per a validar els resultats obtinguts, també es realitzaran assajos en mostres de pacients infectats per *P. vivax* i farem una redefinició *in silico* d'aquesta família.

- Expressar alguns membres de les proteïnes VIR en *P. falciparum* per a determinar la seva localització subcel·lular en aquest model.
- Determinar si en mostres de *P. vivax* obtingudes de diferents pacients també s'observen diferents localitzacions subcel·lulars.
- Validar biològicament un nou mètode computacional que redefineix la família VIR.
- Estudiar les propietats adhesives de les proteïnes VIR expressades en *P. falciparum* mitjançant assajos d'adhesió a cèl·lules que expressen diferents receptors endotelials.
- Determinar les capacitats adhesives de les línies transgèniques de *P. falciparum* i de mostres de *P. vivax* a fibroblasts de la melsa.

Article 1: L'anàlisi funcional de les proteïnes VIR de *Plasmodium vivax* revela que tenen diferents localitzacions subcel·lulars i que presenten adhesió a ICAM-1.

Antecedents

La família multigènica *vir* és la més diversa que podem trobar al gènere *Plasmodium* (Carlton *et al.*, 2008). Per questa raó, els experiments *in silico* van preveure que aquestes proteïnes podrien tenir diferents localitzacions subcel·lulars (Carlton *et al.*, 2008, Fernandez-Becerra *et al.*, 2009). Concretament, només la meitat de proteïnes VIR tenen el domini PEXEL que permet l'exportació d'aquestes proteïnes al citoplasma de l'eritròcit. A més, els membres de la subfamílies A i D presenten similituds estructurals respectivament amb proteïnes de les famílies SURFIN i PfMC-2TM, que presenten localitzacions subcel·lulars diferents (Merino *et al.*, 2006, Carlton *et al.*, 2008). Els membres de la família SURFIN es troben a l'interior del paràsit a mesura que madura i finalment envolten els merozoïts (Winter *et al.*, 2005, Mphande *et al.*, 2008) mentre que la família multigènica PfMC-2TM es troba als Maurer's Clefts (Sam-Yellowe *et al.*, 2004), unes estructures membranoses al citosol de l'eritròcit infectat que permeten l'exportació de proteïnes del paràsit a la membrana.

Objectius

En l'absència del cultiu *in vitro* a llarg termini de *P. vivax,* volem generar línies transgèniques de *P. falciparum* que expressin proteïnes de diferents subfamílies VIR per a determinar la seva localització subcel·lular. En la mesura del possible, es volen fer assajos fenotípics de les diferents línies transgèniques.

Resultats

- Membres de les subfamílies A (VIR17), C (VIR14), i D (VIR10) fusionades a una seqüència triple d'hemaglutinina i/o a GFP (Green Fluorescent Protein) es van expressar satisfactòriament en *P. falciparum*.
- La proteïna VIR17 s'expressa dins el paràsit al llarg del cicle sanguini asexual i no s'exporta al citoplasma de l'eritròcit infectat. En els merozoïts, la proteïna es troba associada a la membrana i a l'extrem apical.

- Les proteïnes VIR10 (subfamília D) i VIR14 (subfamília C), que no presenten els senyals i motius d'export SP (pèptid senyal) i PEXEL, són exportades a la membrana de l'eritròcit infectat mitjançant una via independent de Maurer's Clefts.
- Els assajos d'immunofluorescència utilitzant línies transgèniques sense fixar, van demostrar que malgrat ambdues proteïnes es troben a la membrana de l'eritròcit infectat, només la proteïna VIR14 es troba exposada a la superfície.
- Anticossos generats contra dominis conservats de les proteïnes VIR van demostrar que aquestes presenten diferents localitzacions subcel·lulars en mostres de camp de *P. vivax.* Les diferències en la localització depenen del pacient i de l'estadi de maduració en el cicle sanguini del paràsit.
- La línia transgènica que expressava el VIR14 presenta adhesió en estàtic a cèl·lules CHO que expressen diferents receptors endotelials com CD36, ICAM-1, VCAM i E-selectina. No obstant això, només l'adhesió a ICAM-1 és inhibida pels anticossos que reconeixen dominis VIR conservats.
- En condicions de flux fisiològic, la línia que expressa la proteïna VIR14 només manté l'adhesió a les cèl·lules CHO-ICAM-1.

Conclusions

Les proteïnes VIR presenten diferents localitzacions subcel·lulars tant en les línies transgèniques de *P. falciparum* com en les mostres de pacients de *P. vivax.* A més, la línia transgènica que expressa un membre de la subfamília C presenta adhesió a cèl·lules que expressen el receptor ICAM-1. Cal remarcar que els nostres resultats es veuen reforçats per estudis anteriors que demostren l'adhesió de reticulòcits infectats per *P. vivax* a ICAM-1 (Carvalho *et al.*, 2010).

Article 2: Un nou mètode computacional redefineix la família multigènica VIR de *Plasmodium vivax*

Antecedents

La família multigènica *vir* està composta per 346 gens i 80 pseudogens dividits en 12 subfamílies (Carlton *et al.*, 2008). Aquesta classificació de la família manté gran quantitat de gens sense agrupar dins les seves subfamílies. A més a més, la gran diversitat present en aquesta família multigènica va fer pensar que les proteïnes que la composen podrien tenir diferents localitzacions subcel·lulars i funcions (Merino *et al.*, 2006). Recentment s'ha demostrat que els membres de la família multigènica *vir* presenta diferents localitzacions subcel·lulars (Bernabeu *et al.*, 2012b). Aquest fet fa pensar que potser l'actual classificació de la família no sigui prou acurada.

Objectiu

Aquest article pretén aplicar un nou algoritme (Enright *et al.*, 2002, Joseph *et al.*, 2009, Apeltsin *et al.*, 2011) que millori la classificació de les proteïnes VIR i contribueixi en el seu coneixement.

Resultats

- L'aplicació del nou algoritme dóna lloc a una nova classificació de les proteïnes VIR, validada pel fet que aconsegueix la correcta classificació de les diferents famílies multigèniques de *P. falciparum.*
- Les subfamílies A, D i H ja no poden ser englobades dins la família multigènica VIR. El nostre mètode nou mostra una clara separació entre elles aplicant els mateixos requisits que permeten la correcta separació de les proteïnes de *P. falciparum.* Hem denominat aquestes noves famílies multigèniques: PvPIRA, PvPIRC i PvPIRH.
- Els assajos de localització subcel·lular, en les línies de *P falciparum* que expressen membres de la subfamílies A i D (Bernabeu *et al.*, 2012b) validen la classificació que les separa en noves famílies multigèniques.

- Els membres que composen les (sub)famílies D i H presenten blocs d'homologia propis no compartits amb la resta de proteïnes VIR, fet que també valida la separació d'aquestes subfamílies en noves famílies multigèniques.
- Un dels grans avantatges d'aquesta nova classificació és el fet que permet classificar dins les noves (sub)famílies proposades, proteïnes que abans restaven sense clusteritzar.
- El segon avantatge, es que aquest mètode permet anotar 39 proteïnes, prèviament anotades com a hipotètiques, dins de la família VIR i les noves famílies. A més, la colocalització subtelomèrica amb membres de la seves putatives (sub)famílies fa pensar que la nostra classificació és correcta.

Conclusió

El nostre mètode permet una classificació més refinada de les proteïnes multigèniques de *P. vivax,* a la vegada que permet la classificació de proteïnes prèviament anotades com a hipotètiques. Aquesta nova classificació pot ser aplicada per entendre millor la família multigènica i el paper que pot jugar en la patologia de la malaltia. Per exemple, la majoria dels membres d'algunes noves (sub)famílies estan diferencialment expressats en pacients de *P vivax* (Westenberger *et al.*, 2010). Finalment, el nou mètode aplicat a totes les famílies multigèniques del gènere *Plasmodium* permet establir noves relacions entre elles i inferir possibles funcions comunes.

Resultats no publicats: Proteïnes variants subtelomèriques de *Plasmodium vivax* i citoadhesió a la melsa humana

Antecedents

Recentment han aparegut nombroses evidències que posen en dubte el dogma que afirma que *P. vivax* no presenta adhesió als capil·lars dels òrgans interns (Carvalho *et al.*, 2010, Chotivanich *et al.*, 2012, Machado Siqueira *et al.*, 2012, Mayor *et al.*, 2012). La melsa presenta un paper important en la malària ja que a la vegada que elimina el paràsit, modula l'expressió de les proteïnes variants de *Plasmodium* (Barnwell *et al.*, 1983, David *et al.*, 1983, Hommel *et al.*, 1983, Handunnetti *et al.*, 1987, Gilks *et al.*, 1990, Bachmann *et al.*, 2009). Fa pocs anys, el nostre grup va demostrar que la infecció amb una soca no letal de *P. yoelii,* que també infecta reticulòcits, provoca una modificació de la melsa mitjançant la formació d'una barrera d'origen fibroblàstic que provoca l'acumulació de reticulòcits infectats (Martin-Jaular *et al.*, 2011). En aquesta línia, el nostre grup ja va hipotetitzar al 2004 que *P. vivax* provoca la modificació de l'arquitectura de la melsa creant una barrera d'origen fibroblàstic a la qual el paràsit és capaç d'adherir-se mitjançant les proteïnes VIR, facilitant d'aquesta manera l'encontre amb reticulòcits i evitant l'acció dels macròfags presents a la melsa.

Objectiu

Determinar si *P. vivax* presenta adhesió a fibroblasts de melsa i estudiar si els membres de les famílies variants juguen un paper important en aquesta.

Resultats

- La línia transgènica de *P. falciparum* que expressa la proteïna VIR14 presenta una gran adhesió a fibroblasts de melsa de diferent origen. Aquesta adhesió és específica ja que aquesta línia transgènica no presenta adhesió a fibroblasts de pulmó.
- Aquesta adhesió sembla mediada per la proteïna VIR14, ja que la línia transgènica que l'expressa no presenta cap proteïna PfEMP1 sobre-expressada.

- L'especificitat d'adhesió de la família VIR ve reforçada pel fet que una línia transgènica que expressa un membre de la família Pv-FAM-D no presenta adhesió ni a receptors endotelials ni a fibroblasts de melsa.
- Les mostres de pacients infectats per *P. vivax* també presenten adhesió a fibroblasts de melsa. Els nivells d'adhesió són variables depenent de la mostra i un anticòs generat contra dominis VIR conservats l'inhibeix significativament. Encara que aquesta adhesió és variable, en molts pacients obtenim nivells d'adhesió similars als obtinguts amb la línia transgènica de *P. falciparum*.
- El receptor ICAM-1 no sembla tenir un paper important en aquesta adhesió, ni en les mostres de pacients de *P. vivax* ni en les línies transgèniques de *P. falciparum*.

Conclusió

En aquest estudi hem demostrat que *P. vivax* presenta adhesió a fibroblasts de melsa. A més, tant els assajos realitzats amb mostres de camp, com els que utilitzen les línies transgèniques de *P. falciparum* han demostrat que almenys algunes proteïnes VIR estan implicades en adhesió. ICAM-1 en canvi, no sembla presentar un paper important i tot sembla indicar que un altre receptor desconegut hi podria estar implicat. El fet que *P. vivax* presenti una adhesió tant elevada a aquestes cèl·lules demostra clarament que aquest paràsit té una gran afinitat per la melsa. A més, els resultats obtinguts aquí complementen la troballa de gran acumulació de paràsit que es va trobar a la melsa d'un pacient infectat per *P. vivax* que no havia estat sotmès a tractament antimalàric (Machado Siqueira *et al.*, 2012). D'aquesta manera, sembla que l'adhesió als fibroblasts de melsa podria evitar l'acció filtradora de la melsa i la fagocitosi per part dels macròfags, a la vegada que es facilita una nova infecció mitjançant l'encontre amb reticulòcits.

Discussió

Històricament la infecció per *Plasmodium vivax* ha estat considerada benigna i en conseqüència desatesa (Anstey *et al.*, 2012, Baird, 2013). No obstant això, les últimes dades clíniques i epidemiològiques demostren que existeixen aproximadament les mateixes probabilitats, tant en *Plasmodium falciparum* com en *P. vivax,* de patir una malaltia severa un cop té lloc la infecció (Genton *et al.*, 2008, Tjitra *et al.*, 2008, Kochar *et al.*, 2009, Andrade *et al.*, 2010, Manning *et al.*, 2011, Kaushik *et al.*, 2012, Mahgoub *et al.*, 2012). A més, al contrari del que es pensava, s'està demostrant que el paràsit circula establement pel continent africà (Culleton *et al.*, 2012) i darrerament s'estan produint transmissions estables de *P. vivax* en països com Grècia, on el paràsit ja havia estat eradicat (Andriopoulos *et al.*, 2013). Aquesta percepció errònia de la malaltia, juntament amb la impossibilitat de cultivar el paràsit *in vitro*, han dificultat la recerca en aquesta malaltia. Per aquesta raó, si algun dia volem arribar a eradicar la malària, s'ha d'impulsar la recerca en aquesta espècie (Mueller *et al.*, 2009). Si no, existeix la possibilitat que *P. vivax* esdevingui el darrer paràsit causant de la malària.

Les proteïnes variants de *P. vivax* han estat el focus d'atenció principal d'aquesta tesi. Les famílies multigèniques formades per proteïnes variants estan presents en la majoria de patògens, des de bacteris fins a paràsits passant pels fongs. Mitjançant un mecanisme conegut com a variació antigènica, la majoria de patògens alternen l'expressió de les proteïnes de superfície evitant així el reconeixement per part del sistema immune (Deitsch *et al.*, 2009). El control de l'expressió d'aquestes proteïnes és especialment complex en el cas de paràsits com *Trypanosoma brucei* (Pays *et al.*, 2004, Taylor *et al.*, 2006) o *P. falciparum* (Baruch *et al.*, 1995, Smith *et al.*, 1995, Su *et al.*, 1995), els quals van alternant l'expressió, a la superfície, d'un únic membre de les famílies VSP i PfEMP1, respectivament. Aquest fenomen, anomenat exclusió al·lèlica, està àmpliament regulat per aquests paràsits. Les famílies multigèniques estan també presents en tots els paràsits del gènere *Plasmodium,* malgrat la funció de la majoria d'elles encara és desconeguda.

VIR: Diferents localitzacions cel·lulars, diferents funcions i nova classificació

La família multigènica *vir* és la més diversa que podem trobar al gènere *Plasmodium* (Carlton *et al.*, 2008). El fet que la majoria no presenti motius d'export, juntament amb l'existència de motius diferencials similars a altres famílies de *P. falciparum,* va fer creure que podien tenir localitzacions cel·lulars diferents (Merino *et al.*, 2006, Carlton *et al.*, 2008). A més, estudis anteriors van demostrar que aquests gens no presenten una expressió clonal (Fernandez-Becerra *et al.*, 2005), fet que fa pensar que podrien exercir funcions diferents a part de la variació antigènica (Carlton *et al.*, 2008, Fernandez-Becerra *et al.*, 2009).

En absència d'un cultiu a llarg termini de *P. vivax,* hem decidit estudiar la funció de les famílies multigèniques d'aquest paràsit mitjançant estudis de transfecció heteròloga en *P. falciparum.* La viabilitat i eficàcia d'aquest mètode ja havien estat demostrades anteriorment, bàsicament per a l'estudi de proteïnes implicades en resistència a fàrmacs (O'Neil *et al.*, 2007, Sa *et al.*, 2009, Auliff *et al.*, 2010)

Gràcies a la metodologia descrita en aquesta tesi hem demostrat que els membres de les subfamílies VIR A, C i D presenten localitzacions subcel·lulars diferents (Bernabeu *et al.*, 2012b). A més, aquestes proteïnes tenen funcions diferents ja que només una d'elles presenta adhesió a receptors endotelials. No obstant això, els assajos *in silico,* que vam realitzar posteriorment, van revelar que aquestes diferències eren degudes a que els membres de les subfamílies A i D ja no poden ser considerats dins la família VIR. Així mateix, la subfamília H tampoc pot ser considerada dins els VIR (Lopez *et al.*, 2013). La pregunta que ens fem ara és: la nova família VIR presenta diferents localitzacions subcel·lulars i funcions? El nostre mètode de classificació nou encara mostra una gran diversitat entre els diferents membres, de fet força major que la resta de famílies de *Plasmodium* (Lopez *et al.*, 2013). El fet que famílies molt més conservades, com en el cas de RIFIN (Petter *et al.*, 2007), presentin subgrups amb diferents localitzacions i funcions, fa pensar que els membres dins la família VIR també poden exercir múltiples rols. Per altra banda, també existeix la possibilitat que la nova família VIR tingui una única funció i s'expressi clonalment.

Adhesió a la melsa de *P. vivax* i proteïnes variants

La melsa té un paper molt important en la malària, cosa que queda evidenciada amb el fet que un dels principals efectes secundaris de la malaltia és l'esplenomegàlia (Yadava *et al.*, 1996, Anstey *et al.*, 2012). Aquest òrgan té un paper dual tant en l'eliminació del paràsit com en el control de l'expressió de les famílies multigèniques variants de *Plasmodium* (Barnwell *et al.*, 1983, David *et al.*, 1983, Hommel *et al.*, 1983, Handunnetti *et al.*, 1987, Gilks *et al.*, 1990, Bachmann *et al.*, 2009). En aquesta línia, els paràsits dels pacients sense melsa no presenten ni expressió de proteïnes variants a la superfície ni adhesió als òrgans interns (Bachmann *et al.*, 2009). Per aquest motiu, es creu que en espècies com *P. falciparum*, l'adhesió és un fenomen derivat de la intenció del paràsit d'evitar el pas per la melsa.

En canvi, històricament s'ha cregut que *P. vivax* no presenta adhesió. Malgrat els últims estudis semblen indicar el contrari (Carvalho *et al.*, 2010, Chotivanich *et al.*, 2012, Machado Siqueira *et al.*, 2012, Mayor *et al.*, 2012), les autòpsies de pacients infectats no mostren una clara acumulació de paràsit (Anstey *et al.*, 2012, Lacerda *et al.*, 2012). D'aquesta manera, si aquest paràsit no presenta adhesió, com pot escapar de la melsa? La hipòtesi del nostre grup és que el paràsit indueix una modificació en l'arquitectura de la melsa, generant una barrera d'origen fibrocític (Weiss, 1990, Weiss, 1991, Martin-Jaular *et al.*, 2011) que permet al paràsit escapar del filtratge de la melsa i de l'eliminació per part dels macròfags, a la vegada que facilita l'encontre i la infecció de nous reticulòcits (del Portillo *et al.*, 2004, Fernandez-Becerra *et al.*, 2009). De fet, el nostre grup va validar aquesta hipòtesi utilitzant un model murí de la soca no letal de *P. yoelii* 17X que infecta reticulòcits. Pocs dies després de la infecció, va tenir lloc la reorganització de la melsa del ratolí amb la generació d'una barrera d'origen fibrocític. El paràsit *P. yoelii* es va trobar acumulat en aquesta barrera, evitant així l'activitat macrofàgica (Martin-Jaular *et al.*, 2011).

En aquesta tesi hem demostrat que tant la línia transgènica que expressa VIR14 com *P. vivax* procedent de mostres de pacients presenten adhesió específica a fibroblasts de melsa (Bernabeu et al., resultats no publicats). L'adhesió de mostres de camp de *P. vivax* a talls de melsa ja va ser demostrada amb anterioritat (Ferrer et al., resultats no

publicats). A més, el fet que l'única demostració clara d'acumulació de *P. vivax* en un teixit hagi estat a la melsa (Machado Siqueira *et al.*, 2012), fa difícil argumentar contra aquesta adhesió.

Per altra banda, sembla clar que les proteïnes VIR tenen un paper important en l'adhesió, ja que la línia transgènica de *P. falciparum* que expressa VIR14 no sobre-expressa cap PfEMP1 que pugui mediar la unió, i els anticossos anti-VIR són capaços d'inhibir-la en assajos realitzats en pacients. En canvi, el rol d'ICAM-1 com a receptor no és tan clar. Creiem que aquest receptor podria ajudar i/o intensificar aquesta adhesió però no n'és el principal responsable. Aquest paper secundari d'ICAM-1 és consistent amb el fet que no s'ha observat una clara i àmplia presència del paràsit en autòpsies de pacients de *P. vivax,* ja que ICAM-1 és un receptor àmpliament expressat en molts teixits (Anstey *et al.*, 2012, Lacerda *et al.*, 2012).

Observacions finals

En aquesta tesi hem demostrat que les proteïnes VIR tenen localitzacions cel·lulars i exerceixen diferents funcions. A més, hem millorat la seva classificació revelant que diverses famílies multigèniques composen l'antiga família VIR. Finalment, hem constatat que una de les seves funcions és la de facilitar l'adhesió del paràsit a la melsa, evitant així la seva eliminació per part d'aquest òrgan. La recerca en *P. vivax* ha estat llargament desatesa perquè s'ha subestimat la importància d'aquest paràsit, i per la impossibilitat de cultivar-lo *in vitro.* Si algun dia volem aconseguir l'eradicació d'aquesta malaltia, és imprescindible aconseguir un cultiu estable de *P. vivax* per intensificar la recerca en aquest paràsit. Aquest cultiu, sense cap mena de dubte, facilitarà l'estudi de la funció de les proteïnes de *P. vivax* i la seva relació amb la patologia de la malaltia, cosa que permetrà el disseny de noves estratègies que aconsegueixin l'eliminació d'aquest paràsit.

Conclusions:

Les conclusions principals d'aquesta tesi són les següents.

- Les proteïnes VIR de les subfamílies A, C i D presenten diferents localitzacions subcel·lulars quan s'expressen en *P. falciparum* i exerceixen diferents funcions. Més tard, la nostra redefinició de les proteïnes variants de *P. vivax* ha demostrat que les diferències són degudes a que les subfamílies A i D ja no es poden considerar membres de la família VIR.

- En infeccions naturals, les proteïnes VIR també presenten diferents localitzacions subcel·lulars depenent del pacient i de l'estadi en què es trobi el paràsit.

- La proteïna VIR14 de la subfamília C presenta adhesió, en condicions de flux fisiològic, a cèl·lules CHO que expressen el receptor endotelial humà ICAM-1 i a fibroblasts de melsa.

- Els reticulòcits infectats per *P. vivax* de pacients de l'Amazònia brasilera presenten citoadhesió a fibroblasts de la melsa.

- Almenys alguns membres de la família VIR són els lligands que permeten l'adhesió de *P. vivax* als fibroblasts de melsa. A més, els estudis utilitzant el model de *P. falciparum* demostren que concretament la subfamília C exerceix aquesta funció. El fet que membres de la subfamília Pv-FAM-D no presentin propietats citoadhesives dóna una gran especificitat a la funció adhesiva de les proteïnes VIR.

- El receptor ICAM-1 no està implicat directament en l'adhesió de *P. vivax* a fibroblasts de melsa.

- En absència d'un cultiu de *P. vivax,* el model d'expressió heteròloga de proteïnes de *P. vivax* en *P. falciparum* pot facilitar la recerca en aquest paràsit.

Additional projects

A predicted RNA binding protein containing a single RNA Recognition Motif (RRM) typical of cyanobacteria is targeted to the apicoplast

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Abstract

Malaria parasites harbour an essential vestigial plastid-like organelle, the apicoplast, acquired by a secondary endosymbiotic event. Most of the genes encoding predicted apicoplast proteins have been transferred to the nucleus; yet, the apicoplast still keeps a small circular genome whose genes are all simultaneously and maximally transcribed during schizogony. Of note, inhibition of apicoplast transcription has an immediate killing effect on the parasite but still very little is known about the transcriptional regulation of this organelle. The annotation of the *P. vivax* genome revealed the presence of a putative nuclear encoded RNA binding protein containing a single RNA Recognition Motif (RRM) typical of cyanobacteria. To get insights into its function, we generated a *P. falciparum* transgenic line expressing the *P. vivax* protein fused to a triple hemagglutinine tag. Confocal imaging studies revealed that the transgenic line presented parasite populations with different subcellular localization. Of them, co-localization assays using an antibody against the *P. falciparum* Acyl Carrier Protein demonstrated that this protein is targeted to the apicoplast in some infected Red Blood Cells. Moreover, global transcriptional analysis of highly synchronous parasites during schizogony revealed a

set of unique upregulated genes. Furthermore, phenotypical analysis revealed a significant increase in gametocytes in this transgenic line as compared to the parental line. Last, all attempts to knock-out the orthologous gene in *P. falciparum* have failed. This protein could thus be considered a new antimalarial apicoplast drug target.

Introduction

Nearly all apicomplexan parasites, with the exception of Cryptosporidia's species, have a vestigial plastid-like organelle, the so called apicoplast [1]. This organelle is surrounded by four membranes and it was originated by a secondary endosymbiotic event. Phylogenetic evidences indicated that it was acquired after a protist engulfment and retention of a red alga that contained a plastid obtained by primary endosymbiosis of a cyanobacterium-like prokaryote [2]. Even though its photosynthetic function had been lost, it still harbors essential metabolic pathways that convert this organelle in an attractive drug target [3].

The apicoplast contains a genome consistent of a 35Kb circular and double-strand DNA highly conserved among apicomplexan species. Of note, the sequence of the apicoplast genome gave no clue regarding its function, as most of the genes are apparently involved in organelle housekeeping functions [4]. As with most symbionts, many genes that were encoded by the apicoplast genome were transferred to the parasite nucleus to avoid deleterious mutations of non-recombinant genomes. The trafficking of these nuclear encoded proteins to the organelle is mediated by a bipartite leader at the N-terminus that consist of a signal peptide (SP), that allow entry into the secretory pathway, and a plant-like transit peptide (TP) for subsequent import into the apicoplast. [5-7]. According to the apicoplast prediction algorithms PlasmoAP [5] and PATS [8], more than 500 proteins encoded by the nuclear

genome of *P. falciparum* present an N-terminus bipartite signal [5]. Of those, 350 still remain as hypothetical proteins while the remaining show significant sequence similarity to other proteins with known function or structure [9, 10]. As a consequence, it was revealed that this organelle harbors a collection of plastid-like anabolic pathways such as: (i) fatty acid synthesis with the type II fatty acid synthase (FASII), (ii) isoprenoid biosynthesis via the DOXP/ MEP pathway, (iii) iron-sulfur [Fe-S] cluster synthesis and (iv) heme biosynthesis associated with the mitochondria. Recent gene deletion studies have revealed that the apicoplast FASII pathway is essential in liver stages [11, 12] whereas the DOXP pathway is only essential in blood stages [13]. For this reason, Fosmidomycin, an inhibitor of the second enzyme of the DOXP pathway, is effective in managing the clinical symptoms of malaria associated with the asexual blood-stage parasites [14].

On the other hand, the housekeeping genes encoded by the apicoplast genome are all transcribed [15, 16] and present a highly coordinated expression that peaks during the schizont stage [17]. The synthesized mRNAs are polycistronic, unlike those produced in the parasite nucleus, and almost provides the essential machinery for transcription and translation. The circular plastid encodes large and small ribosomal subunits (LSU rRNA and SSU rRNA), an elongation factor (ef-TufA) and 17 ribosomal proteins. Moreover, 34 tRNAs are present in the genome and it encodes for three subunits of the bacterial-type RNA polymerase (rpoB, rpoC1 and rpoC2). Of the eight identified Open Reading Frames (ORFS), only three encode for genes with predicted function. Finally, DNA replication is performed by nuclear-encoded proteins, as none DNA replication protein is encoded by the the apicoplast genome [4].

An interesting issue of the apicoplast is the “delayed-death” phenomenon. Treatment of *Plasmodium* blood stages with some antibiotics has no effect on the first inthraerythrocytic developmental cycle

and thus released merozoites are able to invade new erythrocytes. However, during the second cycle treated parasites are unable to form functional merozoites capable of egressing from the host cell [18]. As a consequence, parasites dye due to the so called “delayed-death” phenotype [18]. Of interest, most of the antibiotics that present “delayed-death” phenotype target the translation machinery of the apicoplast and are highly active against apicomplexans [19]. In contrast, antibiotics that disrupt the apicoplast anabolic functions result in relatively rapid death of the parasite [6, 14, 20, 21]. Finally, rifampicin and rifampin that target apicoplast transcription also cause a direct death of the parasites [17, 22].

Despite intense research on this organelle, still very little is known about its transcriptional regulation. The annotation of the *P. vivax* genome [23] revealed the presence of a RNA Binding Protein (RBP) (PVX_084415) with a single RNA recognition motif (RRM) putatively target to the apicoplast. Typically, an RRM is approximately 90 amino acids long with a typical $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology that forms a four-stranded β-sheet packed against two α-helices [24]. As a consequence of its “archaic” structure, we hypothesize that this protein may be a relict of cyanobacterial transcription. To get insights into its function, we generated a *P. falciparum* transgenic line expressing the *P. vivax* protein fused to a triple hemagglutinine tag. In here, we show subcellular localization analysis undertaken in this transgenic line, as well as phenotypical analysis and knock-out generation attempts of the orthologous gene in *P. falciparum* .

Materials and methods

Plasmid construct generation

Pvrbp gene (PVX_084415) was amplified from Sal1 cDNA using primers F-Pvrbp: GGATCCGGTACCATGCCATACATCCGCG; R-Pvrbp: GGATCCCTGCAGCGACATGTTTGGCT and was cloned in the KpnI-PstI cloning sites of modified transfection vector pARL1a-3HA [25]. Thus, creating plasmid pARL1a_PvRBP-3HA. To generate plasmid pARL4a_PvRBP-3HA, BSD resistance gene was extracted from plasmid pCC4 and cloned in the BamHI-SfoI site of the plasmid pARL1a_PvRBP-3HA whose hDHFR gene has been previously removed. The recombinant regions of the Knock Out plasmid 108-pCC1-140 were amplified from 3D7 gDNA using primers, al108: atcccgcgGAACTTGCAAGATATATT; and al109: gatactagtCCCCTGAATCTTTAATGTATGG for homologous recombinant region 5' and primers al140 atcgaattcGGACGAAATATGGGACACGG; and al141: gatcctaGGGATTGTTAGATGAAGCCATC for homologous recombinant region 3'. First, the 5' recombinant region was cloned in the SacII-SpeI site of multicloning site I (MCI) of the plasmid pCC1, generating plasmid 108-pCC1. Finally, the 3' recombinant region was cloned in the EcoRI-AvrII site of the multicloning site II (MCSII) of the plasmid 108-pCC1.

P. falciparum culture and transfection generation

Transfection of Plasmid pARL1a_PvRBP-3HA was done by transfecting *P. falciparum* 3D7 parasites as described previously [27]. Briefly, 150 mg of plasmid was used to electroporate 600 ml of uninfected red blood cells and this mix was added to about 10^7 parasites. Plasmids pARL4a_PvRBP-3HA and 108-pCC1-140 were transfected into 3D7 parasites by electroporating ring-stage parasites (> 5% parasitaemia) with 100 µg of purified plasmid DNA (Qiagen) as previously described [26]. All transfections were done using 0.310 kV and 950 F as electroporation conditions. Culture was maintained in either 2,5 nm hDHFR or 2µg/ml BSD and parasites appear 20 to 30 days after drug addition. Transgenic lines were maintained under drug addition and depletion cycles for integration. 5 cycles of drug selection were undertaken in transgenic lines transfected with plasmid 108-pCC1-140.

Western blotting

Trophozoite stage parasites were harvested at >5% parasitaemia, treated with 0.15% saponine and resuspended in PBS with protease inhibitor cocktail (Roche). Samples were boiled and separated on a 12% SDS-PAGE, transferred on to Hybond-C nitrocellulose membrane (Amersham) and blocked in blocking buffer (1 x PBS, 0.1% Tween-20, 5% milk powder) overnight. The blot was washed and incubated for 1 h with primary antibody [rat anti-HA (1:250, Roche) or mouse anti-Hsp70 antibody (1:1000)] in dilution buffer (1 x PBS, 0.1% Tween-20, 1% milk powder). Subsequently, the blots were washed and incubated for 1 h with secondary antibody conjugated to HRP [anti-mouse or anti-rat IgG antibody (1:1500, Molecular Probes)]. Bands were visualized by using ECL detection kit (Amersham).

Indirect immunofluorescence assays

Cultured *P. falciparum* 3D7_R1-3HA and 3D7_R2-3HA transgenic lines, with predominantly rings and young trophozoite stages, were washed in PBS and then fixed with 4% EM grade paraformaldehyde and 0.075% EM grade glutaraldehyde in PBS [28]. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 h at room temperature in 3% PBS-Bovine Serum Albumin (PBS-BSA). Samples were incubated overnight with primary antibody [rabbit anti-HA (1:50, Molecular Probes), rat anti-HA (1:50, Roche), or rabbit anti-PfACP (1:50)] diluted in 3% PBS-BSA followed by 1 h incubation with secondary antibody [anti-rabbit or anti-rat IgG conjugated with Alexa Fluor 488 and/or anti-rabbit IgG conjugated with Alexa Fluor 594 (1:100, Molecular Probes)] diluted in 3% PBS-BSA. Nuclei were stained in the secondary antibody incubation with 4,6-diaminido-2-phenylindole (DAPI, 2 mg/ml diluted in 3% PBS-BSA). Confocal microscopy was performed using a laser scanning con-focal microscope (TCS-SP5; Leica Microsystems), at microscopy scientific and technical services of Universitat de Barcelona. Images were processed using ImageJ image browser software.

Global transcriptional analysis

Parasites were highly synchronized by doing a 70% percoll gradient in cultures with mature stages, followed by a 5% sorbitol synchronization 4 to 5 hours later when merozoite egress had occured. RNA was extracted using the RNeasy Mini Kit (QIAGEN) and RNA purification was assessed using a bioanalyzer an Agillent 2100 Bioanalyzer. A GeneChip Plasmodium/Anopheles Genome Array (Affymetrix) was used and two bilogical replicates were done at IDIBAPS genomic unit. Expression levels were normalized by using RMA[29]. Then, a low intensity filter was applied which removed all of those probes that yielded very low intensities (<5) for all samples. Thus, 2899 probes (222 genes) were selected for downstream analysis. Finally, differentially expressed genes were identified by running SAM (Significance Analysis of Microarrays) [30].

Real-time PCR

Real time was performed as described in [25]. Primers used were:

PF13_0011_F GAAGCGTATCATGAACGACAAGA; PF13_0011_R CTTATTCTTGCTGCTGCGTC;

PFD0310w_F TCAGGTGCCTCTCTTCATGCT; PFD0310w_R GCTGAGTTTCTAAAGGCATTTTGTC;

PF14_0744_F GATGTACCGAAGTATGAGAATGATT and PF14_0744_R GATAACGGCAAGGATATTTCTT

Bioinformatical analysis

Apicoplast protein prediction was made using both PATS [8] and PlasmoAP [5]. The residues and the conditions used by these programs were manually revised. Aligments of the orthologous proteins were made using ClustalW.

Structual Model generation

The 3D model of PVX_084415 was obtained from the iTasser web server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). This software uses multiple-threading alignments with known structural models (in our case, known 3D structures of RRM proteins) followed by iterative template fragment assembly simulations [31-33].

Results and Discussion

The single RRM of the PVX_084415 proteins suggests a cyanobacterial origin

The *P. vivax* genome annotation revealed the presence of 316 proteins predicted to be targeted to the apicoplast. The entire repertoire of sequences presented targeting leader sequences, but of them, only 157 presented plastid-based functions and plastid-located orthologs. Included in that list, it was found and RNA Binding Protein that was described as an RNA Binding Protein (RBP) (PVX_084415). A protein BLAST analysis of this protein revealed that it contains a 90 amino acid motif with two conserved ribonucloeprotein domain consensus sequences whose structural model predicts a RNA-Binding Domain that comprised by the typical $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology and an N-terminal helix encoded by the TP domain (Figure 1). Eukaryotic and prokaryotic RNA Binding proteins present quite different structural organization. In eukaryotic proteins, RRM are often found as multiple copies within a protein (44% have from two to six RRM) and/or together with other protein domains. The association with different types of protein domains confers the RRM domain different affinity and specificity for the RNA sequence [34]. Eukaryotic RBPs have been widely studied and are involved in pre-mRNA splicing, alternative splicing in the nucleus or in the control of mRNA stability and translation efficiency on the cytoplasm [35]. On the other hand, prokaryotic RRM proteins are rather small (about 100 amino acids) and have a single copy of the RRM. In bacteria, RRM proteins are particularly prevalent in cyanobacteria, while other genomes encode only a few or even do not present any RRM signature. Remarkably, the RBP of this study only presents a single RNA recognition motif that may reveal the putative cyanobacterial origin of this protein. Furthermore, a ClustalW aligment of the *P. vivax* protein and its orthologous in *Plasmodium* showed that the protein presents a high level of conservation in the

RRM in all malaria species. Hence, the high conservation of the domain might bring to light that this protein exerts an important function in the plastidial organelle.

Sub-cellular localization of the PvRBP

Even though the protein was annotated as an apicoplast protein in the *P. vivax* genome annotation, ClustalW aligment of the protein and its orthologous in *Plasmodium* revealed that the PvRBP lacks most of the N-terminus bipartite sequence that putatively targets to the plastidial organelle (Figure 2A). However, according to the analysis of predicted apicoplast proteins made in the *P. vivax* genome [23], this protein presents apicoplast targeting sequences as well as orthologous targeted to the plastid.

To decipher its sub-cellular localization, we created *P. falciparum* transgenic lines that expressed the *P. vivax* protein, as previous studies have demonstrated the adequacy of this model to determine the location of *P. vivax* proteins in this organelle [25]. To undertake future Knock Out compensation studies, we generate two transgenic lines resistant to WR99210 and Blasticidin selection drugs. For this reason, we modified the transfection vector pARL1a_PvRBP-3HA by replacing the hDHFR gene for the Blasticidin S Deaminase gene (BSD) from plasmid pCC4 with a BamHI-SfoI digestion (Figure 3B). After transfection and resistant drug preassure, we obtained two transgenic lines that expressed the PvRBP protein fused to 3HA. Of note, the transgenic line resistant to WR99210 (3D7_R1-3HA) presented a bigger expression of the protein than the transgenic line resistant to BSD (3D7_R2-3HA) (Figure 3B).

After generation of stable transgenic lines, indirect immunofluorescence assay (IFA) using both anti-HA and an anti- *P. falciparum* Acyl Carrier Protein (PfACP) revealed and almost complete co-localization in apicoplast (Figure 4B). Yet, some parasites expressed the protein in the parasite cytoplasm (Figure 4C, upper pannel) while others expressed the RBP in a sub-cellular localization that resambled the

parasite parasiophorous vacuole (PV) (Figure 4C middle and lower panel). Interestingly, *P. falciparum* transgenic lines of truncated proteins lacking the SP were found in the cytoplasm of the parasites, whereas proteins lacking the TP were found in the PV [5]. The question thus remains as to how this protein was trafficked to the apicoplast since it was lacking a clear bipartite signal. One explanation might be that an alternative pathway to the bipartite leader sequence is targeting the protein into the organelle. This has been shown with the *P. falciparum* protein PfoTPT that lacks the apicoplast bipartite leader and yet it localizes to the apicoplast. The proposed mechanism of transport of this protein was the insertion of the protein in the endoplasmatic reticulum by the first of its ten transmembrane domains [36]. However, the PvRBP protein has neither a transmebrane domain nor a highly hydrophobic region that could favor this alternative pathway. A second explanation of the plastid localization in some parasites could be the result of a single homologous recombination event in the internal locus of the *P. falciparum* orthologous, after several cycles of drug addition and depletion. However, an argument against this hypothesis is that, despite the protein shows a high level of conservation, a lower conservation is observed at the DNA level due to the differences in codon usage of the two species. Finally, the most plausible explanation for this event is a joined transport of the *P. vivax* protein and the *P. falciparum* orthologous. It has been demonstrated that RRMs appear to make multiple contacts not only with single-stranded RNA, but also with single-stranded DNA and proteins [34]. Thus, the high similarity of the RRM domain may have facilitated a dimerization of the protein and a co-traffic event of the two orthologous. Notably, dimerization of RBPs [37] and RRMs [38] has been demonstrated in human proteins.

Recently, an improved N-terminal protein annotation algorithm of *Plasmodium* proteins revealed that there is a large percentage of missannotated proteins in the genomes of different malarial species [39]. We thus used this algorithm and showed that the PvRBP (PVX_084415) used in this study had been missannotated. The predicted new protein presents a new exon on the 5' end of the gene and extending the N-terminus end of this protein (Figure 2B). The new annotated protein is strongly predicted to be an apicoplast protein by PATS [8] but not by PlasmoAP [5]. Apicoplast prediction by PlasmoAP is based on the presence a highly hydrophobic motif recognized as a signal peptide and a transit peptide stretch highly rich in asparagines (N) and lysines (K) with a net basic charge. Despite the new *P. vivax* reannotated protein and its orthologous in *P. falciparum* are not targeted to the plastid according to PlasmoAP, manual inspection of residues in the transit peptide argues against the result of the algorithm (Table 1, Figure 2). Both proteins present more than 9 N or K in the transit region and a positive global region. In addition, both proteins contain chaperon binding sites (DnaK sites) that would facilitate the transit of the proteins to the apicoplast (Table 1). Moreover, the rodent *Plasmodium* orthologous are clearly predicted to be plastid proteins and subcellular localizations are generally conserved among orthologs [39]. Present efforts are directed to generate transgenic lines expressing the *P. vivax* RBP (PVX_084415) and its orthologous in *P. falciparum* fused to a triple hemagglutinen tag to further confirm the apicoplast location of both proteins.

Dual localization of proteins is a widespread phenomenon found in eukaryotes [40]. Recently, three aminoacyl-tRNA synthetases of *P. falciparum* have been described to localize in the cytosol and the apicoplast [41]. Morever, other examples of dual targeting are found in *Plasmodium* : Glutatione reductase is located in both cytosol and apicoplast, while thioredoxin reductase (TxR) is located in the mitochondria as well as the cytosol [42]. It is tempting to speculate that the PvRBP, instead of being

missannotated, has evolved to present dual targeting to both the cytosol and the apicoplast. Different mechanisms can led to dual targeting: alternative splicing, alternation in transcription or translation initiation sites and the use of an ambiguous N-terminal targeting sequence. Although unlikely, it may be possible that the unclear PlasmoAP prediction of the *P. falciparum* orthologous is promoting an inefficient detection of the TP to promote dual location. On the other hand, the *P. vivax* protein can present dual location by either alternation of transcription or translation initiation sites. Thus, both proteins can be located to both cytosol and apicoplast.

Surprisingly, the transgenic line 3D7_R1-3HA presented a remarkable phenotype. A few weeks after thawing, it presented a significant increase in gametocytes production (2% gametocytes/iRBC). To understand this phenotype we realize a global transcription analysis of highly synchronous cultures of transgenic line 3D7_R1-3HA and the parental strain 3D7. Unexpectedly, none gene involved in gametogenesis was differentially expressed in the two strains. In addition, Real time PCR analysis did not showed significant differences in genes related with gametocytes formation [43-46]. On the other hand, significant differences in several *var* and one *rifin* gene appeared in the global transcription array (Table 2). A probably explanation for appearance of gametocytes and variant genes differentially expressed might be the wrong location of the PvRBP in the parasite Endoplasmatic Reticulum or cytoplasm. RBP specificity to RNA, DNA or other proteins depends on the number of RRM motifs that presents. We can speculate that the phenotype observed in this transgenic line is a consequence of the presence of single RRM in this protein that might confer a low binding specificity for RNA and alterations in transcription regulation.

Finally, the high conservation of the protein sequence among the RRM in its orthologous in *Plasmodium* suggested an important role of this protein (Figure 2A and B). To determine the

essentiality of this protein, attempts to knockout the orthologous protein in *P. falciparum* were done (Figure 5A). After 5 cycles of drug addition/depletion the genomic locus of the PF3D7_1207500 still maintains its length as demonstrated by PCR, thus revealing a possible "essential" role of this protein (Figure 5B). Finally, we also tried to knockout the *P. falciparum* orthologous in the transgenic line that expressed the PvRBP under the pressure of BSD. Surprisingly, no parasites appear after two independent transfections.

Conclusion

In here, we have shown that a predicted RNA binding protein containing a single RNA Recognition Motif (RRM) typical of cyanobacteria is targeted to the apicoplast. Yet, some parasites express this protein outside the apicoplast likely due to its original missannotation which failed to predict a complete N-terminus bipartite signal. Even though the exact sub-cellular localization of this protein remains to be determined, the impossibility to Knockout the protein as well as the fact that the protein resembles the structure of a cyanobacterial RNA-Binding Proteins, encourage further research on it. Undoubtedly, future transfection of the reannotated *P. vivax* PvRBP protein as well as its orthologous will shed light into its role in apicoplast transcription and RNA stability maintenance. Research on the plastid's translation has contributed enormously in the use of novel prokaryotic translation inhibitors in anti-malarial therapy. Indeed, doxycycline is already recommended as a malaria prophylactic for travelers to endemic areas. For this reason, there is no doubt that molecular characterization of proteins with a crucial role in plastid transcription is likely to offer new insights into apicoplast function and help in rational drug design.

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	SP	PATS	Plasmo AP	DnaK Binding Sites	NK	Basics	Acidic	Ratio
P. berghei	Yes	Yes	Yes (5/5)	Yes	13	17	6	0,3
P. yoelii	No	No	Yes (5/5)	Yes	14	17	6	0,3
P. chabaudi	Yes	Yes	Yes (5/5)	Yes	13	17	6	0,3
P. knowlesi	Yes	Yes	No (3/5)	Yes	11	12	5	0,4
P. falciparum	Yes	Yes	No (3/5)	Yes	10	17	6	0,3
P. vivax	No	No	No	Yes	5	8	4	0,5
P. vivax new	Yes	Yes	No (3/5)	Yes	11	19	5	0,21

Table 1. Bioinformatic analysis of the *Plasmodium* orthologous of the PvRBP.

SP: Presence of Signal Peptide; PATS: Result of PATS algorithm; PlasmoAP: Result of PlasmoAP algorithm, in brackets number of tests positive; NK: number of Asparagines and Lysines in the Transit Peptide; Basics: number of basic amino acids in the Transit Peptid; Acidic: number of acidic residues in the Transit Peptide; Ratio: Ratio of acidic and basic residues in the Transit Peptide

Gene	Target Description	3D7	3D7_R1-3HA
PFC1115w	Rifin	4.38 ± 1.04	7.17 ± 0.62
PFD0625c	Erythrocyte Membrane Protein 1 (PfEMP1)	3.48 ±0.08	6.16 ± 0.94
PFB0075c	Plasmodium exported protein (hyp9)	3.16 ± 0.51	6.52 ± 0.65
PFD0615c	Erythrocyte Membrane Protein 1 (PfEMP1)	2.76 ±0.02	7.57 ± 1.94
PFD0630c	Erythrocyte Membrane Protein 1 (PfEMP1)	4.30 ± 0.61	8.90 ± 1.31
PFD1000c	Erythrocyte Membrane Protein 1 (PfEMP1)	2.69 ± 0.44	7.32 ± 1.34
PFB0080	Hypotetical protein RESA-like	3.09 ± 0.01	6.86 ± 0.02
PFD0625c	Erythrocyte Membrane Protein 1 (PfEMP1)	3.98 ± 0.63	8.9 ± 1.34
PFD0635c	Erythrocyte Membrane Protein 1 (PfEMP1)	2.25 ± 0.14	6.7 ± 0.86
PFD0630c	Erythrocyte Membrane Protein 1 (PfEMP1)	2.32 ± 0.01	6.9 ± 0.85
PFB0105c	Plasmodium exported protein (PHISTc)	3.09 ± 0.10	8.47 ± 1.81
PFD1245c	Erythrocyte Membrane Protein 1 (PfEMP1)	3.96 ± 0.70	9.07 ±1.28
PFD0625c	Erythrocyte Membrane Protein 1 (PfEMP1)	3.1 ± 0.33	9.02 ± 1.38
PFB0100c	Knob Associated Histidine-Rich Protein (KAHRP)	4.50 ± 0.47	10.95 ± 0.40

Table 2. Global transcriptional analysis of highly synchronous cultures.

Differential expressed genes in transgenic line 3D7-R1_3HA compared to 3D7 parental strain. Mean values ± standard deviation of two biological replicates are shown.

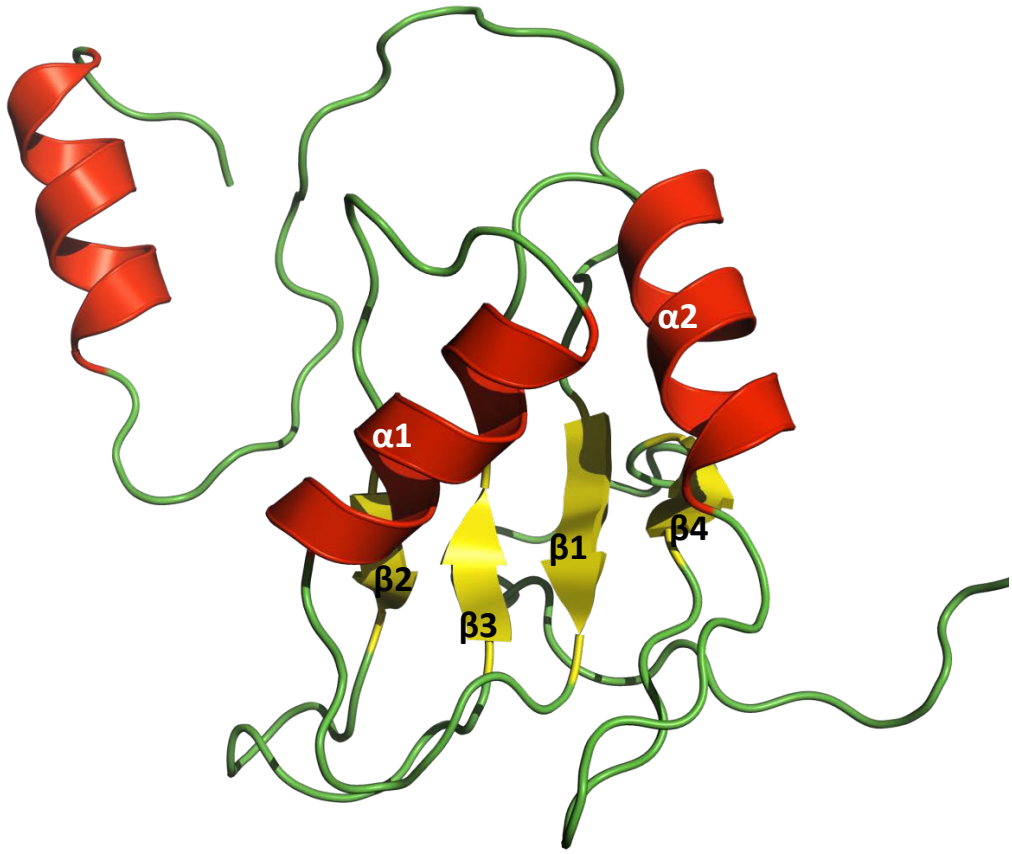

Figure 2. Structural model of the *P. vivax* RNA-Binding Protein.

α-helices are displayed in red and β-strands are in yellow

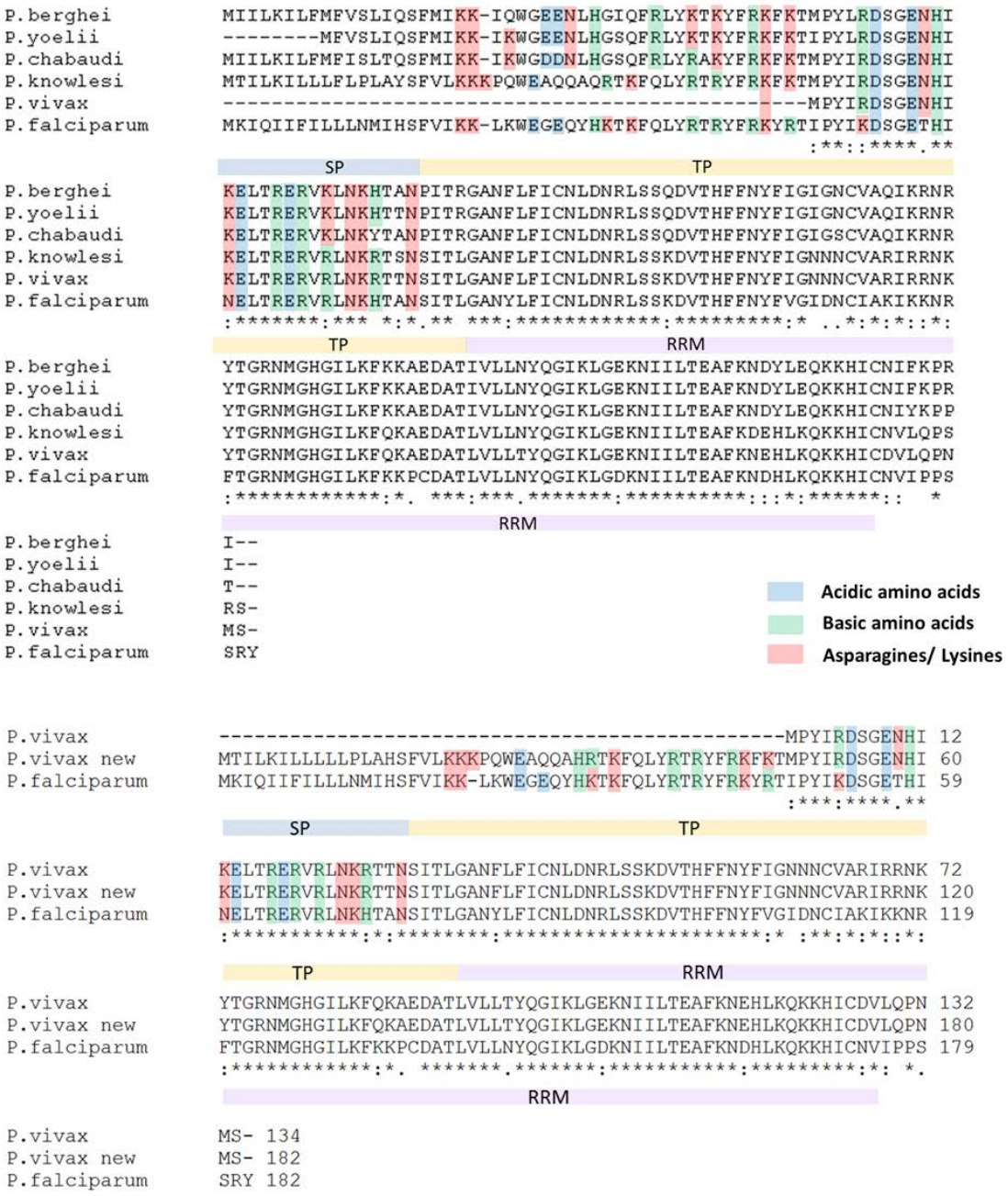

Figure 2. ClustalW algorithm of the orthologous of the *P. vivax* RNA-Binding Protein.

The different domains that compose the protein are shown below the aligment. Asparagines and Lysines of the Transit Peptide are shown in red, Acidic amino acids in blue and basic amino acis (with the exception of Lysines) are shown in green.

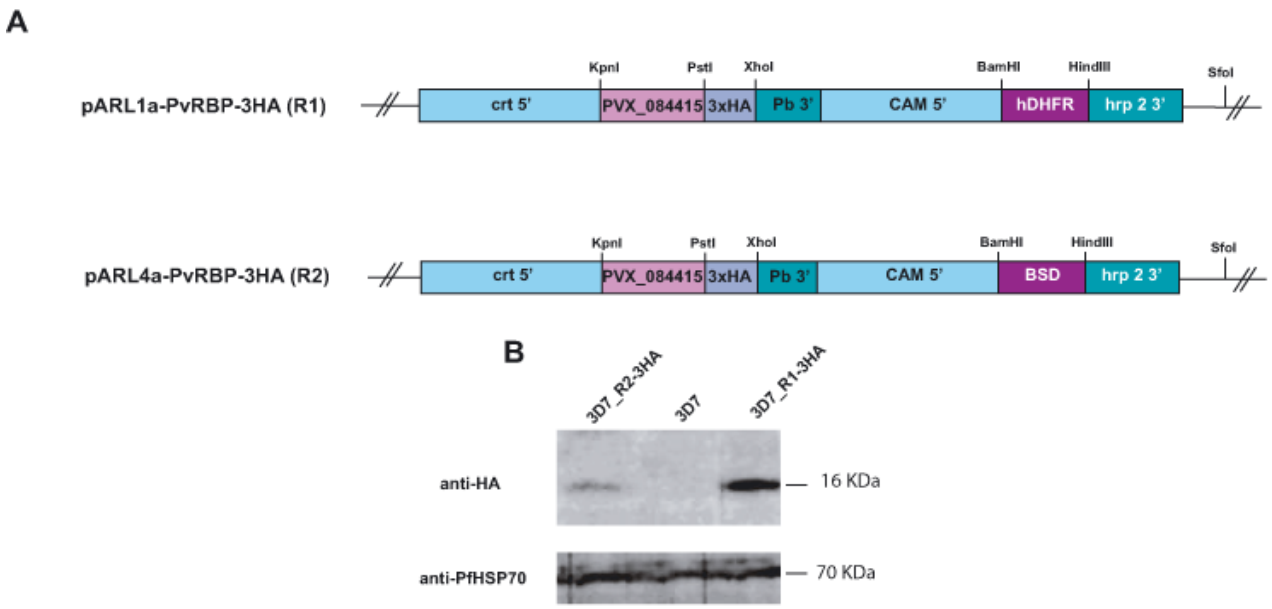

Figure 3. Expression of the *P. vivax* RNA-Binding Protein in *P. falciparum.*

A. Schematic representation of the plasmids used to transfect 3D7.

B. Western blot expression analysis of the RNA-Binding proteins expressed in transgenic P. falciparum. Wild-type *P. falciparum* 3D7 as well as *P. falciparum* transgenic lines 3D7-R1_3HA and 3D7-R2_3HA subjected to western analysis and probed with anti-HA (upper panel) and anti-PfHSP70 as a loading control (lower panel). Molecular weights of P. vivax proteins fused to the tags are given in KiloDaltons (kDa)

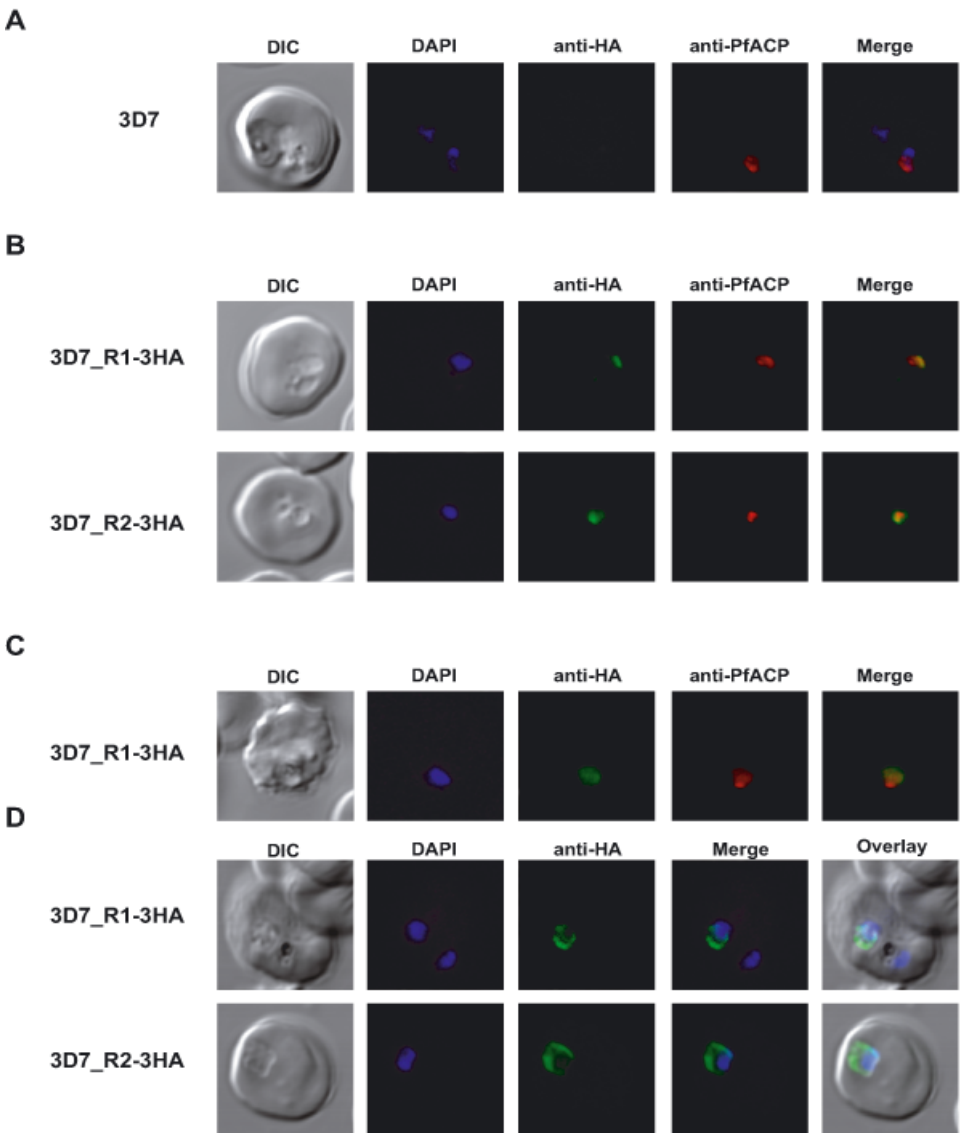

Figure 4. Subcellular localization of PvRBP expressed in the *P. falciparum* transgenic lines

Parasites were labelled with anti-HA (green) or anti-PfACP (red) and DAPI for nuclear staining.

A. Immunofluorescence assay in the 3D7 parental strain.

B. Immunofluorescence assay that shows an apicoplast location in transgenic lines 3D7-R1_3HA and 3D7-R2_3HA.

C. Immunofluorescence assay that show a cytoplasmic location transgenic lins 3D7-R1_3HA.

D. Immunofluorescence assay that shows a parasitophorous vacuole location transgenic lines 3D7-R1_3HA and 3D7-R2_3HA.

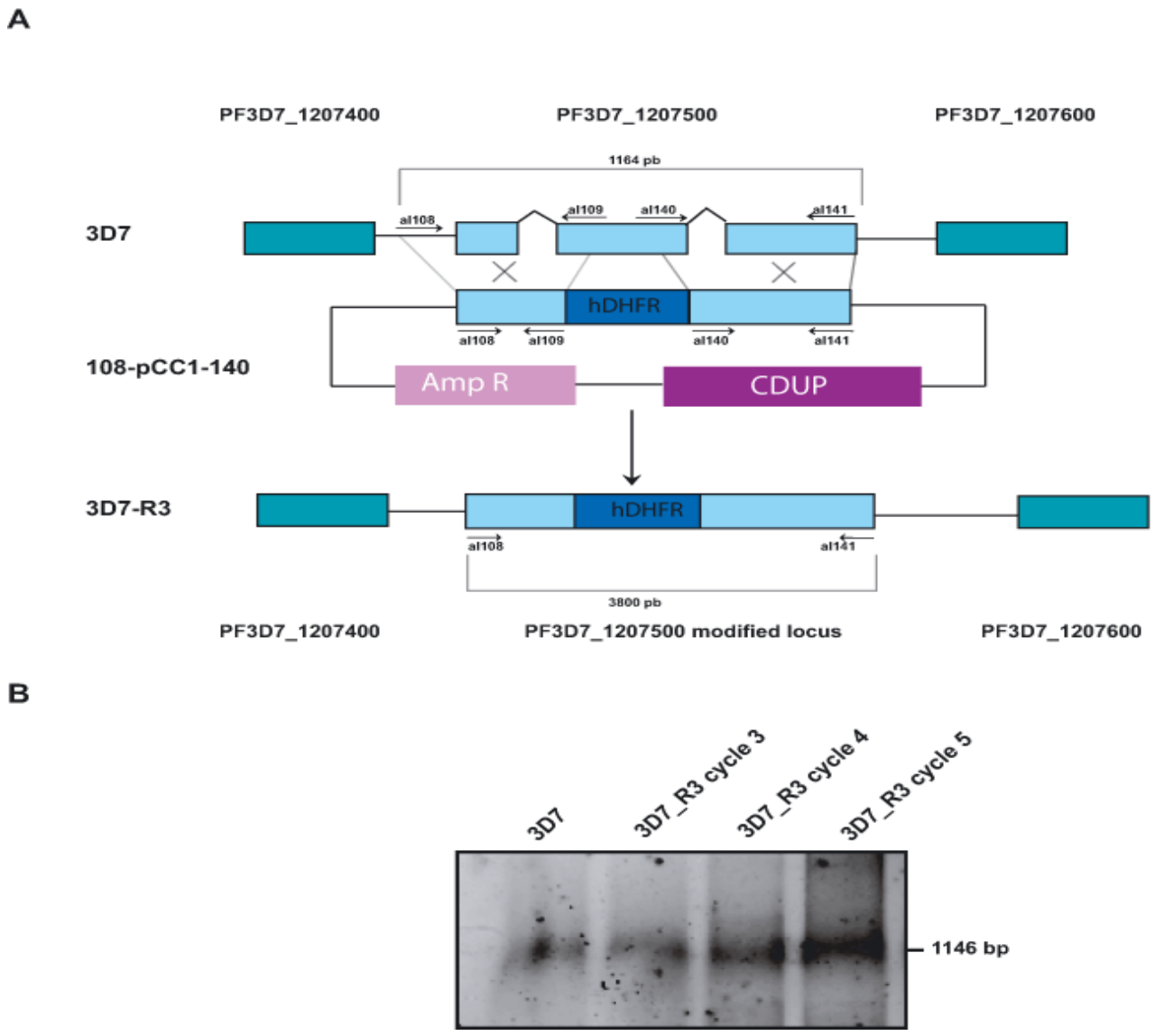

Figure 5. Knock Out attempt of the *P. falciparum* orthologous of the PvRBP (PF3D7_12070500) generating the transgenic line 3D7_R3

A. Schematic representation of the Knock out strategy followed. AmpR, CDUP, and hDHFR are the resistance genes. The arrows represent the primers used for the cloning.

B. PCR of the internal locus using primers al108 and al141

Other contributions

6.3.1 Research article 1:

Bernabeu *et al. Malaria Journal* 2012, **11**:405
http://www.malariajournal.com/content/11/1/405

RESEARCH Open Access

Plasmodium vivax malaria in Mali: a study from three different regions

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Abstract

Background: *Plasmodium vivax* has traditionally been considered virtually absent from Western and Central Africa, due to the absence of the Duffy blood group in most of the population living in these areas. Recent reports, however, suggest the circulation of *P. vivax* in sub-Saharan Africa.

Methods: Giemsa/Field-stained smears from febrile patients recruited in five different cities (Goundam, Tombouctou, Gao, Bourem and Kidal) pertaining to three regions from Northern Mali were examined. Nested-PCR and DNA sequence analyses of selected samples were performed to fully confirm the presence of *P. vivax* infections.

Results: Results demonstrated the presence of *P. vivax* infections in close to 30% of the cases as detected by Giemsa/Field-stained smears and nested-PCR and DNA-sequence analyses of selected samples unequivocally confirmed the presence of *P. vivax*.

Conclusions: The diagnostics of this human malaria parasite should be taken into account in the context of malaria control and elimination efforts, not only in Mali, but also in sub-Saharan Africa.

Keywords: Mali, Sub-Saharan Africa, Plasmodium vivax, Vivax malaria, Nested-PCR, DNA sequencing, SSU RNA, Giemsa-smears

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6.3.2 Research article 2:

Biosynthesis of GDP-fucose and other sugar nucleotides in the blood-stages of *Plasmodium falciparum**

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Abstract

Carbohydrate structures play important roles in many biological processes, including cell-adhesion, cell-cell communication and host-pathogen interactions. Sugar nucleotides are activated forms of sugars used by the cell as donors for most glycosylation reactions. Using a liquid chromatography-tandem mass spectrometry-based method we identified and quantified the pools of UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, GDP-mannose and GDP-fucose in *Plasmodium falciparum* intraerythrocytic life stages. We assembled these data with the *in silico* functional reconstruction of the parasite metabolic pathways obtained from the *P. falciparum* annotated genome, exposing new active biosynthetic routes crucial for further glycosylation reactions. Fucose is a sugar present in glycoconjugates often associated with recognition and adhesion events. Thus, the GDP-fucose precursor is essential in a wide variety of organisms. *P. falciparum* presents homologues of GDP-mannose 4,6-dehydratase (GMD) and GDP-L-fucose synthase (FS) enzymes that are active *in vitro*, indicating that most GDP-fucose is formed by a '*de novo*' pathway that involves the bioconversion of GDP-mannose. Homologues for enzymes involved in a fucose salvage pathway are apparently absent in the *P. falciparum* genome. This is in agreement with *in vivo* metabolic labelling experiments showing that fucose is not significantly incorporated by the parasite. Fluorescence microscopy of epitope-tagged versions of *Pf*GMD and *Pf*FS expressed in transgenic 3D7 parasites show that these enzymes localize in the cytoplasm of *P. falciparum* during the intraerythrocytic developmental cycle. Although the function of fucose in the parasite is not known, the presence of GDP-fucose suggests that the metabolite may be used for further fucosylation reactions.

6.3.1 Review 1:

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On cytoadhesion of *Plasmodium vivax*: *raison d'être*?

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It is generally accepted that Plasmodium vivax, *the most widely distributed human malaria parasite, causes mild disease and that this species does not sequester in the deep capillaries of internal organs. Recent evidence, however, has demonstrated that there is severe disease, sometimes resulting in death, exclusively associated with* P. vivax *and that* P. vivax*-infected reticulocytes are able to cytoadhere in vitro to different endothelial cells and placental cryosections. Here, we review the scarce and preliminary data on cytoadherence in* P. vivax, *reinforcing the importance of this phenomenon in this species and highlighting the avenues that it opens for our understanding of the pathology of this neglected human malaria parasite.*

Key words: *Plasmodium vivax* - malaria - cytoadherence - severe disease

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