



**UNIVERSIDAD DEL ROSARIO**



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DE SALAMANCA**

**DETERMINACIÓN DEL PROTEOMA DE LA CEPA VCG-1 DE *Plasmodium vivax* Y  
CARACTERIZACIÓN DE MOLÉCULAS CANDIDATAS PARA SU INCLUSIÓN EN  
EL DESARROLLO DE UNA VACUNA**

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Tesis de Doctorado presentada como requisito para optar a la doble titulación de Doctor en Ciencias Biomédicas y Biológicas de la Universidad del Rosario y Doctor por la Universidad de Salamanca dentro del programa Salud y Desarrollo en los Trópicos.

**BOGOTÁ D.C., 2017**



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**DOCTORADO EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS**

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## **DEDICATORIA**

*A Dios, esencia de la vida.*

*A mis padres y hermanos, ejemplo de perseverancia.*

*A mi esposa e hijo, mi mayor tesoro.*

## **AGRADECIMIENTOS**

A Dios, por su paciencia y misericordia y por haber cumplido los anhelos de mi vida.

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A mi hijo, quien es y será siempre la motivación de mi vida.

A mis padres, por su ejemplo de perseverancia y por ser los gestores de mis sueños.

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## LISTA DE ABREVIATURAS

<b>Abreviatura</b>	<b>Término</b>
AMA-1	Apical Merozoite Antigen
ARP	Asparagine Rich Protein
DARC	Duffy Antigen Receptor for Chemokines
DBL	Duffy Binding-like
DBP	Duffy Binding Protein
EBA	Erythrocyte Binding Antigen
EBL	Erythrocyte Binding-Like
EBP	Erythrocyte Binding Protein
EST	Expressed Sequence Tags
ETRAMP	Early Transcribed Membrane Protein
EXP	Exported Protein
FIDIC	Fundación Instituto de Inmunología de Colombia
GAMA	GPI-Anchored Micronemal Antigen
GPI	Glycosylphosphatidylinositol
HABP	High-Activity Binding Peptide
HMM	Hidden Markov Models
IFI	Inmunofluorescencia Indirecta
INS	Instituto Nacional de Salud
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
JCVI	J. Craig Venter Institute
LC-MS/MS	Liquid Chromatography Coupled to Tandem Mass Spectrometry
LLINs	Long-Lasting Insecticidal Nets
MHC	Major Histocompatibility Complex
MPAC	Malaria Policy Advisory Committee
MSP	Merozoite Surface Protein
OMS	Organización Mundial de la Salud
PBMC	Peripheral Blood Mononuclear Cells
RBSA	Reticulocyte Binding Surface Antigen
RAP	Rhoptry-Associated Proteins
RBL	Reticulocyte Binding-Like
RBP	Reticulocyte Binding Protein
Rh	Reticulocyte Binding Protein Homologue
RON	Rhoptry Neck Protein
SCU	Sangre de Cordón Umbilical
SMC	Seasonal Malaria Chemoprevention
TIGR	The Institute for Genomic Research
TRAg	Tryptophan-Rich Antigen
VCG-1	Vivax Colombia Guaviare 1
WGCF	Wheat Germ Cell-Free System

## RESUMEN

La identificación y caracterización de proteínas que utilizan los merozoitos de *Plasmodium* para invadir a su célula hospedera, representan una estrategia importante para desarrollar un método de control contra estos parásitos. A pesar de ello, la investigación básica en *P. vivax* está retrasada por su difícil propagación *in vitro*, debido a la preferencia que tiene el parásito por invadir reticulocitos, los cuales se encuentran en escaso porcentaje en sangre periférica de humanos adultos (1-2%) y son difíciles de obtener con alta pureza, en suficiente cantidad y totalmente viables. Como consecuencia de lo anterior, el conocimiento del número de moléculas que expresa *P. vivax* y cuáles de ellas son candidatas para componer una vacuna, es escaso.

En este estudio, se evaluó el proteoma de una cepa de *P. vivax* adaptada a primates y se caracterizaron moléculas antigénicas y con capacidad de adhesión a reticulocitos humanos. En el análisis del proteoma de la cepa VCG-1 de *P. vivax*, se detectaron 734 proteínas, algunas esenciales en los pasos clave para establecer la invasión del merozoito a su célula diana. Además, se identificaron 811 componentes de eritrocitos (hospederos vitales de *Plasmodium*) del primate *A. nancymaae*, de los cuales 51 son proteínas integrales de membrana, 7 descritas como receptores de *Plasmodium*. Por otro lado, se identificó la presencia, transcripción y expresión de los genes codificantes de tres moléculas de *P. vivax*: *PvARP*, *PvRBSA* y *PvGAMA*, así como su antigenicidad. De particular interés, se encontró que *PvRBSA* y *PvGAMA* se unen en mayor proporción a reticulocitos que expresan el receptor CD71 de forma abundante (CD71<sup>hi</sup>), lo que sugiere que estas moléculas pueden estar participando en la selección preferencial que tienen los merozoitos de *P. vivax* por los reticulocitos humanos.

Este es el primer estudio en Colombia donde se determina la composición proteica de una cepa de *P. vivax* adaptada a primates, así como la de eritrocitos de *A. nancymaae*. Como resultado más importante, se caracterizaron moléculas de *P. vivax* que son candidatos idóneos a ser evaluados como componentes de una vacuna contra la malaria causada por esta especie parasitaria.

## ABSTRACT

Identifying and characterising proteins which use *Plasmodium* merozoites to invade host cells represents an important strategy for developing a method for controlling these parasites. However, basic *P. vivax* research has been delayed due to difficulties in propagating it *in vitro* as the parasite prefers to invade reticulocytes; there is a low percentage of these in adult human peripheral blood (1%-2%) and they are difficult to obtain with high purity, in a sufficient amount and totally viable. Consequently, knowledge is scarce regarding the amount of molecules being expressed by *P. vivax* and which of them represent good candidates for inclusion in an effective vaccine.

This study has been aimed at evaluating the proteome of a primate-adapted *P. vivax* strain; antigenic molecules able to bind to human reticulocytes have been characterised. Analysing the *P. vivax* VCG-1 strain proteome led to detecting 734 proteins, some of them essential in key steps for establishing merozoite invasion of target cells. Furthermore, 811 *A. nancymaae* primate erythrocyte components (vital *Plasmodium* hosts) were identified; 51 of them were integral membrane proteins, 7 described as *Plasmodium* receptors. The presence, transcription, expression and antigenicity of genes encoding three *P. vivax* molecules (*PvARP*, *PvRBSA* and *PvGAMA*) were identified. Particularly interesting was the finding that a higher percentage of *PvRBSA* and *PvGAMA* bound to reticulocytes abundantly expressing the CD71 receptor (CD71<sup>hi</sup>), thereby suggesting that these molecules could be participating in *P. vivax* merozoite preferential selection for human reticulocytes.

This the first study in Colombia which has determined the protein composition of a primate-adapted *P. vivax* strain as well as *A. nancymaae* erythrocytes. More importantly, *P. vivax* molecules were characterised which appear to be suitable candidates for being evaluated as components of a vaccine against malaria caused by the parasite species.

# INTRODUCCIÓN

## La malaria: epidemiología y agente etiológico.

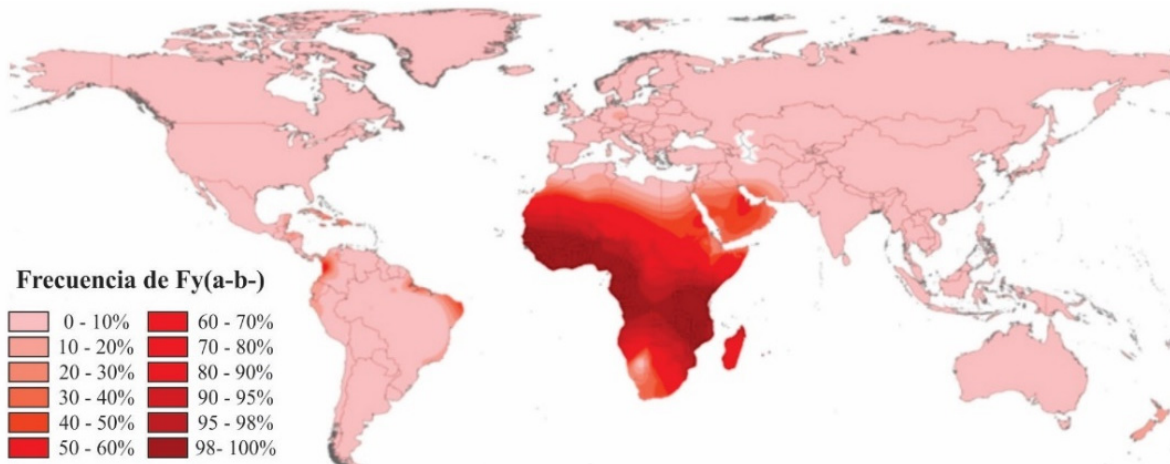
La malaria es una enfermedad parasitaria que continúa siendo una de las más importantes en el mundo, ya que afecta la salud de miles de individuos que viven en países localizados en regiones tropicales y sub-tropicales (1). De acuerdo con la Organización Mundial de la Salud (OMS), 104 países son endémicos de malaria (Figura 1), de los cuales 97 de ellos presentan transmisión y siete están en la fase de prevención de reintroducción desde el año 2013 (1). En el año de inicio de esta investigación, se registraron 207 millones de infecciones por paludismo y 627.000 muertes de niños y adultos en todo el mundo, principalmente en África, áreas de Oriente Medio y Asia (1). En la actualidad, aunque el estimado de muertes por la enfermedad disminuyó a 429.000, esta cifra sigue siendo alarmante (2). A lo anterior se suma que el parásito está desarrollando resistencia frente a medicamentos anti-maláricos (3, 4) y además, hay un incremento de la resistencia a insecticidas por los vectores transmisores (5).



Figura 1. Países endémicos de malaria según la OMS.

La enfermedad es causada en humanos por cinco especies de protozoos pertenecientes al género *Plasmodium*: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* y *Plasmodium knowlesi*. La primera de ellas es la más importante por su

letalidad y predominio, principalmente en África (1), mientras que *P. vivax* causa una morbilidad alta en Asia y América, lo cual se debe en parte, a que la presencia del antígeno Duffy (Fy), esencial para la invasión de *P. vivax*, es poco común en los africanos (6) (Figura 2), mientras que es más frecuente en individuos que viven en zonas tropicales de Sudamérica y el Sudeste asiático.



**Figura 2.** Distribución global del fenotipo Duffy negativo. Imagen modificada de Howes *et al.*, 2011 (6).

Según la literatura, 2,85 billones de personas se encuentran en riesgo de contraer la infección por *P. vivax*; 91% (2,59 billones) viven en el Centro y Sureste de Asia, 5,5% (160.000 millones) en América y 3,5% (100.000 millones) en el continente africano (7). Price y su grupo reportaron que *P. vivax* genera entre 132 a 391 millones de casos nuevos anualmente (8). Aunque se piensa que *P. vivax* es una especie causante de enfermedad benigna, durante los últimos 15 años se han incrementado los reportes de casos de infección grave y muerte por el parásito, principalmente en India, USA, Indonesia y Pakistán (9).

En Colombia, según el informe del Instituto Nacional de Salud (INS) de Bogotá, se notificaron 288.200 casos de malaria en total durante los últimos 5 años, de los cuales, el 55,1% corresponden a *P. vivax* (10). Los departamentos con mayor número de casos de paludismo causado por dicha especie fueron Antioquia, Chocó, Córdoba, Bolívar y Nariño. Hasta la fecha se notificaron 16 casos de malaria grave por *P. vivax* en los municipios Colombianos de Tumaco, Cali y Buenaventura (11). A pesar de lo anterior, los reportes epidemiológicos, la carga global y la gravedad de la enfermedad son subestimados, debido a la falta de notificación de los casos clínicos en los sistemas de información del país (12).

## Prevención y control

El comité asesor de políticas para controlar la malaria de WHO (MPAC: *Malaria Policy Advisory Committee*) estableció varias estrategias para la prevención del paludismo (1). Dentro de ellas, se encuentra el uso de mosquiteras impregnadas con insecticidas de larga duración (LLINs: *Long-Lasting Insecticidal Nets*), la fumigación residual de interiores (IRS: *Indoor Residual Spraying*), la creación de sedes entomológicas para estudiar y controlar el vector, el tratamiento intermitente preventivo (IPT: *Intermittent Preventive Treatment*), la quimioprevención de malaria estacional (SMC: *Seasonal Malaria Chemoprevention*) y el diagnóstico y tratamiento oportuno (*Diagnosis and treatment of malaria*) (1). El uso de dichas estrategias redujo la incidencia y mortalidad de la enfermedad en zonas endémicas en un 21% y 29% respectivamente, en todos los grupos de edad, y la mortalidad en niños menores de 5 años en un 35% (2). Pese a ello, la cantidad de casos de paludismo continúan siendo alarmantes, más aún si se tiene en cuenta que la población neonatal es la principalmente afectada.

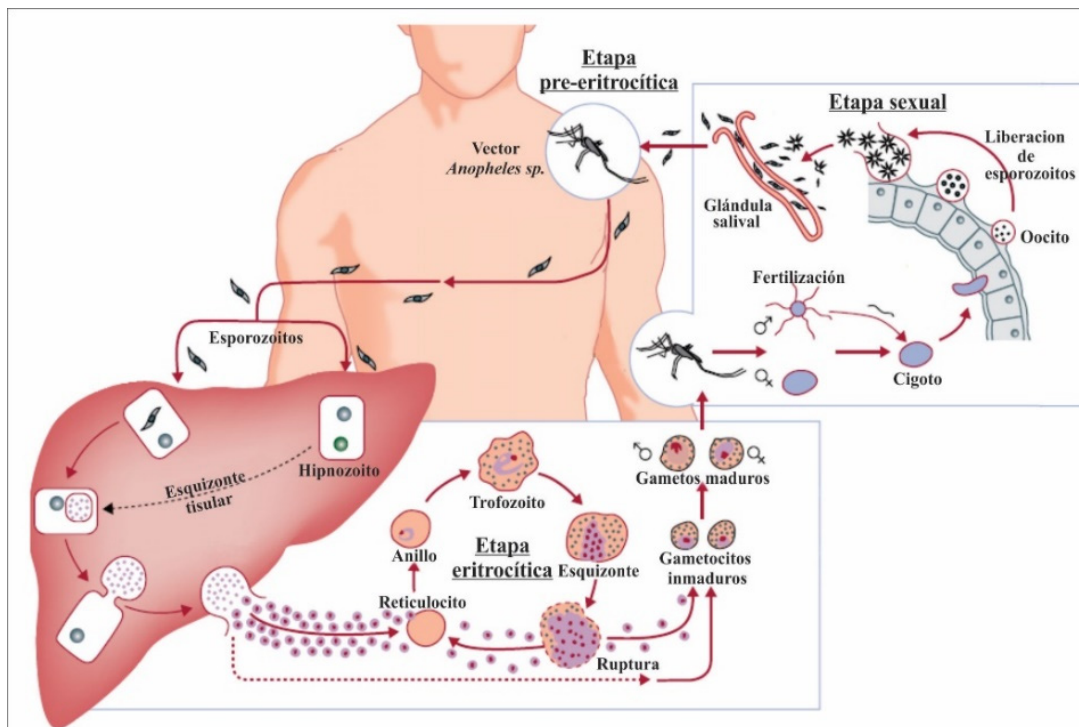
Dado que las estrategias de control y prevención de la malaria no son suficientes, distintos grupos de investigación concentran sus esfuerzos en desarrollar una vacuna, la cual es considerada como una alternativa eficiente. Este enfoque mostró ser útil en el control y erradicación de enfermedades infecciosas como ocurrió en el caso de la vacunación contra la poliomielitis, la cual redujo la incidencia global en un 99.9% entre los años 1998-2002 (13). Por lo tanto, para poder desarrollar una vacuna contra la malaria, es necesario conocer el ciclo de vida del parásito y establecer los puntos de intervención apropiados.

## Ciclo de vida de *Plasmodium*

La infección en humanos por parásitos del género *Plasmodium* se inicia tras la inoculación de 30 a 200 formas infectivas denominada esporozoitos, a través de la picadura de un mosquito hembra del género *Anopheles* (Figura 3) (14). Durante dicho estadio denominado **pre-eritrocítico**, los esporozoitos se transportan en el torrente sanguíneo e invaden los hepatocitos, en los cuales, el parásito se replica en miles de merozoitos por célula, dando lugar a la formación de esquizontes tisulares. Una particularidad de *P. vivax*, es que éste puede permanecer en estado latente en las

células hepáticas (forma conocida como hipnozoito) y puede reactivarse aún después de haber desaparecido el parásito de la sangre (Figura 3) (15, 16).

Posteriormente, se produce la ruptura de los esquizontes hepáticos y la liberación de los merozoitos al torrente sanguíneo, los cuales rápidamente invaden los eritrocitos (o reticulocitos en el caso de *P. vivax*), dando inicio a la **etapa eritrocítica**. Una vez infectado el eritrocito, los parásitos inician una división asexual para generar nuevas formas parasitarias, pasando por la etapa de anillo, trofozoito y finalmente esquizonte, la cual dura aproximadamente 48-72 horas dependiendo de la especie parasitaria. En esta última etapa, las células hospederas se rompen y liberan nuevos merozoitos con capacidad infectiva, los cuales repetirán el proceso de invasión, multiplicándose de forma exponencial.



**Figura 3.** Ciclo de vida de *Plasmodium*. Imagen modificada de Mueller *et al.*, 2016 (14).

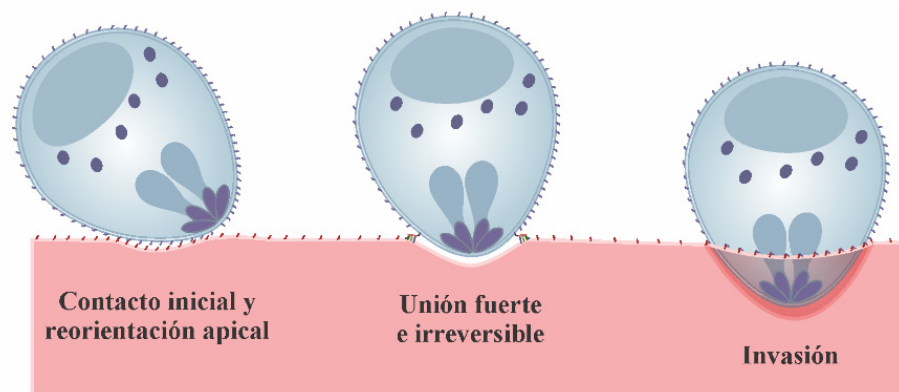
Por otro lado, algunos merozoitos se diferencian sexualmente en gametos maduros masculinos o femeninos (**etapa sexual**). En *P. vivax*, estos se generan antes que los individuos infectados sean sintomáticos, lo que favorece la transmisión temprana del parásito (14). Los merozoitos diferenciados sexualmente son ingeridos por el vector cuando se alimenta, y dentro de éste, se da el ciclo sexual que involucra la fertilización de los gametos y el paso del cigoto a través



del intestino medio. Posteriormente, el cigoto se transforma en un oocito adherente, dentro del cual se forman esporozoitos que son liberados y migran a las glándulas salivales del mosquito, los cuales serán posteriormente inoculados durante una nueva picadura (Figura 3).

### **Invasión de los merozoitos de *Plasmodium* a los eritrocitos**

Durante esta etapa ocurre la multiplicación de los merozoitos, y su liberación está directamente relacionada con la sintomatología clínica y la gravedad de la enfermedad. Este proceso ha sido ampliamente estudiado en *P. falciparum*, dado que se tiene establecido un cultivo continuo *in vitro* para este parásito. Según diversos estudios, la invasión del merozoito a la célula ocurre rápidamente (en menos de 40 segundos) y se divide en varias fases: contacto inicial y reorientación hacia el polo apical, formación de una unión fuerte e irreversible y, por último, la invasión a los eritrocitos (Figura 4) (17). Durante dichas fases, participan numerosas proteínas del merozoito presentes en su superficie o secretadas por los órganos incluidos en el complejo apical (roptrias y micronemas), las cuales se unen a los componentes integrales de la membrana de los eritrocitos, para facilitar la invasión (18, 19).



**Figura 4.** Fases de invasión de merozoitos de *Plasmodium* a eritrocitos humanos. Imagen modificada de Koch *et al.*, 2016 (18).

#### ***Contacto inicial y reorientación apical.***

La unión inicial del merozoito a la superficie del eritrocito es un proceso reversible y de baja afinidad, mediado por moléculas pertenecientes a la familia de proteínas de la superficie del merozoito (MSPs: *Merozoite Surface Proteins*). La proteína más abundante y mejor estudiada es MSP-1, la cual sirve de plataforma para el ensamblaje de un complejo molecular compuesto por

otras proteínas de superficie como MSP-6, MSP-7, MSP-9 y MSPDBL (*Duffy binding-like*) (20, 21). Estas moléculas se exponen a anticuerpos cuando el merozoito se libera al torrente sanguíneo y, al ser polimórficas, ayudan al parásito a evadir la respuesta inmune del hospedero. El bloqueo de la unión del merozoito a su célula diana, utilizando anticuerpos dirigidos contra MSP-1, valida la función que desempeña la molécula durante la invasión (19).

### ***Formación de unión fuerte e irreversible.***

Las proteínas que participan en esta fase son adhesinas provenientes de las roptrias y micronemas, las cuales tienen una mayor afinidad por los receptores celulares. Algunas moléculas vinculadas en este proceso pertenecen a la familia de las EBAs (*Erythrocyte Binding Antigens*), localizadas en micronemas, y las Rhs (*Reticulocyte Binding Protein Homologues*), localizadas en roptrias, que son liberadas cuando se inicia el contacto merozoito-célula diana. Estas moléculas permiten a *P. falciparum* invadir las células a través de rutas alternas, lo que le confiere al parásito un mecanismo para contrarrestar la respuesta inmune humoral del hospedero (22).

Por otro lado, se conoce un complejo multimérico esencial en la invasión, dado que establece la interacción fuerte e irreversible entre el merozoito y su célula diana, conformado por el antígeno apical de membrana 1 (AMA1: *Apical Membrane Antigen 1*) y algunas proteínas del cuello de las roptrias (RON: *Rhoptry Neck Proteins*) (RON2, RON4 y RON5). La depleción genética de AMA-1 es letal para *P. falciparum*, siendo esta molécula crítica en la invasión parasitaria (23). Rh5 es otra molécula importante, debido a que la eliminación del gen es letal para el parásito (24) y además, se ha demostrado que anticuerpos dirigidos contra la proteína, logran bloquear la invasión de múltiples cepas de *P. falciparum* a eritrocitos humanos (25).

### ***Invasión a los eritrocitos.***

Una vez establecida la unión fuerte, el contenido proteico del complejo apical es liberado por un mecanismo aún desconocido, y se inicia la formación de la vacuola parasitófora, a medida que el parásito se internaliza dentro de la célula diana (17).

Según lo descrito hasta ahora, las proteínas más importantes para el contacto inicial e invasión del parásito a los eritrocitos se localizan en la superficie o en los organelos apicales. En

este sentido, es importante conocer las proteínas que expresa *P. vivax* al final del ciclo intraeritrocítico y, principalmente, aquellas que se unen a la célula hospedera, con el fin de establecer estrategias para controlar su adhesión e invasión.

### Ciencias ómicas en *P. vivax*

En los últimos años, ha surgido gran interés por obtener datos genéticos y moleculares de los microorganismos, mediante el uso de las ciencias ómicas. Estas herramientas son de utilidad para conocer la biología y entender la compleja maquinaria que utilizan algunos microorganismos para invadir sus células diana. Parte de la investigación a nivel mundial para el desarrollo de vacunas contra la malaria, se enfoca principalmente en *P. falciparum*, la cual es soportada de forma importante por el desarrollo del cultivo *in vitro* del parásito, sumado a los estudios de identificación a gran escala de genes (26), transcritos (27) y proteínas (28, 29). Con base en la caracterización de moléculas que permitieron los estudios anteriores, se pudo avanzar en la determinación del papel que juegan algunas de ellas en el proceso de invasión de *P. falciparum* a los eritrocitos y, como consecuencia, se han evaluado como candidatas a vacuna en estudios clínicos (30).

En el caso de *P. vivax*, la investigación orientada a conocer su biología está notablemente retrasada, debido en gran parte a la difícil propagación del parásito *in vitro*, por la preferencia que tiene de invadir reticulocitos, los cuales son escasos en sangre periférica de humanos adultos (1-2%) y difíciles de obtener de forma viable y en suficiente cantidad (31). Como consecuencia de lo anterior, los estudios enfocados a determinar el genoma, transcriptoma y proteoma en *P. vivax* no tienen la misma cronología con respecto a la investigación en *P. falciparum* (Tabla 1).

**Tabla 1.** Cronología de la investigación en ciencias ómicas de *P. vivax* vs *P. falciparum*.

Tipo de estudio/Especie	<i>P. falciparum</i> - Año (Ref)	<i>P. vivax</i> - Año (Ref)
<b>Genoma</b>	2002 (26)	2008 (32)
<b>Transcriptoma</b>	2003 (27)	2005 (33), 2008 (34)
<b>Proteoma</b>	2002 (28,29)	2009 (35), 2011 (36, 37)

La secuenciación completa del genoma de *P. vivax* realizada por el *J. Craig Venter Institute* (JCVI) permitió hacer estudios comparativos *in silico* entre las diferentes especies de *Plasmodium* (32). Este estudio fue el punto de partida para conocer la mayoría de genes homólogos entre las

especies de *Plasmodium*, nuevas familias de genes en *P. vivax*, y algunos de los ligandos de unión más relevantes, pertenecientes a la familia de genes codificantes de las RBPs (*Reticulocyte binding proteins*). Estos hallazgos soportan la idea de que *P. vivax* puede usar rutas de invasión adicionales, al igual que lo hacen los parásitos *P. falciparum* y *Plasmodium yoelii* (32).

En transcriptómica hay dos estudios. Cui y su grupo construyeron una librería de ADNc proveniente de parásitos de *P. vivax* mezclados en los distintos estadios sanguíneos (anillos, trofozoitos y esquizontes), para conocer los genes codificantes de la especie y acelerar su anotación (33). Al comparar las secuencias expresadas (EST: *Expressed Sequence Tags*) con la base de datos pública del Instituto para Investigación en Genómica (TIGR: *The Institute for Genomic Research*), encontraron que la mayoría de sus secuencias eran similares a las de *P. falciparum*. Más adelante, Bozdech y su grupo cultivaron el parásito en forma sincrónica para estudiar la expresión estadio-específica de los genes (34). El estudio permitió conocer los genes que se expresan de manera abundante en las distintas etapas de desarrollo intraeritrocítico. De interés, se encontró una expresión diferencial de las RBPs según el aislado analizado y, además, se identificaron algunas proteínas homólogas a las de otras especies de *Plasmodium* para las cuales había evidencia experimental de su función de adhesión celular.

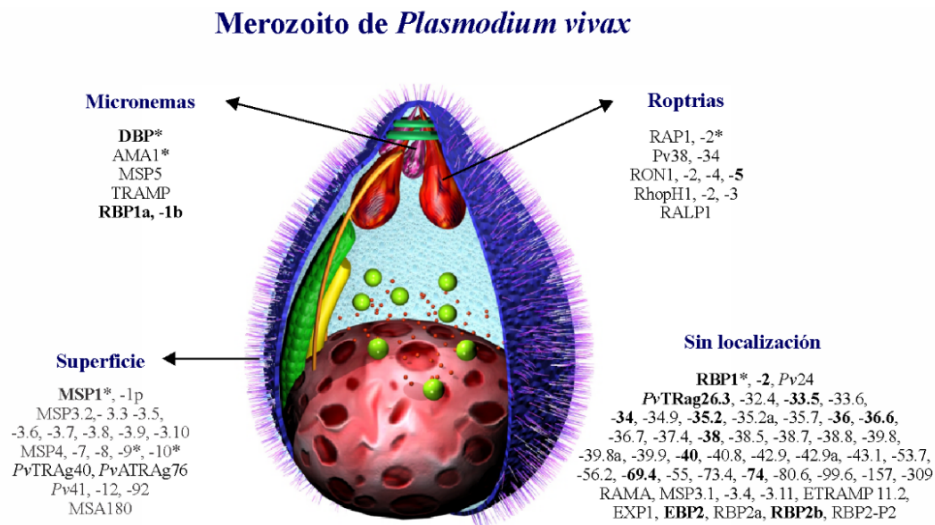
En el estudio de proteoma de esquizontes de *P. vivax*, se utilizó la técnica de separación de proteínas en 2D y su posterior análisis por espectrometría de masas (MS: *Mass Spectrometry*). En un primer estudio, Acharya y su grupo, identificaron 154 proteínas en parásitos de *P. vivax* provenientes de aislados clínicos; algunas fueron proteínas hipotéticas, otras enzimas metabólicas, chaperonas y moléculas involucradas en virulencia (37). En el mismo año, Roobsoong y colaboradores, identificaron 316 proteínas en muestras de parásito extraídas de pacientes sintomáticos; las funciones encontradas más comunes fueron: unión, síntesis, metabolismo y transporte celular (36). Cabe destacar que, al momento de iniciar esta investigación, no había ningún reporte del proteoma de parásitos de *P. vivax* adaptados a primates no humanos.

La inmunoproteómica se usó en los últimos años para determinar la capacidad de varias proteínas para desencadenar una respuesta antigénica durante la infección natural. Usando la tecnología de microarreglos de proteínas obtenidas mediante el sistema WGCF (*Wheat Germ Cell-Free System*), Chen y su grupo reportaron la inmunoreactividad frente a 18 proteínas (de las cuales

11 no tenían evidencia funcional) de un total de 86 (38), mientras que el grupo de Lu mostró la reactividad frente a 44 moléculas de 152 (39). Las moléculas inmunoreactivas se consideran relevantes para evaluarlas como componentes de una vacuna, dado a que éstas logran desencadenar una respuesta inmune natural, que a menudo se encuentra relacionada con la disminución de la parasitemia (22). Por todo lo expuesto, los anteriores estudios son de gran utilidad, debido a que ofrecen información acerca de las proteínas que generan una respuesta inmune en *P. vivax*, pudiendo así ser consideradas como candidatas a vacuna.

### Proteínas caracterizadas en *P. vivax*

Hasta el año 2012, se había reportado la caracterización de 26 moléculas de la fase intraeritrocítica (Figura 5) (40). En general, las primeras moléculas de *P. vivax* caracterizadas se seleccionaron según su homología a las de otras especies de *Plasmodium*, cuyo papel en invasión celular se había determinado experimentalmente. Sin embargo, recientemente se tienen en cuenta otros criterios como la elevada expresión al final del ciclo de vida intraeritrocítico (mayor a 35 horas), la predicción de un péptido señal o localización extracelular, la presencia de dominios de interacción entre proteínas y/o secuencias transmembranales o de anclaje a GPI (41). En cuanto a la validación por biología molecular, se utiliza el parásito obtenido de sangre de pacientes infectados naturalmente o de primates infectados experimentalmente.



**Figura 5.** Proteínas del estadio intraeritrocítico de *P. vivax* caracterizadas a la fecha. Los asteriscos indican las moléculas evaluadas previamente a nivel pre-clínico. Los nombres en negrita corresponden a las moléculas con capacidad de unión a reticulocitos.

En la actualidad, se han reportado 87 proteínas de *P. vivax*, excluyendo las caracterizadas en este trabajo: algunas son miembros de las familias multigénicas como las MSPs (42-49), TRAGs (*Tryptophan Rich Antigens*) (50-54), EXPs (*Exported Proteins*), ETRAMPs (*Early Transcribed Membrane Proteins*), así como también las RBPs (*Reticulocyte Binding Proteins*) (55-57) y DBPs (*Duffy Binding Proteins*) (58, 59), relacionadas a las Rh y EBAs de *P. falciparum*, respectivamente, y por último las RONS (60-63) (Figura 5).

### ***Proteínas de P. vivax con actividad de unión a reticulocitos.***

A pesar del avance logrado en la caracterización de moléculas de *P. vivax*, la función de unión a eritrocitos se ha descrito para pocas de ellas. A continuación, se describen las distintas técnicas que se utilizan para estudiar la interacción entre las proteínas de *P. vivax* (Figura 5) y los reticulocitos humanos. La primera aproximación para determinar la unión de moléculas de *P. vivax* a las células fue mediante el ensayo de rosetas. DBP fue la primer molécula de *P. vivax* estudiada, de la cual se conocía su interacción con eritrocitos que expresan el antígeno Duffy sobre su superficie, también conocido como DARC (*Duffy Antigen Receptor for Chemokines*) (64, 65). DBP se expresó en varios fragmentos sobre la superficie de células COS-7, las cuales se incubaron con una muestra de sangre de humanos adultos. Como resultado, se encontró que los eritrocitos Duffy positivo eran capaces de unirse sólo a las células COS-7 que expresaban la región II de la proteína DBP (DBP-RII), considerándose como el dominio de unión de la molécula (66).

Años más tarde, se estudiaron las interacciones proteína-célula diana mediante un ensayo de unión a eritrocitos *in vitro* (EBA: *Erythrocyte-Binding Assay*). En resumen, proteínas marcadas con metionina <sup>36</sup>S, provenientes del sobrenadante de un cultivo de merozoitos de *P. vivax*, se incubaron con dos tipos de muestras: una enriquecida y otra depletada de reticulocitos. El análisis mediante SDS-PAGE y fluorografía de las proteínas eluidas con un *buffer* salino, permitió identificar dos bandas de alto peso molecular (250 y 280 kDa), cuya intensidad se correlacionaba con el porcentaje de reticulocitos de cada muestra independiente del fenotipo Duffy, y fueron denominadas RBP-1 y RBP-2. Con este hallazgo, los autores propusieron que los merozoitos de *P. vivax* contienen otras adhesinas diferentes a DBP, que interactúan con los reticulocitos humanos (55).

Posteriormente, se usó una estrategia altamente sensible para identificar los fragmentos de las moléculas de *P. vivax* que se unen específicamente a sus células hospederas. Esta metodología implicó la síntesis de proteínas parasitarias en péptidos de 20 aminoácidos de largo, y su posterior incubación con eritrocitos de un individuo sano o un individuo que sufría de una  $\beta$ -talasemia, y cuyo porcentaje de reticulocitos en sangre periférica excedía el 85%. Los ensayos de competición en los que se usaron péptidos radiomarcados con  $^{125}\text{I}$ , permitieron encontrar péptidos de alta capacidad de unión (HABPs: *High Activity Binding Peptides*) específica para reticulocitos. Así mismo, se identificaron los residuos críticos en la unión de los HABPs, mediante un cribado con análogos de glicina. De esta manera, se demostró que DBP, MSP-1 y RBP-1 contienen varios péptidos que se unen fuertemente a reticulocitos y que tienen residuos críticos en la unión (67-69), lo que soporta que el parásito utiliza regiones específicas de sus moléculas, para poder interactuar con los receptores de sus células diana. Pese a lo anterior, no se pudo continuar con este enfoque, debido a la dificultad de obtener muestras enriquecidas en reticulocitos.

En la actualidad, se evalúa la interacción proteína-célula diana con técnicas como inmunoprecipitación, inmunofluorescencia indirecta (IFI) o citometría de flujo. En el primer caso, un fragmento de 33 kDa de la proteína PvRON5 expresado en *Escherichia coli* y purificado (rPvRON5-33kDa), se incubó con una muestra de reticulocitos enriquecidos mediante un gradiente de Percoll. Posteriormente, rPvRON5-33kDa se eluyó con cloruro de sodio, se inmunoprecipitó utilizando anticuerpo anti-histidinas y perlas de sefarosa conjugadas con proteína G, evaluándose por Western blot. Por otro lado, rPvRON5-33kDa se incubó con una muestra de sangre de cordón umbilical que contiene 5-6% de reticulocitos, y la interacción proteína-célula diana se determinó por IFI usando los anticuerpos anti-histidinas (marcador de la proteína) y anti-CD71 (marcador de reticulocitos). Los resultados de Western blot y microscopía, revelaron que rPvRON5-33kDa se une a eritrocitos maduros e inmaduros. Sin embargo, el análisis de la intensidad media de fluorescencia asociada a las células, mostró mayor unión de rPvRON5-33kDa a reticulocitos, lo que soporta que la molécula está involucrada en la adhesión del merozoito de *P. vivax* a células rojas inmaduras (60).

En el caso de la citometría de flujo, se utilizó sangre de cordón umbilical total o reticulocitos enriquecidos por *sorting* inmunomagnético con perlas CD71 o solución de Nycodenz.

La interacción proteína-célula diana se determinó usando Reticount (marcador de ARN/ADN) y el anticuerpo anti-histidinas acoplado a Alexa fluor 647. Como resultado, se ha descrito la unión a reticulocitos para algunas moléculas como: TRAgS (*PvTRAg* (TRAg40), *PvTRAg26.3*, *PvTRAg33.5*, *PvTRAg34*, *PvTRAg35.2*, *PvTRAg36*, *PvTRAg36.6*, *PvTRAg38*, *PvTRAg69.4* y *PvTRAg74* (50, 51)), EBP2 (*Erythrocyte Binding Protein 2*) (59), RBP-1a, RBP-1b (56) y RBP-2b (57).

### **Vacunas contra *P. vivax***

Cien años después del inicio de la vacunología por Edward Jenner, la “prueba de concepto” se estableció con la investigación de Louis Pasteur, quien demostró que la inoculación de un microorganismo atenuado o inactivado en humanos, conlleva al desarrollo de una respuesta inmune (similar a la natural) que protege contra la infección (70). Actualmente, las vacunas se agrupan en tres categorías de acuerdo a la metodología empleada: de primera, segunda y tercera generación, siendo las de primera generación aquellas que emplean el microorganismo completo (70). Siguiendo la misma metodología para *P. vivax*, Clyde y su grupo inmunizaron un individuo con esporozoitos de la especie mediante la picadura de 1.000 mosquitos irradiados, los cuales desencadenaron una respuesta protectora de corta duración contra la enfermedad, mostrando así su seguridad e inmunogenicidad (71). A pesar de lo anterior, varias dificultades técnicas se presentan al trabajar con este tipo de vacunas de primera generación para *P. vivax*: no se cuenta con una fuente rica en gametocitos que pueda usarse como precursor de esporozoitos viables, es difícil estimar la cantidad de esporozoitos requerida para desarrollar una respuesta inmune protectora, la producción en masa y preservación de la vacuna continúa siendo un reto, y por último, el procedimiento no es práctico, dado que involucra la picadura por más de 1.000 mosquitos en el individuo.

Las vacunas de segunda generación están compuestas por moléculas purificadas que desencadenen una fuerte respuesta inmunológica protectora (70). Esta metodología no ha tenido éxito en *Plasmodium*, dada la alta variabilidad antigénica de los parásitos (40, 72). Del total de proteínas descritas en la fase intraeritrocítica de *P. vivax*, tan solo 7 de ellas (DBP, AMA-1, MSP-1, MSP-9, MSP-10, RBP-1 y RAP2 (*Rhoptry-Associated Protein*)) han sido evaluadas en primates o roedores (Figura 5) (73-81), y ninguna ha sido ensayada todavía a nivel clínico.



Se demostró que anticuerpos dirigidos contra DBP-RII, generados en el modelo murino o primate, son capaces de inhibir la unión del dominio de la proteína al receptor *in vitro* (74, 77). Además, dos epítomos de DBP inducen una respuesta de tipo Th1/Th2 (con secreción de IFN- $\gamma$  e IL-6) en individuos que tienen malaria (82, 83). En cuanto a MSP-1, dos fragmentos emulsificados en adyuvante de Freund desencadenaron una respuesta inmune protectora que osciló entre el 50% y 80% en los animales inmunizados, la cual se relaciona con altos títulos de anticuerpos y elevados niveles de producción de IFN- $\gamma$  (73, 81). AMA-1 generó una fuerte respuesta inmune humoral en ratones BALB/c al ser formulada en adyuvante de Freund, hidróxido de aluminio, Quil A, Saponina QS-21, CpG ODN 1826 o TiterMax (75).

La inmunización de monos *Aotus* con la región recombinante III de RBP-1, formulada con adyuvante de Freund, desencadenó la producción de anticuerpos y estimulación de linfocitos T; pese a ello, la respuesta inmune no fue protectora frente al reto experimental con la cepa VCG-1 de *P. vivax* (79). Por otro lado, la vacunación de monos con la proteína MSP-10 fue inmunogénica al formularse en adyuvante de Freund, Montanide ISA 720 o hidróxido de aluminio. Sin embargo, tampoco protegió frente al reto experimental (76).

Un ensayo realizado con RAP2 recombinante, mostró protección parcial de los monos, representada en la baja parasitemia encontrada en el grupo de primates inmunizados con respecto al grupo control (80). Por otro lado, dos recombinantes de MSP-9, una que incluye la región N terminal (PvMSP-9-Nt) y otra que contiene un bloque repetitivo (MSP-9-RepII), se inocularon en roedores (78). Aunque ambas formulaciones fueron inmunogénicas, tan solo aquella que contenía la región N-terminal estimuló la producción de INF $\gamma$  e IL-5 en células mononucleares de sangre periférica (PBMC: *Peripheral Blood Mononuclear Cells*) de los animales inmunizados.

La tercera generación de vacunas se basa en tecnologías como vacunología reversa, biología estructural y vacunas sintéticas (70). Estas últimas, se componen de proteínas o fragmentos de ellas que utiliza el parásito para unirse a su célula diana y se caracterizan por ser multivalentes, dado que incluyen más de un antígeno. Esta metodología ha sido el enfoque principal de la Fundación Instituto de Inmunología de Colombia (FIDIC) desde hace más de 30 años. La investigación permitió establecer los principios para el diseño lógico y racional de vacunas sintéticas multi-antigénicas y multi-estadio basadas en subunidades contra *P. falciparum*

(72, 84). A diferencia del enfoque clásico, la metodología seguida por la FIDIC involucra la identificación de HABPs, los cuales son conservados entre las distintas cepas de parásito. Posteriormente, los residuos críticos de unión a los eritrocitos son modificados para permitir un mejor anclaje en el complejo mayor de histocompatibilidad de clase II (MHC: *Major Histocompatibility Complex*) y así volverlos altamente inmunogénicos e inducir una respuesta protectora en el modelo experimental (72). Mientras que en *P. falciparum* se han analizado más de 58 moléculas siguiendo la metodología previamente descrita, en *P. vivax* sólo se han estudiado las proteínas DBP (69), MSP-1 (67) y RBP-1 (68), debido a la dificultad de obtener reticulocitos puros y en suficiente cantidad para realizar los ensayos de unión.

## PREGUNTAS DE INVESTIGACIÓN

De acuerdo a lo descrito anteriormente, la investigación realizada en *P. vivax*, en relación a la composición proteica del parásito, es mínima en comparación con lo reportado para *P. falciparum*. Por otro lado, la mayoría de moléculas de *P. vivax* identificadas por bioinformática y caracterizadas por biología molecular, carecen de validación experimental en cuanto a función de unión a las células diana. Por lo tanto, nos planteamos las siguientes preguntas:

1. ¿Es similar el número de proteínas expresadas por *P. vivax* durante el ciclo intraeritrocítico, al referido para *P. falciparum*?
2. ¿Se ha comprobado experimentalmente que las moléculas seleccionadas por predicción bioinformática, tienen características adecuadas para considerarlas como buenas candidatas a vacuna?

Para dar respuesta a las preguntas de investigación, nos planteamos un objetivo general que se desarrolló en tres objetivos específicos.

## OBJETIVOS

### Objetivo general

Estudiar la composición proteica de la cepa VCG-1 de *Plasmodium vivax* y caracterizar algunas moléculas con posible función en invasión a reticulocitos.

### Objetivos específicos

Determinar el proteoma de la cepa VCG-1 de *P. vivax*.

Seleccionar y caracterizar moléculas con potencial papel en invasión celular.

Evaluar la antigenicidad de las proteínas caracterizadas durante la infección natural por *P. vivax*.

## INTRODUCCIÓN A LOS CAPÍTULOS

Las enfermedades infecciosas son un problema de salud pública importante por las altas tasas de mortalidad que generan, principalmente en países en vías de desarrollo. Dentro de éstas, la malaria es la tercera causa de muertes en el mundo (1) y aunque se han desarrollado varias estrategias de control, los índices de morbi-mortalidad continúan siendo excesivamente altos, lo que ha llevado a varios grupos de investigación a enfocarse en desarrollar vacunas como una alternativa para prevenir la enfermedad.

El primer paso clave para poder hacer una vacuna, es identificar y caracterizar las proteínas involucradas en la unión de merozoitos de *Plasmodium* a sus células diana y posteriormente inhibir dicha interacción parásito-célula. Pese a lo anterior, el proceso de adhesión celular e invasión de *P. vivax* a sus células diana es poco conocido. Por lo tanto, se han llevado a cabo dos enfoques con la finalidad de conocer en profundidad la biología de *P. vivax*: el análisis mediante ciencias ómicas y la predicción *in silico* y validación por biología molecular de las proteínas candidatas a vacuna.

Dentro de las técnicas altamente eficientes empleadas para identificar cientos de proteínas, se encuentra la proteómica (85); esta estrategia permite hacer análisis a gran escala de la composición de diversos organismos o microorganismos, sirviendo además de fuente para realizar predicciones *in silico* de la función que estas proteínas puedan tener. A la fecha de inicio de este trabajo, este enfoque había permitido la identificación de tan solo un tercio de las moléculas que *P. vivax* expresa en su estadio intraeritrocítico (36, 37), comparado con lo reportado previamente para *P. falciparum* (28).

En cuanto a la caracterización de proteínas en la especie más estudiada, *P. falciparum*, se cuenta con la descripción de 58 moléculas que se expresan al final del ciclo intra-eritrocítico y que están implicadas en la invasión a los eritrocitos (84). En el caso de *P. vivax*, mediante los estudios comparativos con otras especies de *Plasmodium* y la adaptación de varias cepas del parásito en primates, se habían podido describir 25 moléculas al momento de iniciar esta investigación, de las cuales solamente 3 tenían evidencia de participar en la adhesión celular (36, 39, 42-49, 52-55, 58, 61-63, 86-96) (Anexo 1). Esto se debe a la carencia de un cultivo *in vitro* que soporte el crecimiento continuo del parásito, lo cual, no solo afecta al avance en el conocimiento de la biología de *P.*

*vivax* en cuanto a las proteínas relacionadas con la invasión celular, sino también al desarrollo de una vacuna contra esta especie de *Plasmodium* (40).

Teniendo claro que el conocimiento de la biología de *P. vivax* es importante para entender el mecanismo de invasión del parásito a su célula hospedera, en este proyecto se propuso estudiar la composición proteica de la cepa VCG-1 de *P. vivax* y caracterizar otras moléculas (distintas a las ya identificadas) que fuesen interesantes candidatas para evaluar su utilidad a futuro, en el diseño de una vacuna contra el parásito. Para abordar los componentes descritos y cumplir con el objetivo general de esta investigación, se desarrollaron tres objetivos específicos, cuyos resultados se describen a continuación, en tres secciones que comprenden la introducción a los capítulos.

### **Proteómica en *P. vivax***

Según la literatura, en el año 2013 se habían identificado 457 proteínas en total en *P. vivax* mediante proteómica e inmunoproteómica, lo cual era poco más de un tercio de las moléculas detectadas para *P. falciparum* en los diferentes estadios intraeritrocíticos del parásito (1.289 moléculas, de las cuales 714 son del estadio asexual, 931 de gametocitos (célula sexual germinal) y 645 de gametos (célula sexual madura), siendo 651 moléculas comunes entre estadios) (28). Para investigar si *P. vivax* expresa un número similar de moléculas a las reportadas para *P. falciparum* y dar respuesta a la primera pregunta de investigación, se analizó el proteoma de la cepa VCG-1 de *P. vivax* propagada en primates, cuyos resultados se describen en el **capítulo 1** de este documento. Este objetivo se desarrolló analizando los péptidos de las proteínas parasitarias por LC-MS/MS. Como resultado, se detectaron 1.309 moléculas en total, 56,1% de *P. vivax* y 43,2% de primates.

En comparación con estudios previos de proteoma e inmunoproteoma (35-39), se detectaron 504 moléculas nuevas y 230 en común, lo que permitió incrementar el número de proteínas identificadas en *P. vivax* a 960 en total. Es de gran interés que el 27% de las proteínas detectadas participan en procesos clave de invasión como el contacto inicial con eritrocitos, reorientación y formación de la unión estrecha e internalización del parásito dentro de su célula. Según el análisis *in silico*, se predijeron 16 proteínas que participan en una sola ruta metabólica del parásito y no tienen ortólogos en humanos. La carencia de este grupo de proteínas en

mamíferos, las hace una diana ideal para el diseño de nuevos medicamentos antimaláricos. Por otra parte, se identificaron 31 proteínas con posible papel en la invasión celular, de las cuales 7 habían sido caracterizadas previamente por la FIDIC, mediante técnicas bioquímicas y de biología molecular.

Por otro lado, el conocimiento de las características proteómicas de los eritrocitos de *A. nancymaae* representa un área fascinante de estudio, ya que estas células son la principal diana de invasión de los *Plasmodium*. Sin embargo, y aunque el *Aotus* es uno de los modelos de primates no humanos considerados más apropiados para la investigación biomédica en malaria, el conocimiento de su proteoma y en particular el de sus eritrocitos era desconocido. Teniendo en cuenta lo anterior y tomando los resultados obtenidos en este estudio, se realizó la identificación comparativa de proteínas de los eritrocitos de *A. nancymaae* utilizando la información disponible para *Homo sapiens*.

Se reportó por primera vez el proteoma de eritrocitos de *A. nancymaae* (Anexo 2). Hubo alta similitud de los péptidos del primate encontrados por LC-MS/MS con aquellos de *H. sapiens*, lo cual soporta la estrecha relación filogenética entre las dos especies. Se identificaron 1.138 moléculas en total, de las cuales 811 son componentes de eritrocitos maduros. De estos, 51 correspondieron a proteínas integrales de membrana. Siete de ellas son receptores para merozoitos de *Plasmodium*, de acuerdo a lo observado experimentalmente en estudio previos. Este análisis preliminar, fortalece la idea que el primate es un modelo apropiado para continuar con estudios de investigación biomédica básica y aplicada, orientados a desarrollar una vacuna totalmente efectiva contra la malaria.

### **Selección y caracterización de proteínas de *P. vivax***

La caracterización de proteínas relacionadas con la invasión celular se considera un paso clave para diseñar vacunas efectivas contra los agentes patógenos (*Plasmodium* en nuestro caso). Sin embargo, la información disponible para *P. vivax* es escasa en relación a las moléculas que utiliza el parásito para invadir sus células diana. Esto se debe principalmente a la difícil propagación continua del parásito *in vitro*. Consecuentemente, la bioinformática se ha utilizado

desde hace varios años para identificar proteínas candidatas a vacuna en *P. vivax* mediante análisis comparativos.

Por ejemplo, Restrepo-Montoya y su grupo identificaron 45 genes de *P. vivax* que codifican para moléculas candidatas a vacuna, utilizando un perfil probabilístico de Modelos Ocultos de Markov (HMM: *Hidden Markov Models*), entrenando los clasificadores con proteínas de varias especies de *Plasmodium* (diferentes a *P. vivax*) involucradas en la invasión celular (97). Por otro lado, Frech y su equipo, encontraron 8 genes exclusivos de *P. vivax* (posiblemente codificantes de proteínas que participan en la adhesión a sus células diana) en un *cluster* no sinténico sobre el cromosoma 6, mediante la comparación de los genes del parásito con la anotación del genoma de otras especies de *Plasmodium* (98).

Teniendo en cuenta lo anterior y explorando la información del proteoma de *P. vivax*, se realizó un análisis *in silico* con el fin de predecir proteínas que compartan características de un buen candidato a vacuna como las descritas (y confirmadas experimentalmente) para *P. falciparum*, tales como: la expresión del gen en la forma infectiva del parásito (merozoitos) a las células rojas y la presencia de una secuencia señal de secreción, ya sea para llevar la proteína a la superficie del parásito, o a sus organelos apicales (roptrias o micronemas). Además, se tuvo en cuenta la presencia de regiones transmembranales o de anclaje GPI, la determinación de dominios de interacción proteína-proteína o la función de adhesión en otras especies de *Plasmodium*. De acuerdo a los anteriores criterios, se seleccionaron tres moléculas de *P. vivax*: una proteína rica en asparagina (ARP: *Asparagine Rich Protein*), el antígeno de superficie de unión a reticulocitos (RBSA: *Reticulocyte Binding Surface Antigen*) y el antígeno de micronemas anclado a GPI (*Glycosylphosphatidylinositol*) (GAMA: *GPI-Anchored Micronemal Antigen*). Vale la pena destacar que PvRBSA sólo estaba presente en especies del parásito que infectan reticulocitos humanos (como *P. vivax* o *P. cynomolgi*).

La caracterización de las proteínas seleccionadas se realizó siguiendo los parámetros clásicos del dogma central de la biología molecular, como se describe en el **capítulo 2** y en el anexo 3. Inicialmente, se confirmó la presencia y transcripción de los genes *arp*, *rbsa* y *gama* en el genoma de la cepa VCG-1 de *P. vivax*. Además, se verificó el patrón de localización de los productos codificantes, ya sea en la superficie (en el caso de PvARP y PvRBSA) o en los organelos



apicales (en el caso de *PvGAMA*). A pesar de lo anterior, en este punto no hay suficiente evidencia para sugerir que *PvARP*, *PvRBSA* y *PvGAMA* pueden ser componentes de una vacuna. Por ello, se evaluaron otras magnitudes para fundamentar que las proteínas puedan llegar a ser buenos candidatos a vacuna, como la antigenicidad y la capacidad de unión a las células hospederas.

## **Antigenicidad y adhesión celular de proteínas de *P. vivax***

### ***Antigenicidad***

Durante la liberación de los merozoitos de *Plasmodium* al torrente sanguíneo, se desencadena una respuesta inmune por parte del hospedero, la cual a menudo se correlaciona con la inmunidad adquirida naturalmente (57, 99-101). De esta manera, conocer las moléculas antigénicas de *P. vivax* y en particular, aquellas que utiliza el parásito para adherirse e invadir a sus células diana, tiene implicaciones importantes para el desarrollo de vacunas sintéticas. La evidencia experimental del papel antigénico y de adhesión de *PvARP*, *PvRBSA* y *PvGAMA* a las células diana permite dar respuesta a la segunda pregunta de investigación planteada en este trabajo.

Se han detectado anticuerpos anti-*PvARP*, *PvRBSA* y *PvGAMA* en sueros de individuos que habían sufrido malaria por *P. vivax* (**capítulo 2**), sugiriendo la antigenicidad de estas moléculas durante la infección natural. Además, se evaluó si las regiones repetitivas (RR) estaban implicadas en la evasión de la respuesta inmune, como se indica en estudios previos (102, 103), utilizando los fragmentos amino (Nt) y carboxilo (Ct) terminal de *PvGAMA*. Se observó una mayor reactividad de los sueros contra *PvGAMA*-Ct que contiene la RR. Hay que poner de manifiesto que la respuesta de anticuerpos anti-*PvGAMA*-Ct no inhibió la unión del fragmento a reticulocitos humanos (ver más adelante), lo que soporta la idea de que las RR pueden ser distractores de la respuesta inmune. Estos datos sugieren que la respuesta inmune del hospedero está dirigida contra regiones no importantes en la adhesión celular (como las RR), lo cual puede ser la explicación de por qué *PvARP* y *PvGAMA*, las cuales tienen RR, no presentaron asociación entre los niveles de anticuerpos y la reducción del riesgo de presentar enfermedad clínica, como se había descrito previamente (104).

### ***Unión a reticulocitos humanos***

Como objetivo adicional a los inicialmente planteados en esta investigación, se determinó la actividad de unión de las moléculas recombinantes a reticulocitos humanos, dada la importancia que presentan las proteínas tipo adhesinas en el diseño de una vacuna. Teniendo en cuenta que la principal dificultad de estudiar *P. vivax* es no poder propagar continuamente el parásito, debido a que el crecimiento de éste en células sanguíneas se produce preferentemente en reticulocitos (105), se realizó una revisión de las fuentes ricas en dicho tipo de células para escoger la más apropiada y utilizarla en un ensayo que permitiera cuantificar la interacción proteína-célula (Anexo 4). Según lo descrito en la literatura, se escogió la sangre de cordón umbilical (SCU) de niños recién nacidos, por su mayor porcentaje de reticulocitos (6-7%) y por su facilidad en la obtención y procesamiento.

La cuantificación de la interacción proteína recombinante-célula diana se realizó por la técnica de citometría de flujo, utilizando una muestra de SCU con fenotipo Duffy positivo (molécula esencial para la invasión de *P. vivax* a reticulocitos (66)) y proteína obtenida de forma soluble utilizando el sistema de expresión en *E. coli* (manuscrito en preparación). PvARP es insoluble y aunque se extrajo a partir de cuerpos de inclusión y se dializó exhaustivamente, ésta no se unió a las células, posiblemente por la ausencia de una apropiada conformación estructural. Por otro lado, PvRBSA, expresada sin el péptido señal ni la región transmembranal, se obtuvo de forma soluble y se unió en un mayor porcentaje a reticulocitos en comparación con los eritrocitos maduros.

PvGAMA se utilizó en la validación de una nueva estrategia diseñada para identificar regiones conservadas en moléculas de *P. vivax* que están sujetas a restricción funcional. Lo anterior se debe a que las moléculas del parásito contienen regiones de unión funcionales que se conservan entre distintas especies de *Plasmodium* y evolucionan de una manera más lenta. De acuerdo al análisis de selección natural, PvGAMA tiene dos regiones altamente conservadas entre especies, las cuales se unieron en mayor porcentaje a reticulocitos que a eritrocitos maduros, validando así el modelo propuesto. Este resultado confirma la utilidad del método de selección natural para identificar regiones conservadas de las moléculas y de esta manera evitar respuestas alelo específicas, lo cual es contraproducente para el desarrollo de una vacuna ampliamente protectora.

Como se demostró, PvRBSA y los fragmentos de PvGAMA se unieron a reticulocitos en un mayor porcentaje comparado con eritrocitos maduros, lo cual sugiere que estas moléculas tienen preferencia de unión a dicha población celular. Con base en esto y teniendo en cuenta el tropismo de los merozoitos de *P. vivax* por invadir la población de reticulocitos que expresan el receptor CD71 de forma abundante (CD71<sup>hi</sup>) (105), se determinó si PvRBSA y PvGAMA interactúan con dicha población de células. Al analizar la unión de las moléculas en función de la intensidad de la señal para el marcador CD71, se encontró que PvRBSA y tres fragmentos de PvGAMA se unen en mayor proporción a las células que expresan CD71<sup>hi</sup>, confirmando así la unión de las proteínas al estadio más inmaduro de los reticulocitos.

Esta unión también se demostró en otros estudios en los que se evaluaron proteínas de *P. vivax* localizadas en superficie (como MSP-1 (67)), en micronemas (como DBP (69) y RBP1a, RBP1b y RBP2b (56, 57)) o en roptrias (como PvRON5 (60)), cuyos homólogos en *P. falciparum* son particularmente importantes durante el proceso de contacto inicial, reorientación y formación de la unión fuerte. Se ha sugerido que las RBPs están implicadas en la pre-selección de reticulocitos por los merozoitos de *P. vivax*. Sin embargo, en un estudio receptor-ligando en el cual se utilizaron péptidos de 20 aminoácidos de largo, derivados de la proteína MSP-1, se encontró que varios de ellos se unen fuertemente a reticulocitos, pero no a eritrocitos maduros, lo que destaca el papel de la proteína en la selección celular durante el contacto inicial con la célula. Según lo anterior y teniendo en cuenta los resultados obtenidos, se sugiere que no solo DBP, las RBPs y MSP-1 participan en la selección de reticulocitos, sino que PvRBSA y PvGAMA también intervienen en este proceso.

## **CAPÍTULO 1**

“Determinación del proteoma del estadio sanguíneo de la cepa VCG-1 de *Plasmodium vivax*”

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## Determining the *Plasmodium vivax* VCG-1 strain blood stage proteome



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### ABSTRACT

*Plasmodium vivax* is the second most prevalent parasite species causing malaria in humans living in tropical and subtropical areas throughout the world. There have been few *P. vivax* proteomic studies to date and they have focused on using clinical isolates, given the technical difficulties concerning how to maintain an *in vitro* culture of this species. This study was thus focused on identifying the *P. vivax* VCG-1 strain proteome during its blood lifecycle through LC-MS/MS; this led to identifying 734 proteins, thus increasing the overall number reported for *P. vivax* to date. Some of them have previously been related to reticulocyte invasion, parasite virulence and growth and others are new molecules possibly playing a functional role during metabolic processes, as predicted by Database for Annotation, Visualization and Integrated Discovery (DAVID) functional analysis. This is the first large-scale proteomic analysis of a *P. vivax* strain adapted to a non-human primate model showing the parasite protein repertoire during the blood lifecycle. Database searches facilitated the *in silico* prediction of proteins proposed for evaluation in further experimental assays regarding their potential as pharmacologic targets or as component of a totally efficient vaccine against malaria caused by *P. vivax*.

#### Biological significance

*P. vivax* malaria continues being a public health problem around world. Although considerable progress has been made in understanding genome- and transcriptome-related *P. vivax* biology, there are few proteome studies, currently representing only 8.5% of the predicted *in silico* proteome reported in public databases. A high-throughput proteomic assay was used for discovering new *P. vivax* intra-reticulocyte asexual stage molecules taken from parasites maintained *in vivo* in a primate model. The methodology avoided the main problem related to standardising an *in vitro* culture system to obtain enough samples for protein identification and annotation. This study provides a source of potential information contributing towards a basic understanding of *P. vivax* biology related to parasite proteins which are of significant importance for the malaria research community.

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## 1. Introduction

Malaria remains a disease causing concern for public health in countries located in the world's tropical and subtropical regions. The World Health Organization (WHO) has estimated that 207 million cases and 627,000 deaths, mostly in children under 5 years of age, occurred in endemic countries during 2012 [1]. Most of the global burden concerning parasitic disease is caused by *Plasmodium falciparum* and *Plasmodium vivax* species; the latter predominates on the Asian and American continents and is responsible for causing significant morbidity in endemic communities [2]. Several studies have showed that *P. vivax* infection can cause complicated malaria [3,4] thereby making it a potential menace. Developing effective control strategies has therefore become a worldwide public health priority.

Although several groups worldwide are focused on studying *P. vivax*, basic research regarding this species has been delayed by its biological complexity. For instance, it has a preference for invading reticulocytes, a small percentage of which are found in peripheral blood [5], making it difficult to standardise an *in vitro* continuous culture for obtaining large amounts of parasite [5]. Regarding vaccine design, the molecules involved in invasion are highly polymorphic, i.e. the Duffy binding protein (DBP) [6], apical merozoite antigen 1 (AMA-1) [7], reticulocyte binding proteins (RBPs) [8,9] and merozoite surface protein 1 (MSP-1) [10]. The picture is further complicated as latent liver forms (hypnozoites) generate new parasites which are genetically different from those found during the primary infection [11,12].

Just 42 molecules from the *P. vivax* haematic phase [13–43], 3 from the liver stage [44,45] and 3 from the sexual stage have been identified and characterised using classical molecular biology. A few of them are currently being evaluated in preclinical and clinical studies [45]. Identifying the proteins expressed by *P. vivax* is an important step in understanding disease pathogeny and also in studying their role as biomarkers [46], pharmacologic targets [47] or candidates for an antimalarial vaccine [48,49]; *P. vivax* complexity means that other methods should be used to expand knowledge regarding its protein repertoire to find new molecules which can be characterised in further functional studies.

Bioinformatics tools have been used for identifying *P. vivax* proteins by comparing their encoding genes with genomic annotation from other *Plasmodium* species. Restrepo-Montoya et al. used probabilistic profile hidden Markov models (HMMs) trained with several *Plasmodium* species proteins for which the role in invasion has been experimentally determined. The methodology allowed identifying 45 *P. vivax* genes whose encoded proteins might have a potential role in invasion [50]. Frech et al. found eight *P. vivax* exclusive genes in a non-syntenic cluster on chromosome 6, suggesting that their encoded proteins might play a role in invasion of reticulocytes [51]. Although *in silico* analysis is a useful tool for selecting molecules having a possible adhesion function, experimental validation is required.

On the other hand, earlier proteomic studies have helped to characterise the protein composition of *P. vivax*. Acharya et al. identified 154 proteins in clinically isolated *P. vivax* parasites from information derived from mass spectrometry (MS); some were hypothetical proteins, metabolic enzymes, chaperones and

molecules involved in virulence [47,52]. Roobsoong et al. identified 316 proteins in schizont-enriched parasite samples obtained from symptomatic malaria patients. After separating the complex sample on a 2D gel and digesting it, analysis revealed proteins having different functions, such as binding, synthesis, cell transport and metabolism [35]. Two immunoassay-based studies for identifying *P. vivax* antigenic proteins have also been developed. Chen et al. used the wheat germ cell-free system (WGCF) for the mass expression of 86 molecules; 18 of them were recognised by sera from *P. vivax* infected patients (11 of them having no functional evidence) [53]. Lu et al. expressed 152 proteins using the same WGCF expression system, 44 of which were immunoreactive [43]. The proteomic and immunoproteomic studies described above led to identifying 457 *P. vivax* proteins, this being a third of the *P. falciparum* molecules detected during different parasite stages (1289 proteins, of which 714 have been identified in asexual blood stages, 931 in gametocytes and 645 in gametes) [54].

More recently, the human serum proteome has been evaluated for identifying the host immune response to *P. vivax* malaria infection. Serum biomarkers (serum amyloid A and haptoglobin) allowing *P. vivax* infection to be discriminated from that produced by *P. falciparum* have been found when sera from patients with non-complicated malaria were compared to healthy volunteers' sera by classical 2D gels and novel 2D-DIGE technology followed by MALDI-TOF/TOF MS analysis [55,56]. Comparison with *P. falciparum* or leptospiral (febrile control) infected patients' serum proteome revealed that the *Plasmodium* parasite altered serum proteins involved in the host's physiological pathways.

Given that the *P. vivax* proteome has only been analysed using parasite samples obtained from clinical isolates, this research was thus aimed at a large-scale study of a primate model-adapted *P. vivax* strain (VCG-1) proteome for increasing knowledge about parasite protein composition. MS/MS analysis of *P. vivax* enriched blood stages (i.e. ring, trophozoite and schizont forms) complemented earlier work by adding a significant number of new proteins to the available information for the species. Proteins were categorised according to GO term and potential drug target and vaccine candidates were predicted *in silico*. Further experimental analysis of some molecules dealt with here will provide deeper knowledge of *P. vivax* biology.

## 2. Materials and methods

### 2.1. Reagents

ACN, methanol, formic acid (FA) and water were obtained from Fisher Scientific. Chloroform, DTT, ammonium bicarbonate (AB) and tris(2-carboxyethyl)phosphine (TCEP) hydrochloride were obtained from Sigma-Aldrich. Urea and 2-iodoacetamide (IAA) were purchased from Merck. Lys-C was obtained from Wako and trypsin from Promega. All reagents had high purity or were HPLC grade.

### 2.2. Animal handling

Monkeys kept at Fundación Instituto de Inmunología de Colombia (FIDIC)'s primate station (Leticia, Amazon) were

handled in accordance with Colombian Law 84/1989 and resolution 504/1996 and EU Directive 2010/63/EU for animal experiments and followed established guidelines for the care and use of laboratory animals (National Institute of Health, USA). The animals were constantly supervised by a primatologist. The bleeding procedure for *Aotus* monkeys was approved by the Ethics Committee of FIDIC's Primate Experimental Station and carried out in line with the conditions stipulated by CorpoAmazonia (resolution 00066, September 13th 2006). Nine *Aotus* monkeys were experimentally inoculated with  $2.5 \times 10^6$  reticulocytes infected with the Vivax Colombia Guaviare-1 (VCG-1) strain parasites, according to a previously described protocol [57]. Infection progress was monitored daily throughout the entire study (up to day 18) using acridine orange staining which allowed red-orange brilliant fluorescence to be observed in parasite cytoplasm with an ochre background. Parasite density was determined using the following formula: (no. of infected cells/total cells)  $\times$  100. The *P. vivax* infected blood samples were collected for proteomic studies once parasitaemia percentage was found to be between 2 and 5. Monkeys were treated with paediatric doses of chloroquine (10 mg/kg on the first day and 7.5 mg/kg/day until the fifth day) and primaquine (0.25 mg/kg/day from the third to the fifth day) at the end of the study to guarantee parasite clearance from blood. Once experiments were over, CorpoAmazonia officers supervised the primates' return to their natural habitat in excellent health.

### 2.3. Isolating *P. vivax* blood stages

A sample from each *P. vivax* stage was collected when that stage represented more than 70% of all stages on a particular slide. The readings were taken and recorded by an expert/experienced microscopist using acridine orange staining. A 3 mL blood sample containing parasite-infected cells from its different stages was thus collected in a heparin tube and sent to FIDIC's molecular biology laboratory, along with a record of the percentage for each parasite form observed (Table 1).

Leukocytes and platelets were removed by filtering through a CF11 column, as previously described by Sriprawat et al. [58] and parasite percentage was confirmed again using acridine staining (Table 1). Samples enriched in each stage (ring, trophozoite and schizont) were pooled accordingly and selected for proteomics analysis. Ring and/or trophozoite stages could not be enriched to >90% purity since no density gradient protocol was available for such purpose; however, schizonts were enriched using a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden), as previously described [59]. Parasites were isolated from cells by incubating them for 5 min in 0.02 mM saponin buffer containing 7 mM  $K_2HPO_4$ ,

1 mM  $NaH_2PO_4$ , 11 mM  $NaHCO_3$ , 58 mM KCl, 56 mM NaCl, 1 mM  $MgCl_2$  and 14 mM glucose, pH 7.5 and then were washed intensively with PBS pH 7.0.

### 2.4. Protein extraction and precipitation

Whole proteins obtained from each *P. vivax*-enriched stage were extracted following an established *P. falciparum* protocol [60]. Briefly, parasites were disrupted by three cycles of freezing/thawing and sonicated in digestion buffer (4 M urea, 0.4% Triton X-100, 50 mM Tris-HCl, 5 mM EDTA, 10 mM  $MgSO_4$ , pH 8.0) supplemented with protease inhibitor (1 mM PMSF, 1 mM IAA, 1 mM EDTA and 1 mg/mL leupeptin). Samples were spun at 13,000 rpm for 20 min at 4 °C and the supernatant was recovered and stored at -70 °C until use. Protein extracts were purified by precipitating them using the methanol/chloroform method. The dried pellet was homogenised in buffer containing 8 M urea and 50 mM AB. Precipitated proteins were quantified with a micro BCA protein assay kit (Thermo scientific) using a bovine serum albumin (BSA) curve as reference and stored at -20 °C until use.

### 2.5. Protein digestion and purification

Two micrograms of each parasite lysate obtained from different blood development stages were reduced with 5 mM TCEP at 37 °C for 1 hour. Cysteines were alkylated with 20 mM IAA at room temperature (RT) for 30 min in the dark and excess reagent was quenched with 10 mM DTT for 5 min at RT. Samples were enzymatically digested at 37 °C for 2 hours with Lys-C protease in a 1:50 enzyme:protein ratio (w/w) followed by dilution to less than 1 M urea and trypsin digestion at 37 °C for 16 hours at an enzyme:substrate ratio of 1:20 (w/w); the peptide mixture was then frozen at -20 °C until use. Digestion product was re-dissolved in 0.5% FA and desalted using  $C_{18}$  StageTips columns [61]. Purified peptides were eluted from the tips 50% ACN/0.5% FA (v/v). The samples were dried until reaching 1  $\mu$ L and stored at -20 °C until being analysed by LC-MS/MS.

### 2.6. Mass spectrometry

Peptides were analysed by reversed-phased LC-MS/MS using a nanoAcquity UPLC (Waters Corp., Milford, MA) coupled with an LTQ-Orbitrap Velos (Thermo-Fisher, San Jose, CA). Separations were done in a BEH 1.7  $\mu$ m, 130 Å, 75  $\mu$ m  $\times$  250 mm C18 column (Waters Corp., Milford, MA) at a 250 nL/min flow rate. Injected samples were trapped on a Symmetry, 5  $\mu$ m particle size, 180  $\mu$ m  $\times$  20 mm C18 column (Waters Corp., Milford, MA)

**Table 1 – Average percentage parasitaemia of *P. vivax*-infected samples before and after passage through CF11.**

Enriched blood stage	Parasitaemia		Blood stage		
	Initial blood sample	CF11 treatment	Rings	Trophozoites	Schizonts
Ring	5.0%	4.2%	87.2%	12.6%	0.2%
Trophozoite	3.8%	2.7%	29.6%	70.0%	0.4%
Schizont	4.0%	3.2%	5.0%	5.0%	90.0%

and washed with 3% buffer (B) containing 0.1% FA in ACN at 7  $\mu$ L/min flow rate for 3 min before starting the gradient. Peptides were eluted off the column with a four-step gradient using 3–7% B 1 min, 7–25% B 180 min, 25–35% B 30 min and 35–55% B 9 min.

The LTQ-Orbitrap Velos was operated in a data-dependent MS/MS mode using Xcalibur 2.1.0.1140 software (Thermo-Fisher, San Jose, CA) at 2.10 kV spray voltage, 325 °C and 60% S-lens RF level. Survey scans were acquired in the mass range 400 to 1600  $m/z$  with 60,000 resolution at  $m/z$  400 with lock mass option enabled for the 445.120025 ion [62]. The 20 most intense peaks having  $\geq 2$  charge state and above 500 intensity threshold were selected in the ion trap for fragmentation by collision-induced dissociation with 35% normalised energy, 10 ms activation time,  $q = 0.25$ ,  $\pm 2$   $m/z$  precursor isolation width and wideband activation. Maximum injection time was 1000 ms and 50 ms for survey and MS/MS scans, respectively. AGC was  $1 \times 10^6$  for MS and  $5 \times 10^3$  for MS/MS scans. Dynamic exclusion was enabled for 90 s. All samples were analysed in quadruplicate.

### 2.7. Peptide identification by database search

The Mascot algorithm [63] was used for searching the acquired MS/MS spectra, using Thermo Scientific Proteome Discoverer software (v. 1.4.0.288) against a custom database of *P. vivax* parasite (5389 amino acid sequences) in silico reference proteome, New World Monkey family (42,013 molecules) and common contaminant sequences (e.g., human keratins, trypsin, Lys-C and BSA), from the Uniprot protein database, release April 2014. Search parameters were as follows: fully-tryptic digestion with up to two missed cleavages, 10 ppm and 0.8 Da mass tolerances for precursor and product ions, respectively, carbamidomethylation of cysteines, variable oxidation of methionine and N-terminal acetylation. Peptides having MASCOT scores of less than 20 were not considered for analysis. One percent false discovery rate using the Percolator was used for peptide validation [64,65]. Only proteins with at least two significant peptides were considered for analysis.

Identified proteins were compared with previously reported proteome studies [35,47]. Transcription time for schizonts was estimated according to Bozdech's study and the available information in PlasmoDB database [66,67]. Proteins for which there was no transcription evidence were searched using more recent *P. vivax* lifecycle transcription analysis [68].

### 2.8. Protein annotation

The Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.7) 2003–2014 from the National Institute of Allergy and Infectious Diseases (NIAID) [69] was used for functional annotation. The parameters selected here were as follows: GOTERM\_BP\_ALL or GOTERM\_MF\_ALL from the Gene Ontology section. The analysis involved a count of 2 and EASE score threshold was set at 0.05. Results were saved in Microsoft Excel and txt format. Enriched Map with DAVID output was generated using Cytoscape 3.1 software [70]. Analysis parameters involved a 0.05  $p$  value, FDR = 0.1 and overlap coefficient = 0.6. Clusters were circled manually and labelled to highlight the prevalent biological functions

amongst a set of related gene-sets. Parasite proteins having orthologues in humans were searched using the Kyoto Encyclopedia of Genes and Genomes ortholog clusters (KEGG OC) database for drug target analysis [71].

### 2.9. In silico protein characterisation

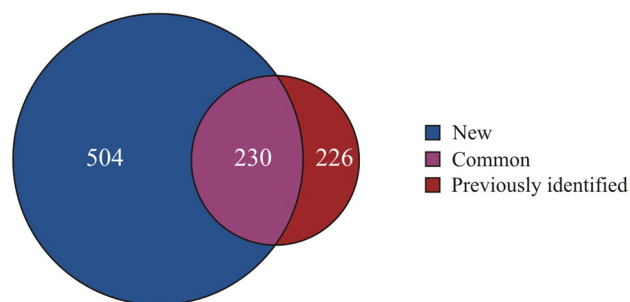
SignalP 4.1 [72] secretion signal sequence prediction and cell localisation predicted by BaCellLo [73] were considered when selecting proteins destined for the secretory pathway. The Interpro database [74] was scanned in the search for putative domains in the whole protein sequence. The presence of transmembrane and glycosylphosphatidylinositol (GPI) anchor sequences was determined by using Phobius [75] and FragAnchor [76] tools, respectively. Adhesine-like proteins were predicted using MAAP software, using  $>0.7$  score, according to the recommendations [77].

## 3. Results

### 3.1. *P. vivax* VCG-1 strain proteome

*P. vivax* VCG-1 strain samples, enriched during different blood stages, were analysed by LC-ESI-MS/MS. A total of 1309 molecules were identified by MASCOT search with a high level of confidence (all having 1% FDR, as estimated by Percolator: Supplementary Data 1). Eighty-six proteins had N-terminal acetylation (supported by 101 peptides). Although 43 additional molecules were identified using the semi-tryptic digestion as a search parameter instead of the tryptic digestion (Supplementary Data 1), these molecules were not considered for further analysis, since we intended to use highly stringent parameters.

When evaluating the molecules' description, 56.1% agreed with *P. vivax* asexual stage proteins and 43.2% with the monkeys' proteome; the latter was due to the presence of the primate material remaining after protein extraction; on the other hand, there was minimal contamination with human proteins (less than 1%). Of the 734 *P. vivax* asexual stage molecules confidently identified here, 504 were new and 230 proteins were common when compared to previous *P. vivax* proteomics and immunoproteomics studies (Fig. 1) (Supplementary Data 2) [35,43,47,53]. This analysis has led to increasing the overall number of reported *P. vivax* molecules to 960,



**Fig. 1** – *P. vivax* proteins identified to date. Venn diagram showing the proteins identified in this study compared to early proteomic and immunoproteomic studies.



comprising 17.8% of the *in silico* predicted reference proteome reported in the Uniprot database.

22.9% of the *P. vivax* VCG-1 strain proteome consisted of hypothetical proteins according to PlasmoDB database (Supplementary Data 3). On the other hand, 69 molecules were found which have been previously described as participating in biological processes which are essential for establishing *Plasmodium* infection or its development within cells, such as cellular invasion (protein processing, initial contact, reorientation and moving junction formation and red blood cell (RBC) internalisation) [78,79], haemoglobin degradation [80], intracellular transport [78,79,81–84], heat shock response [85–87], antigenic variation and immune evasion [88], erythrocyte modification [89] and drug resistance [90] (Fig. 2) (Table 2).

New members of the *Pv-fam* family (not found previously) predicted in the *P. vivax* genome *in silico* analysis [91] were detected (Supplementary Data 3). Rhoptry (RAP-1 (PVX\_085930), -2 (PVX\_097590), RON2 (PVX\_117880), Clag (PVX\_121885)) and surface (MSP-8 (PVX\_097625), -9 (PVX\_124060), Pv41 (Pfs230) (PVX\_000995) and Pv12 (PVX\_113775)) proteins which have already been identified and considered as good candidates for inclusion in a *P. falciparum* vaccine were also identified [92–94]. A recently reported pre-erythrocytic (liver stage antigen (PVX\_091675)) protein was found; although this molecule is immunogenic, its role during blood cycle has not been studied [44].

### 3.2. *P. vivax* VCG-1 strain proteins GO function

GO terms were initially used for categorising whole proteins identified in the *P. vivax* VCG-1 strain through gene-annotation enrichment analysis using DAVID software. A total of 314 proteins were related to biological processes; the enrichment map revealed that most of them were functionally-involved in four processes (statistical significance:  $p < 0.05$ ): protein metabolism and biosynthesis, nucleotide metabolism and biosynthesis, cellular transport and localisation and DNA organisation (Fig. 3) (Supplementary Data 4). On the other hand, 310 molecules were predicted as being related to a molecular function; the most significant related functions derived from DAVID analysis were: structural molecule activity (67 proteins,  $p = 1.26E^{-10}$ ), structural constituent of ribosome (58 proteins,  $p = 8.28E^{-09}$ ), unfolded binding protein (22 proteins,  $p = 2.17E^{-06}$ ), hydrolytic (12 proteins,  $p = 3.14E^{-05}$ ) and translation (24

proteins,  $p = 4.41E^{-04}$ ) activity, and nucleotide binding (149 proteins,  $p = 7.92E^{-04} - 2.03E^{-03}$ ) (Supplementary Data 4). Some proteins could not be classified by DAVID, which may have been because most were not seen to be similar to molecules for which biological knowledge has been reported in databases.

### 3.3. Transcript of protein comparison, according to *P. vivax* stage

There was transcript evidence for 99.2% of the *P. vivax* proteins found here when compared to the *P. vivax* transcriptome profile published by Bozdech et al. [66] (Supplementary Data 5). A total of 329 proteins from ring-enriched, 238 from trophozoite-enriched and 727 from schizont-enriched samples were identified when analysing *P. vivax* extracts separately; 217 proteins were common to all three stages, whilst 2, 16 and 107 molecules were detected in rings/trophozoites, trophozoites/schizonts and rings/schizonts, respectively. Some molecules were only found in one stage: 3 in rings, 2 in trophozoites and 386 in schizonts (Supplementary Data 5).

Interestingly, 6 proteins were found for which there was no evidence of transcripts in Bozdech's study; one hypothetical conserved protein (accession number PVX\_086055) was identified in a later study by Westenberger et al. [68]. The remaining 5 proteins consisted of three hypothetical proteins (PVX\_091652, PVX\_091992 and PVX\_118162), one HAM1 domain-containing protein (PVX\_096292) and one putative arginyl-tRNA synthetase (PVX\_123597) (Supplementary Data 5).

### 3.4. Pharmacological target prediction

Proteins having pharmacological potential were searched by using previously described rules and sequence-derived properties [95]; molecules participating in parasite metabolism which have no orthologues in humans and are possibly involved in just one metabolic pathway were the criteria for drug target prediction.

Proteins participating in KEGG pathways were initially predicted using the DAVID program. The enrichment method grouped 80 *P. vivax* proteins into two categories: 20 proteasome proteins ( $p = 8.3E^{-07}$ ) and 60 ribosome proteins ( $p = 1.5E^{-11}$ ) (Supplementary Data 6). Despite this, all molecules were orthologous to human proteins as predicted using the KEGG OC database.

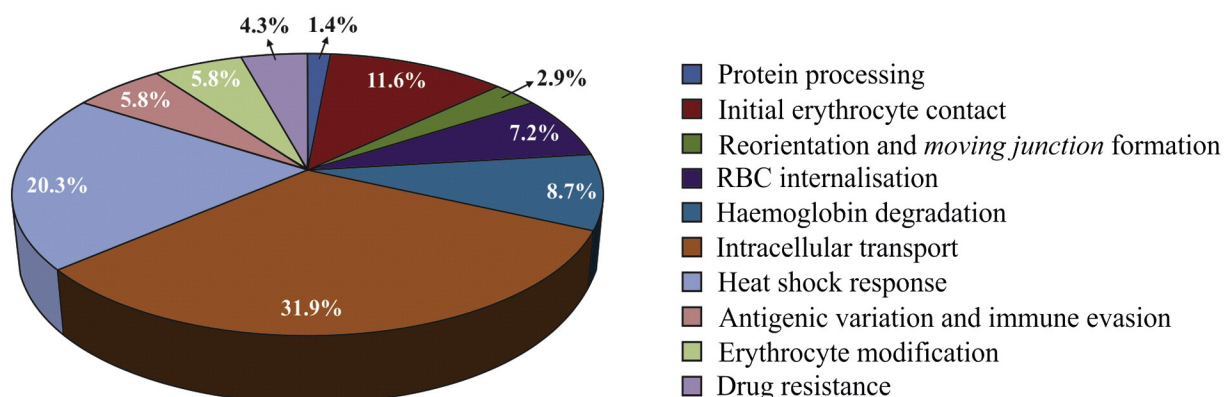


Fig. 2 – Pie chart showing the *P. vivax* proteins distribution related to functional classes.

**Table 2 – Proteins related to *Plasmodium* parasite invasion and cell infection.**

Biological process	Protein name and PlasmoDB ID	References
Protein processing	Subtilisin-like protease (PVX_097935)	[78,79]
Initial erythrocyte contact	MSP-1 (PVX_099980), -7 (PVX_082675), -7H (PVX_082680), -7I (PVX_082685) <sup>a</sup> ; SERA (PVX_003805), -3 (PVX_003840) <sup>a</sup> , -4 (PVX_003825) and -5 (PVX_003810) <sup>a</sup>	
Reorientation and moving junction formation	AMA-1 (PVX_092275) and RON5 (PVX_089530)	
RBC internalisation	Merozoite capping protein 1 (PVX_111355), actin (PVX_101200), myosin A (PVX_083030), actin depolymerising factor (PVX_097745) <sup>a</sup> and myosin-like protein (PVX_113830)	
Haemoglobin degradation	Falcilysin (PVX_115000), vivapain 1 (PVX_240290) <sup>a</sup> , -2 (PVX_091415 and PVX_091405 <sup>a</sup> ), -3 (PVX_091410) <sup>a</sup> and plasmepsin IV (PVX_086040)	[80]
Intracellular transport	EXP 1 (PVX_091700), EXP 2 (PVX_116915), small GTP-binding protein (PVX_089930) <sup>a</sup> , rab GDP dissociation inhibitor beta (PVX_101040) <sup>a</sup> , small GTPase Rab1 (PVX_080550) <sup>a</sup> , -1A (PVX_080610) <sup>a</sup> , -2 (PVX_124195), -5 (PVX_002970) <sup>a</sup> , -5c (PVX_081430), -6 (PVX_092850), -7 (PVX_098605), -11 (PVX_122840) <sup>a</sup> , -11b (PVX_082950) <sup>a</sup> , -18 (PVX_088180) <sup>a</sup> , Sec22 (PVX_095230) <sup>a</sup> , -23A (PVX_089235) <sup>a</sup> , -24 (PVX_115015) <sup>a</sup> , PfSec31p (PVX_002830) <sup>a</sup> , -61α (PVX_083205), -61β (PVX_089275) <sup>a</sup> , -62 (PVX_118580) and -63 (PVX_122755)	[78,79,81–84]
Heat shock response	HSP (PVX_098815 <sup>a</sup> , PVX_002875 <sup>a</sup> , PVX_118295 <sup>a</sup> and PVX_122065), -hslv (PVX_124160), -40 Pfj2 (PVX_091110), -40 Pfj4 (PVX_084600) <sup>a</sup> , -60 (PVX_095000), -70 (PVX_092310), -86 (PVX_087950), -90 (PVX_091545), -101 (PVX_091470), -110 (PVX_083105) and -110c (PVX_087970)	[85–87]
Antigen variation and immune evasion	vir (PVX_096975 and PVX_096980) and vir-12 (PVX_002485 <sup>a</sup> and PVX_022185 <sup>a</sup> )	[88]
Erythrocyte modification	etramp (PVX_003565, PVX_086915 <sup>a</sup> , PVX_090230 and PVX_096070)	[89]
Drug resistance	mrp-1 (PVX_080100), -2 (PVX_118100) and ABC transporter (PVX_124085) <sup>a</sup>	[90]

<sup>a</sup> Proteins identified for the first time in this study. MSP (merozoite surface protein), SERA (serine-repeat antigen), AMA (apical merozoite antigen), RON (rhostry neck protein), EXP (exported protein), HSP (heat shock protein), vir (variable surface protein), *etramp* (early transcribed membrane protein), and *mrp* (multidrug resistance protein).

A total of 177 proteins participating in 87 metabolic pathways were found by using a recently updated PlasmoDB application designed for such purpose in a second analysis [67]; 36 proteins did not have human orthologues and 16 of them were participating in only one pathway (Table 3). The M1-family aminopeptidase (PVX\_122425) was common with the drug targets identified in the *P. vivax* studies reported by Acharya et al. [47]. S-adenosyl-L-homocysteine hydrolase (PVX\_080200), malate:quinone oxidoreductase (PVX\_113980) and leucine aminopeptidase (PVX\_118180) have previously been considered as attractive drug targets for *P. falciparum* [96–98]. Other molecules have been predicted representing major metabolic pathways required for *P. falciparum* parasite replication and growth: adenosine deaminase (PVX\_111245) and phosphoethanolamine N-methyltransferase (PVX\_083045) involved in purine salvage [99] and glycerophospholipid metabolism [100].

### 3.5. In silico predicted vaccine candidates

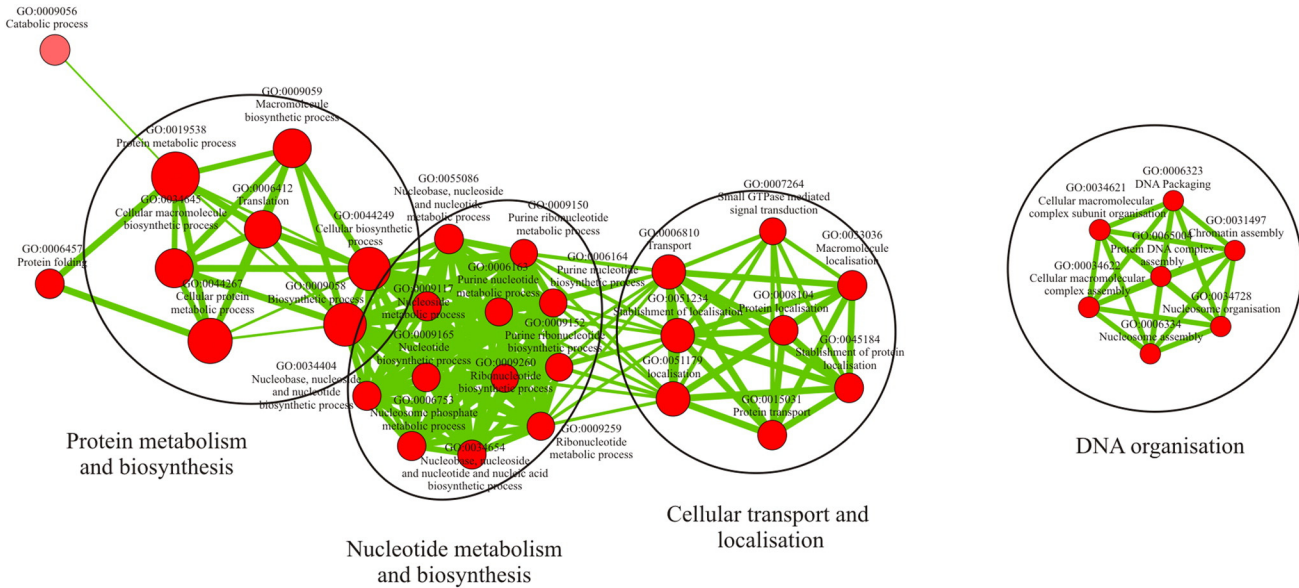
Vaccine candidate molecules were identified, taking the following parameters into account: high expression at the end of the blood lifecycle (>35 hours) (required), prediction of being secreted (required), the presence (or not) of transmembrane regions or GPI-anchors, and the presence (or not) of domains relevant for protein-protein interaction or adhesion function, as determined by the MAAP algorithm. Proteins having domains linked to intracellular functions determined by Interpro scan were excluded.

The analysis led to identifying 31 molecules having the characteristics described above (Table 5). The MSP-1 had

previously been studied in pre-clinical assays [101], others had already been described as surface (Pu12 and Pu41) [26,38] and rhostry (PuRON2) [34] proteins, 8 were hypothetical proteins and other rhostry proteins not described as yet. Six hypothetical proteins have not been studied in any *Plasmodium* species; PVX\_001780 had a domain involved in proteolysis, PVX\_092070 appeared to be restricted to the *Plasmodium* genus and PVX\_099710 had a domain characteristic of extracellular proteins which are cell binding ligands (Table 5). Proteins linked to parasite invasion and growth (subtilisin-like protease, EXP, and SERA proteins) and components of multigene families (MSP-7, *Pv-fam* and *etramp*) were also predicted as vaccine candidates.

## 4. Discussion

The *P. vivax* early proteomic study strategy has involved analysing schizont stages isolated from several human blood samples infected with the parasite. However, no attempt has been made to date to analyse the *P. vivax* protein repertoire using parasite samples from a source having low variability or using different blood life cycle stages. This study has evaluated a primate model-adapted *P. vivax* strain proteome. An attempt was also made to enrich the parasite during different intra-reticulocytes stages (rings, trophozoites and schizonts) to analyse the proteins expressed during different stages, report their annotation and predict in silico potential drug targets and vaccine candidate molecules.



**Fig. 3 – Enrichment map for the *P. vivax* proteins identified here. Map displaying *P. vivax* proteins grouped according to their function. The size of the red node represents the number of proteins by term.**

A total of 734 proteins were confidently identified; 504 were new molecules which led to increasing the number of known *P. vivax* proteins to 960, which is now closer to the 1289 proteins reported for *P. falciparum* in intra-erythrocyte stages [54]. It is worth noting that more than a third of the proteins identified by previous *P. vivax* studies were not recognised here (Supplementary Data 2), probably due to the high sample variability once these had been isolated from infected patients and then mixed and analysed by MS [35,47].

Proteins having N-terminal acetylation were also found. This represents a major post-translational modification which is prevalent in enzymes catalysing intermediate metabolism in human cells [102]. Further analysis of these

proteins is thus needed to study their role in regulating metabolic processes concerning *P. vivax*.

Twenty-five proteins identified here had been shown to be antigenic in earlier immunoproteomic studies [43,53]; these included AMA-1 and MSP-1 as the most studied *P. vivax* antigens and other molecules such as MSP-7, -8, Pv41, Pv12, EXP, aspartic protease PM5, *etramp* and *Pv-fam* protein families and hypothetical proteins (Supplementary Data 3, shown with an asterisk). Taking into account that antigenicity is one of the parameters considered when selecting vaccine candidates [48], added to the antigenic potential previously described for the above proteins, additional experiments aimed at analysing the potential of the above-mentioned proteins (mainly those which have not been

**Table 3 – In silico prediction of potential drug targets.**

Metabolic pathway	PlasmoDB ID	Description
Phenylalanine, tyrosine and tryptophan biosynthesis (ec00400)	PVX_098815	ATP-dependent heat shock protein, putative
Glyoxylate and dicarboxylate metabolism (ec00630)	PVX_111055	Haloacid dehalogenase, putative
Fructose and mannose metabolism (ec00051)	PVX_099200	6-Phosphofructokinase, putative
Cysteine and methionine metabolism (ec00270)	PVX_080200	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase), putative <sup>a</sup>
Pyruvate metabolism (ec00620)	PVX_113980	Malate:quinone oxidoreductase, putative <sup>a</sup>
Glutation metabolism (ec00480)	PVX_118180	Leucine aminopeptidase, putative <sup>a</sup>
	PVX_118545	2-Cys peroxiredoxin, putative
	PVX_122425	M1-family aminopeptidase, putative <sup>a</sup>
	PVX_123435	Thioredoxin peroxidase2, putative
Glycerophospholipid metabolism (ec00564)	PVX_083045	Phosphoethanolamine N-methyltransferase, putative <sup>a</sup>
	PVX_088015	PST-A protein
Aminoacyl-tRNA biosynthesis (ec00970)	PVX_002940	Asparagine-tRNA ligase, putative
	PVX_082520	Glutaminyl-tRNA synthetase, putative
	PVX_088145	Tyrosyl-tRNA synthetase, putative
Methane metabolism (ec00680)	PVX_116710	Vacuolar ATP synthase subunit g, putative
Purine metabolism (ec00230)	PVX_111245	Adenosine deaminase, putative <sup>a</sup>

<sup>a</sup> Proteins which have been suggested as being good drug targets in *P. falciparum*.

studied to date) as components of an anti-malarial vaccine against *P. vivax* should be undertaken. On the other hand, although the number of proteins now identified for *P. vivax* has substantially increased, further investigation is required to discover these molecules' importance regarding the parasite's biological functions, such as antigenic variability, immune evasion, virulence, invasion process, pathogenicity and resistance to drugs.

Comparing stages led to finding a difference between the quantity of proteins detected in ring and trophozoite stages vs. schizonts. This could be explained by there being fewer parasites during early lifecycle phases (early/late rings and trophozoite) and therefore low protein amount and a greater abundance of primate molecules masking *P. vivax* peptide detection (49% for ring-enriched and 66% for trophozoite-enriched samples) (Table 4), this being consistent with one of the main difficulties in proteome analysis [103]. On the other hand, most proteins were found in 2 out of the 3 stages (Supplementary Data 5: see expression time) which might have been because the MS technique used here allows peptides to be detected but does not measure their abundance. Thus the annotation of all proteins identified here could only be determined, which provided an insight into cellular processes in which some proteins participated during parasite development inside a target cell (Fig. 3), whilst no functional preference by stage could be evaluated. A quantitative proteomic analysis is required for determining whether there was a correlation between proteins identified by stage of their encoding mRNA abundance.

Some proteins identified here had no transcript evidence when compared to transcriptomic studies [66,68]. Previous studies have shown a significant difference in the total mRNA levels of 249 genes in three *P. vivax* clinical isolates from Thailand [66] and in gene expression profiles when compared to Peruvian *P. vivax* isolates [68]. The discrepancy between VCG-1 and the *P. vivax* clinical isolates could thus be explained by their different transcriptional profiles during the intra-reticulocyte cycle; however, a gene transcription profile study regarding *P. vivax* VCG-1 strain is thus needed to confirm such hypothesis.

The search for therapeutic targets against malaria has become an important line of research, given that resistant *P. vivax* strains continue emerging and threatening the health of millions of people in endemic areas [104]. Sixteen candidates were predicted in this study, some of them being orthologous to *P. falciparum* proteins which have been considered potential pharmacological targets (Table 3). Although several molecules have been suggested as possible

*P. vivax* drug targets by Acharya et al. [52] not all were identified here because such proteins did not meet the inclusion criteria established for this study [95]. The absence of these predicted proteins in mammals makes them ideal targets for designing novel antimalarial drugs. However, further assays orientated towards evaluating structural homology with other human proteins and the toxicity of the drugs used against these targets in *in vitro* controlled trials are needed to ascertain pharmacological potential.

The difficulties in studying the role of *P. vivax* molecules in invasion when working with this parasite species in the laboratory [5] have highlighted bioinformatics tools as an interesting alternative for selecting and characterising potential vaccine candidates [45]. It was particularly interesting that several vaccine candidates predicted *in silico* could induce an immune response during natural infection, according to previous immunoproteomic studies (Table 5) [43].

The *in silico* prediction led to identifying *Pv-fam-a* proteins in which some members have been shown to bind erythrocytes [105], *Pv-fam-d* for which there is no functional evidence data to date and *etramps* orthologues to *P. falciparum* proteins whose red blood cell binding role has been shown (Table 5) [106]. Other important proteins found were two MSP-7, two SERA and five malarial adhesins, which have been considered good vaccine candidates as they mediate cell binding [79,107]. One *Pv-fam* (PVX\_112685) and one *etramp* (PVX\_096070) proteins were predicted by MAAP, as well as one hypothetical protein (PVX\_084720), the MSP-1 (PVX\_099980) which has been extensively studied in *Plasmodium* species, and one conserved rhoptry protein (PVX\_096245) which is important but not essential for *P. falciparum* invasion, as shown in a gene knockout study [108].

Rhoptry and surface proteins are important candidates given that they are required for host cell attachment and parasite invasion [109,110]; therefore, RON-2 (PVX\_117880), -3 (PVX\_101485), -5 (PVX\_089530), the rhoptry protein above mentioned (PVX\_084720), one member of the cytoadherence protein family (PVX\_121885), and Pv12 (PVX\_113775) and Pv41 (PVX\_000995) could be good candidates.

Interestingly, according to the PlasmoDB information, 2 *Pv-fam* family proteins (PVX\_112685 and PVX\_121910) and one hypothetical protein (PVX\_096055) had no orthologues in *P. falciparum* but were present in *Plasmodium cynomolgi*, a monkey parasite which is a closely *P. vivax*-related species and also infects reticulocytes (Table 5) [111]. This supports the notion that these proteins are possibly related to *P. vivax* cellular preference for invasion. Further characterisation of all the aforementioned molecules should be considered for testing their role in reticulocyte adhesion or invasion.

**Table 4 – Proteins recognised by stage and their amount.**

Stage	Total proteins	<i>P. vivax</i>	Primate	Contaminants <sup>a</sup>
Ring	661	330 (50%)	323 (49%)	8 (1%)
Trophozoite	731	238 (33%)	485 (66%)	8 (1%)
Schizont	1042	727 (70%)	310 (29%)	6 (1%)

Numbers in brackets indicate the percentage of total proteins detected by stage.

<sup>a</sup> Main contaminants were human keratins.

## 5. Conclusions

This is the first proteomic analysis involving a *P. vivax* strain adapted to a non-human primate infection model for evaluating its protein repertoire during blood stages. A total of 504 new *P. vivax* proteins not reported in earlier studies were found here, thus providing relevant data concerning the biology of the *P. vivax* VCG-1 strain related to proteins

**Table 5 – Predicted *P. vivax* vaccine candidates in silico.**

PlasmoDB ID	Description	MET	SP	BacellO	Interpro Scan	Phobius	MAAP	GPI-anchor
PVX_000995 <sup>a</sup>	Transmission-blocking target antigen Pfs230, putative (P41)	35	x	x	s48/45 domain (IPR010884)	–	–	–
PVX_001780	Hypothetical protein, conserved	35	x	x	Aspartic peptidase domain (IPR021109)	1	–	–
PVX_003805 <sup>a</sup>	Serine-repeat antigen (SERA), putative	35	x	x	Papain domain (IPR000668)	–	–	–
PVX_003810	Serine-repeat antigen 5 (SERA), putative	35	x	x	–	–	–	–
PVX_082675	Merozoite surface protein 7 (MSP7)	40	x	x	Merozoite surface protein, C-terminal (IPR024781)	–	–	–
PVX_082680 <sup>a</sup>	Merozoite surface protein 7 (MSP7), putative	35	x	x	–	–	–	–
PVX_084720 <sup>a</sup>	Hypothetical protein, conserved	40	x	x	–	–	x	–
PVX_086915	Early transcribed membrane protein (ETRAMP)	35	x	x	<i>etramp</i> family (IPR006389)	1	–	–
PVX_089530	Rhoptry neck protein 5, putative (RON5)	35	x	x	–	2	–	–
PVX_090230	Early transcribed membrane protein (ETRAMP)	35	x	x	<i>etramp</i> family (IPR006389)	2	–	–
PVX_090945	Hypothetical protein, conserved	35	x	x	–	1	–	–
PVX_091700 <sup>a</sup>	Circumsporozoite-protein related antigen, putative (EXP1)	40	x	x	Circumsporozoite-related antigen family (IPR009512)	1	–	–
PVX_092070 <sup>a</sup>	Hypothetical protein, conserved	40	x	x	Protein of unknown function DUF3271 (IPR021689)	–	–	–
PVX_096055 <sup>b</sup>	Hypothetical protein	43	x	x	–	2	–	–
PVX_096070	Early transcribed membrane protein (ETRAMP)	43	x	x	<i>etramp</i> family (IPR006389)	1	x	–
PVX_096245	Rhoptry-associated leucine zipper-like protein 1	35	x	x	–	–	x	–
PVX_096950	Tryptophan-rich antigen (Pv-fam-a)	43	x	x	Tryptophan/threonine-rich domain (IPR022089)	–	–	–
PVX_096990	<i>Pv-fam-d</i> protein	40	x	x	–	1	–	–
PVX_097935	Subtilisin-like protease precursor, putative	40	x	x	S8/S53 domain (IPR000209)	–	–	–
PVX_099710	Hypothetical protein, conserved	40	x	x	Calycin-like domain (IPR011038)	–	–	–
PVX_099980 <sup>a</sup>	Major blood-stage surface antigen Pv200	35	x	x	EGF domain (IPR010901, IPR024730, IPR024731)	1	x	HP
PVX_101485	Rhoptry neck protein 3, putative (RON3)	35	x	x	–	3	–	–
PVX_112665	Tryptophan-rich antigen ( <i>Pv-fam-a</i> )	43	x	x	Tryptophan/threonine-rich domain (IPR022089)	–	–	–
PVX_112685 <sup>a,b</sup>	Tryptophan-rich antigen ( <i>Pv-fam-a</i> )	40	x	x	–	–	x	–
PVX_113225	Plasmodium exported protein, unknown function	40	x	x	Protein of unknown function DUF3671 (IPR022139)	3	–	–
PVX_113775 <sup>a</sup>	6-cysteine protein (P12)	35	x	x	s48/45 Domain (IPR010884)	1	–	HP
PVX_117880	Rhoptry neck protein 2 (RON2)	35	x	x	–	3	–	–
PVX_121885	Cytoadherence linked asexual protein, CLAG, putative	35	x	x	Cytoadherence-linked asexual protein family (IPR005553)	2	–	–
PVX_121910 <sup>a,b</sup>	<i>Pv-fam-d</i> protein	40	x	x	–	2	–	–
PVX_122910	Hypothetical protein, conserved	43	x	x	–	1	–	–
PVX_124090	Hypothetical protein, conserved	35	x	x	–	1	–	–

MET: maximum expression time; SP: signal peptide; GPI: glycosylphosphatidylinositol; HP: highly probable.  
<sup>a</sup> Previous evidence of antigenicity.  
<sup>b</sup> Proteins with orthologues in *P. cynomolgi* only.

involved in parasite growth, antigenic variability, invasion and others having a GO term linked to metabolic pathways. The study has presented an important source of information for molecule selection, providing the potential for establishing

suitable control strategies aimed at preventing or treating *P. vivax* malaria infection. Further studies are needed to confirm the potential use of the *in silico* predicted drug targets and vaccine candidates here described.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2014.10.003>.

## Competing interests

The authors have declared that no competing interests exist.

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## **CAPÍTULO 2**

“Caracterización de proteínas de *Plasmodium vivax* de potencial utilidad en el desarrollo de una vacuna antimalárica”

RESEARCH

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# Characterizing *PvARP*, a novel *Plasmodium vivax* antigen

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## Abstract

**Background:** *Plasmodium vivax* continues to be the most widely distributed malarial parasite species in tropical and sub-tropical areas, causing high morbidity indices around the world. Better understanding of the proteins used by the parasite during the invasion of red blood cells is required to obtain an effective vaccine against this disease. This study describes characterizing the *P. vivax* asparagine-rich protein (*PvARP*) and examines its antigenicity in natural infection.

**Methods:** The target gene in the study was selected according to a previous *in silico* analysis using profile hidden Markov models which identified *P. vivax* proteins that play a possible role in invasion. Transcription of the *arp* gene in the *P. vivax* VCG-1 strain was here evaluated by RT-PCR. Specific human antibodies against *PvARP* were used to confirm protein expression by Western blot as well as its subcellular localization by immunofluorescence. Recognition of recombinant *PvARP* by sera from *P. vivax*-infected individuals was evaluated by ELISA.

**Results:** VCG-1 strain *PvARP* is a 281-residue-long molecule, which is encoded by a single exon and has an N-terminal secretion signal, as well as a tandem repeat region. This protein is expressed in mature schizonts and is located on the surface of merozoites, having an apparent accumulation towards their apical pole. Sera from *P. vivax*-infected patients recognized the recombinant, thereby suggesting that this protein is targeted by the immune response during infection.

**Conclusions:** This study showed the characterization of *PvARP* and its antigenicity. Further assays orientated towards evaluating this antigen's functional importance during parasite invasion are being carried out.

**Keywords:** *Plasmodium vivax*, Protein, Invasion, Antigenicity, Vaccine

## Background

Malaria is a tropical disease that causes millions of deaths per year around the world. The World Health Organization's (WHO) Malaria Report 2011 indicated that there were 216 million cases and 655,000 deaths, mainly in children aged less than five years [1]. In spite of the incidence of cases worldwide and mortality index having become substantially reduced by 17% and 25% between 2000 and 2010, respectively, the figures regarding cases of malaria continue to be alarming. This is due to two main aspects impeding the total eradication of the disease: a gradual increase of parasite strains which are resistant to anti-malarial drugs [2] and populations of the mosquito vector which are insecticide-resistant [3].

*Plasmodium vivax* stands out as the most widespread parasite species causing malaria in humans; it is found throughout tropical and subtropical areas of the world and causes the disease's highest morbidity indices on the Asian and American continents [4]. Even though it has been thought that *P. vivax* was a benign species, recent studies have shown that infection caused by this parasite could cause severe clinical symptoms [5,6], similar to those found in *Plasmodium falciparum* infection, thereby making it a potential menace.

Synthetic vaccines have been considered a good choice among control strategies when combating infectious diseases. Regarding malarial blood stages, vaccine development has been focused on the recombinant expression of parasite antigens (MSP-1 [7-9] and AMA-1 [10,11] having been the most studied) or on using synthetic peptides

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[12,13]; however, no fully effective vaccine against any species has been reported to date.

Recent work has established that the key to achieving an effective vaccine lies in blocking the interaction of parasite ligands which facilitate adhesion to target cell receptors [14]; this means that molecules localized on parasite surface and apical organelles (rhoptries and micronemes) must be identified. Unfortunately, data regarding the *P. vivax* proteins involved in invasion of reticulocytes that have been functionally characterized to date lag behind that available for their *P. falciparum* counterparts [15]. The foregoing has been due to the difficulty of standardizing an *in vitro* culture given poor reticulocyte recovery from adult human total blood [16]. Such experimental limitation has led to several study alternatives having been suggested; probabilistic techniques have been most useful when predicting possible vaccine candidates. A recent study involving hidden Markov models for analyzing the transcriptome of the *P. vivax* Sal-1 strain's intra-erythrocyte life-cycle has led to the identification of 45 proteins that play a potential role in invasion; the role in cell adhesion for 13 of them (localized in merozoite rhoptries or on their surface) had previously been determined [17]. It was particularly interesting that an asparagine-rich protein (ARP) was found, this being conserved throughout the *Plasmodium* genus [17]. Only its *P. falciparum* orthologue has been described to date, called the apical asparagine-rich protein (*PfAARP*) [18]. The *PfAARP*-encoding gene has a prominent expression pattern towards the last intra-erythrocyte parasite development stage (48 hours post-invasion), which has been shown by real-time PCR and Northern blot. Antigenicity assays have shown that the N-terminal protein's region (*PfAARP*-N) obtained as a recombinant is recognized by antibodies from patients who have been naturally infected by *P. falciparum*. Rabbit antibodies directed against *PfAARP*-N have been able to significantly inhibit parasite invasion of RBC *in vitro*. The foregoing, together with an RBC binding assay involving the expression of the complete protein on COS cell surface, has highlighted this antigen's functional role in parasite binding to and invasion of target cells [18].

The present study was thus aimed at characterizing the asparagine-rich protein orthologue for *PfAARP* in *P. vivax*. Molecular biology assays and immunochemistry techniques were used to demonstrate *Pvarp* gene transcription, protein expression and localization, as well as the ability to induce an antigenic response in patients who had suffered episodes of *P. vivax* malaria.

## Methods

### Selecting the gene and designing the primers and synthetic peptides

*PvARP* was selected, bearing in mind the *in silico* study by Restrepo-Montoya *et al.* [17] of *P. vivax* proteins playing a potential role in invasion. The PlasmoDB [19]

database was then scanned to obtain the *Pvarp* gene sequence from the Salvador 1 (Sal-1) reference strain and to analyze adjacent genes' synteny in different *Plasmodium* species. Specific primers were designed manually using Gene Runner software (version 3.05). B-cell lineal epitopes were predicted with AnthProt software [20] using the deduced amino-acid (aa) sequence. A tBlastn analysis of the predicted B-cell epitopes was then carried out to select peptide sequences exclusive for the *P. vivax* ARP.

### Animal handling

The experimental animals used were handled in accordance with Colombian Law 84/1989 and resolution 504/1996. *Aotus* monkeys kept at FIDIC's primate station (Leticia, Amazon) were handled following established guidelines for the care and use of laboratory animals (National Institute of Health, USA) under the constant supervision of a primatologist. All experimental procedures involving *Aotus* monkeys had been previously approved by the Fundación Instituto de Inmunología's ethics committee and were carried out in agreement with the conditions stipulated by CorpoAmazonia (resolution 00066, 13 September, 2006). An *Aotus* monkey was experimentally infected with the Vivax Colombia Guaviare 1 (VCG-1) strain and monitored daily to assess infection progress throughout the entire study (up to day 18) using Acridine Orange staining. The monkey was treated with paediatric doses of chloroquine (10 mg/kg on the first day and 7.5 mg/kg/day until the fifth day) and primaquine (0.25 mg/kg/day from the third to the fifth day) at the end of the study to guarantee parasite clearance from total blood. Once experiments were over, CorpoAmazonia officers supervised the primate's return to its natural habitat in excellent health.

### Isolating the *Plasmodium vivax* parasite

VCG-1 strain parasites were maintained *in vivo* according to previously described methodology [21]. A *P. vivax*-infected blood sample (3 mL) was passed through a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden) according to an already established protocol [22] for obtaining schizont-stage enriched parasite. The sample was then used as RNA, genomic DNA (gDNA) and total protein source.

### Extracting RNA and cDNA synthesis

Total RNA was extracted from the schizont-enriched sample using the Trizol method and treated with RQ1 (RNA-qualified) RNase-free DNase (Promega, Wisconsin, USA) according to the manufacturer's recommendations. Complementary DNA (cDNA) was synthesized using a SuperScript III enzyme (RT+) (Invitrogen, California, USA) in the following conditions: 65°C for 5 min, 50°C for 1 hour and 70°C for 15 min. An additional reaction

without the SuperScript III enzyme (RT-) was made for use as control. Following 15 min' incubation at 37°C with RNase (Promega, USA) the product was stored at -70°C until its later use.

#### Cloning, sequencing and bioinformatics analysis

The cDNA RT + and RT- samples, as well as the gDNA obtained using a DNA Wizard Genomic purification kit (Promega), were used as template in 10 µL PCR reactions containing 0.5 U/µL Accuzyme DNA polymerase (Bioline), 1x AccuBuffer, 2 mM MgCl<sub>2</sub>, 0.5 mM dNTP, 0.5 µM primers and DNase-free water for completing the reaction volume. Specific primers were designed for amplifying a region containing the entire *Pvarp* gene (direct 5'- CATTGATCAGAGACGAC -3' and reverse 5'- TTGGCACTTTTGTCACGA -3'), or the encoding sequence without the signal peptide (direct 5'- atgTGCAACACAAATGGGA AAA -3' and reverse 5'- CACGCCAAACAGCTTCA -3'); the protein expression start codon was included in the direct primer's 5' end. A set of primers which had been previously designed for amplifying the *Pvron1-a* region (direct 5'- atgGCGAAGGAGCCCAAGTG-3' and reverse 5'- ATCCCTAGCAATGCTTCG -3') [23] was used as control for cDNA contamination with gDNA. The PCR for the *Pvarp* gene began with a denaturing step at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 52°C for 10 sec and 72°C for 1 min. *Pvron1-a* PCR began with a denaturing step at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 56°C for 10 sec and 72°C for 1.5 min. A Wizard PCR preps kit (Promega) was used for purifying *Pvarp* gene amplicons obtained from independent PCRs done with the RT + sample, once quality had been evaluated by 1% agarose gel. Pure products were then ligated to the pEXP5 CT/TOPO expression vector and transformed in TOP10 *Escherichia coli* cells (Invitrogen). Various clones were grown to purify the plasmid, using an UltraClean mini plasmid prep purification kit (MO BIO laboratories, California, USA); insert integrity and its correct orientation were confirmed by sequencing using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, California, USA). VCG-1 strain *Pv*ARP was characterized *in silico* using SignalP 3.0 [24], FragAnchor [25], XSTREAM [26], tools and the Interpro database [27] to search for secretion signal or GPI-anchor sequences, tandem repeats and putative domains, respectively. Clustal W software was used for aligning genes and pertinent encoding sequences [28].

#### Recombinant protein expression and purification

The pEXP5-*Pv*ARP recombinant plasmid which encodes the entire *Pv*ARP sequence without the signal peptide (confirmed by sequencing) was transformed in *E. coli* BL21-AI (Invitrogen), according to the manufacturer's recommendations. A protocol described by Sivashanmugam and his group [29] with some modifications, was used for

improving expression yield. Briefly, the cells were grown overnight at 37°C in 10 mL Luria Bertani (LB) medium containing 100 µg/mL ampicillin and 0.1% (w/v) D-glucose. The initial inoculum was then seeded in 100 mL LB volume with the same amount of the aforementioned ampicillin and D-glucose and left to grow at 37°C using ~300 rpm until reaching 0.5 OD<sub>600</sub>; 0.2% L-arabinose (w/v) was used for five hours to induce expression. The culture was spun at 13,000 rpm for 30 min and lysed in extraction buffer (EB) (6 M urea, 12 mM imidazole, 10 mM Tris-Cl, 100 mM NaH<sub>2</sub>PO<sub>4</sub> and 10 mg/mL lysozyme) supplemented with protease inhibitors (1 mM PMSE, 1 mM iodoacetamide, 1 mM EDTA and 1 mg/mL leupeptin). *Pv*ARP recombinant expression (r*Pv*ARP) was verified by Western blot and the protein was then purified by solid-phase affinity chromatography using Ni<sup>+2</sup>-NTA resin (Qiagen, California, USA) following the manufacturer's recommendations. Briefly, total lysate was incubated with the resin pre-equilibrated with EB overnight at 4°C. The r*Pv*ARP mixture coupled to the resin was placed on a column and then washed several times with EB to eliminate weakly bound proteins. The recombinant protein was eluted with EB containing imidazole at differing concentrations (20, 100, 250 and 500 mM) in 3 mL fractions, which were analyzed by Coomassie blue staining to verify the presence of a single band and then dialyzed in PBS, pH 7.0. A micro BCA protein assay kit (Thermo Scientific) was used for quantifying every fraction so obtained; a bovine serum albumin (BSA) curve was used as reference.

#### Peptide synthesis and obtaining polyclonal antibodies

A 20 aa-long peptide (predicted to be a good B-cell epitope), located at the N-terminus of *Pv*ARP (CG-LDNLKAKESPSSNDDGVYAKG-GC), was synthesized according to a previously-established methodology [30], polymerized, lyophilized and characterized by RP-HPLC and MALDI-TOF MS. Five mg of peptide (called 38582 herein) were immobilized on a CNBr-activated Sepharose 4B column, according to the manufacturer's recommendations. A pool of fifteen sera taken from patients who had suffered previous *P. vivax* malarial episodes (stored in FIDIC's serum-bank, see the 'Sample source' section) was incubated with the peptide coupled to a Sepharose 4B column overnight at 4°C with constant shaking to purify specific antibodies against peptide 38582 (anti-*Pv*ARP<sub>38582</sub>). The retained antibodies were eluted with gradients of increasing salt concentration (50 mM-0.3 M NaCl); they were then dialyzed in PBS, pH 7.8, and stored at -20°C until use.

#### SDS-PAGE and Western blot

Five µg r*Pv*ARP and 50 µg total parasite proteins were separated on 12% SDS-PAGE and then transferred to nitrocellulose membranes. After having been blocked with

5% skimmed milk in PBS-0.05% Tween for one hour, each membrane was cut into strips and individually analyzed as follows: strips with the recombinant protein were incubated for two hours at room temperature (RT) with anti-*Pv*ARP<sub>38582</sub> serum fractions (1:100 dilution) in a solution of 5% skimmed milk in PBS-0.05% Tween to assess which of them contained anti-*Pv*ARP specific antibodies; one strip was incubated with an anti-histidine monoclonal antibody coupled to peroxidase (1:4,500) as positive control for Western blot. Serum fractions recognizing the recombinant protein were then used to detect *Pv*ARP in total parasite lysate in the aforementioned conditions. Once antibody reactivity had been eliminated by incubating anti-*Pv*ARP<sub>38582</sub> serum with peptide 38582 for one hour at 37°C, then this solution was used as control. Following three washes with PBS-0.05% Tween (5 min per wash), the strips were incubated for one hour with phosphatase-conjugated goat anti-human IgG as secondary antibody (1:5,000) at RT. The blots were revealed with a VIP peroxidase (Vector Laboratories, Burlingame, Canada) or BCIP/NBT colour development substrate kits (Promega), according to the manufacturers' indications.

#### Indirect immunofluorescence assay (IFA)

*Plasmodium vivax*-parasitized reticulocytes were washed thrice with PBS and then diluted in this solution until obtaining five to seven schizonts per field evaluated by staining with Acridine orange. Twenty µL of the sample were fed per well on eight-well multitest glass slides (Biomedicals, Inc) and the supernatant was removed 10 min later. Once the samples were dry, they were fixed with 4% formaldehyde for 5 min at RT. Following five washes with PBS, the sample was incubated with 1% Triton X-100 for 5 min in the previously described conditions. After 10-min blocking at RT with 1% (v/v) skimmed milk in PBS, each sample was incubated for one hour at RT with anti-*Pv*ARP<sub>38582</sub> antibodies (20 µL). The samples were then incubated with FITC-conjugated anti-human IgG antibody (Sigma) at 1:30 dilution for 45 min in the dark. The DNA was stained with DAPI (0.5 µg/mL) for 10 min at RT and the excess was removed by washing several times with PBS-0.05% Tween. Once the slides had been examined under an Olympus BX51 fluorescence microscope (using 100× oil immersion objective), Volocity software (version 5.3.2) was used for superimposing the images.

#### Enzyme-linked immunosorbent assay (ELISA)

*Pv*ARP antigenicity was evaluated in triplicate using serum from patients who had been living in malaria-endemic areas in Colombia and had presented episodes of such infection. Sera taken from healthy individuals who had never suffered the disease were used as negative controls. Briefly, 96-well polysorb plates were covered with

1 µg/mL *rPv*ARP overnight at 4°C and then incubated at 37°C for one hour. The dishes were blocked with 200 µL 5% skimmed milk - PBS-0.05% Tween for one hour at 37°C. Antibody reactivity against the recombinant protein was evaluated by incubating the plates with a 1:100 dilution of each human serum in 5% skimmed milk -PBS-0.05% Tween for one hour at 37°C. Following incubation of the dishes with peroxidase-coupled anti-human IgG secondary antibody (1:10,000) diluted in 5% skimmed milk - PBS-0.05% Tween for one hour at 37°C, a peroxidase substrate solution (KPL Laboratories, WA, USA) was added to reveal the reaction, according to the manufacturer's recommendations. Optical density (OD) was detected at 620 nm with an MJ ELISA multiskan reader and then calculated by subtracting the OD value obtained from the control well (no antigen). A 0.11 cut-off value for evaluating the positivity threshold was determined by taking the average of the OD plus twice the standard deviation ( $2 \pm SD$ ) of healthy individuals' sera reactivity.

#### Statistical analysis

Differences in average OD for *rPv*ARP recognition by *P. vivax*-infected patients' sera and in the control group were evaluated using the Kruskal-Wallis rank-sum test. A 0.05 significance level was used for testing a stated hypothesis.

#### Sample source

Sera were obtained from 38 patients who were living in malaria-endemic areas of Colombia and who had suffered previous episodes of *P. vivax* malaria (but not *P. falciparum*), as well as from 15 healthy individuals who had never been affected by the disease. All individuals signed an informed consent form after receiving detailed information regarding the study's goals.

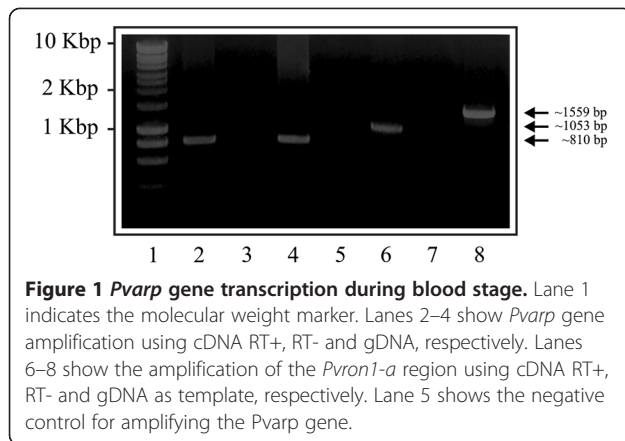
#### Accession number

The nucleotide and aa sequences used here have been reported in the GenBank database, under accession number KC514070.

## Results and discussion

### Analyzing the *arp* gene in *Plasmodium* species

The *P. vivax* proteins identified as playing a potential role in invasion by profile hidden Markov models [17] led to *Pv*ARP being selected. According to the information provided by the PlasmoDB database, the *Pv*arp gene (access number: PVX\_090210) was found to be located between base pairs 1,230,371 and 1,231,228 in chromosome 5 of the Sal-1 strain. Similar genes were also found in the genome of other *Plasmodium* species known to be causing malaria in humans (*P. falciparum* and *Plasmodium knowlesi*), apes (*Plasmodium cynomolgi*) and rodents (*Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium*



**Figure 1** *Pvarp* gene transcription during blood stage. Lane 1 indicates the molecular weight marker. Lanes 2–4 show *Pvarp* gene amplification using cDNA RT+, RT- and gDNA, respectively. Lanes 6–8 show the amplification of the *Pvron1-a* region using cDNA RT+, RT- and gDNA as template, respectively. Lane 5 shows the negative control for amplifying the *Pvarp* gene.

*chabaudi*). When analyzing alignment, the *Pvarp* gene codified product was 61.19%, identical to its orthologue in *P. knowlesi* (PKH\_052690), 53.15% to its orthologue in *P. cynomolgi* (PCYB\_053680) and 33.68% to its orthologue in *P. falciparum* (PF3D7\_0423400), while identity ranged from 23.61% to 22.22% regarding orthologues in *P. chabaudi* (PCHAS\_052400), *P. yoelii* (PY06454) and *P. berghei* (PBANKA\_052380). Such genes were located in a syntenic region, as corroborated by their open reading frame orientation and exon-intron structure. The foregoing supported the idea that the *Pvarp* gene has been derived from a common ancestor; however, experimental evidence concerning the functional role that the encoded protein might have in different parasite species remains to be determined.

#### The *Pvarp* gene is transcribed in schizonts

The presence of *Pvarp* gene transcripts in the *P. vivax* VCG-1 strain was confirmed by PCR using the cDNA from a parasite sample as template. Figure 1 shows the *Pvarp* gene amplification products (excluding the signal peptide-encoding region) (lanes 2–4) and the *Pvron1* gene's *a*

region (lanes 5–8) from cDNA and gDNA. A ~810 bp band (Figure 1; lane 2) obtained from cDNA amplification (RT+) showed that the *Pvarp* gene was transcribed in the schizont-enriched sample, similar to that reported in the transcriptional profile for the Sal-1 strain showing a maximum transcription level after 35 hours of intra-erythrocyte life cycle [31]. It was also confirmed that the *Pvarp* gene was encoded by a single exon once the sequences obtained from cDNA and gDNA products (Figure 1; lanes 2 and 4) had been aligned. The presence of a single ~1,053 bp band in *Pvron1-a* PCR (Figure 1; lane 6) indicated that the cDNA had not been contaminated by gDNA given that the expected product for the latter would have been ~1,559 bp (Figure 1; lane 8). No amplification was observed in the negative controls for each PCR (Figure 1; lanes 3 and 7 (RT-), and 5 (DNA-free water)).

Comparing *Aotus* monkey-adapted VCG-1 strain *Pvarp* gene sequences to those from the Sal-1 reference strain led to identifying four synonymous mutations, two non-synonymous ones producing aa changes (i.e., methionine (M) for asparagine (N) and glycine (G) for N in aa position 217 and 219, respectively) and a 12-base pair deletion related to an asparagine-methionine-asparagine-glycine (NMNG) repeat block (Table 1). It has been found that parasite proteins have both highly polymorphic and conserved regions; the former are the target for an immune response while conserved sequences implicated in interaction with cell receptors are usually not antigenic [32]. Considering that the latter regions might be suitable targets for blocking parasite entry to host cells, further studies aimed at evaluating *Pvarp* gene polymorphism in different isolates are required to determine which sequences could be used as components of a vaccine against malaria caused by *P. vivax*.

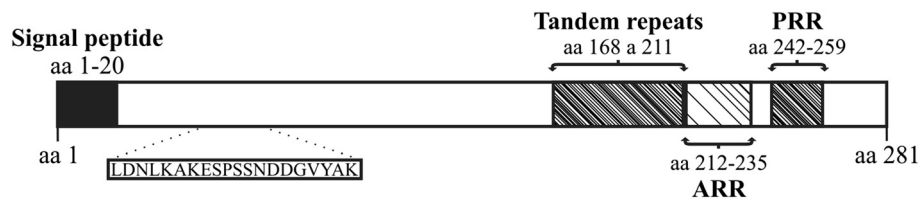
#### Characterizing *PvARP* in silico

The VCG-1 strain *Pvarp* gene encoded a 281 aa long protein having ~30 kDa molecular mass, this being 64 residues

**Table 1** Mutations found in VCG-1 strain *PvARP* nucleotide and amino acid sequences regarding the reference strain (Sal-1)

Base pairs*	Amino acids*	Mutations/Deletions	Changes in <i>Pvarp</i> nucleotide sequences in <i>P. vivax</i> strains		Changes in the <i>PvARP</i> amino acid sequences in <i>P. vivax</i> strains	
			Sal-I	VCG-1	Sal-I	VCG-1
456	152	Synonymous	CAT	CAC	H	-
600-611	200-204	Deletion	CATGAACGGAAA	-	NMNG	-
650	216	Synonymous	TAT	TAA	N	-
651	217	Non-synonymous	GAA	CAA	M	N
655-656	218	Synonymous	CGG	CAA	N	-
657	219	Non-synonymous	AAA	CAA	G	N
663	221	Synonymous	AAC	AAT	N	-

\*Nucleotide and amino acid positions are numbered according to *PvARP* in the Sal-I reference strain.



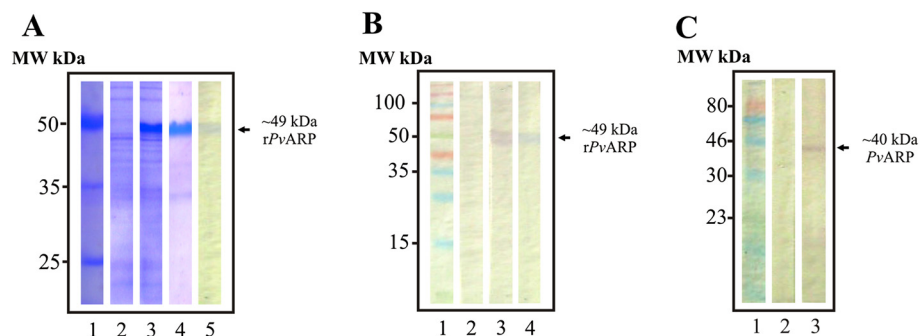
**Figure 2** *In silico* characterization of PvARP, showing signal peptide localization, tandem repeats (TR), asparagine (ARR) and proline (PRR) amino acid repeat regions and the peptide selected for the antibody purification assay (shown in the box).

longer when compared to its homologue *PfAARP* (217 aa) [18]. *PvARP* consists of 20% asparagine residues and has a signal peptide with a cleavage site between aa TNG-KS (Figure 2). A post-translational modification false positive consisting of a C-terminal glycosylphosphatidylinositol (GPI) anchor sequence has been predicted [17], differing to its *P. falciparum* homologue which has a true positive one. Asparagine- and proline-rich regions were found towards the C-terminal extreme of the protein sequence; the first of these covered residues 212 to 235, while the another one was found downstream between aa 242 and 259 (Figure 2). Additionally, a tandem repeat region (TR), a feature shared with other vaccine candidates described to date, was also found using XSTREAM software [26] (Figure 2); this region consisted of 11 repeat blocks from the (D/N/S)(V/M)NG consensus sequence found in aa 168 to 211. The sequence was seen to be exclusive for *P. vivax* and had mutations (two substitutions and four deletions), thereby suggesting that it was under pressure from the immune system. TR have been common in several *P. vivax* antigens described to date, which are mainly located on the surface or in apical organelles; these would include the circumsporozoite protein (CSP) [33], merozoite surface protein 9 (MSP-9) [34], *Pv34* [35] and rhoptry neck proteins 1 and 2 [23,36]. Even

though several studies have shown that the tandem repeats of *PvCSP* trigger an immune response when inoculated in primates and humans [33,37,38], the response so produced did not completely inhibit infection caused by the parasite. It has been shown in other *Plasmodium* species that TR could act as a smokescreen against the immune system, thereby diverting strong reactions towards functionally-relevant regions [39]; however, their exact role in *P. vivax* antigens remains unknown.

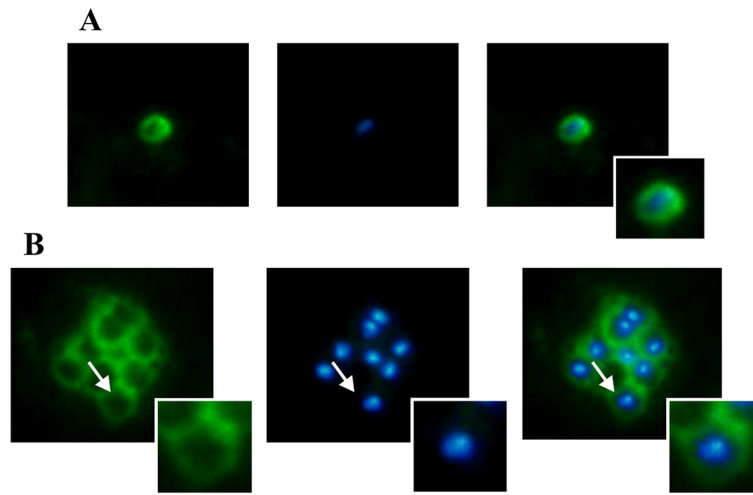
#### **PvARP expression in schizonts and subcellular localization**

Specific human antibodies against an N-terminal *PvARP* synthetic peptide (Figure 2) were used for checking protein expression and localization in the schizont-enriched sample. *PvARP* was recombinantly expressed excluding the signal peptide and then purified (Figure 3A). Once human anti-*PvARP*<sub>38582</sub> antibody ability to detect the recombinant protein in Western blot assays had been checked (Figure 3B), they were then used for detecting the protein on a blot containing parasite total lysate (Figure 3C). Both the parasite and recombinant *PvARP* proteins were detected above the expected weight (~40 and ~49 kDa, respectively), probably due to the presence of acidic aa (aspartic acid and glutamic acid) thereby causing



**Figure 3** Detecting recombinant and parasite protein by human antibodies. (A) Recombinant protein expression and purification. Lanes 2–3 show non-induced and induced cell lysate, respectively (Coomassie staining). Lanes 4–5 show purified rPvARP stained with Coomassie or analyzed by Western blot using anti-polyhistidine antibodies, respectively. (B and C) Antibody ability to recognize recombinant and parasite *PvARP* by Western blot, respectively. Lane 2 shows the absence of human serum reactivity after being pre-incubated with peptide 38582. Lane 3 indicates *PvARP* recognition. Lane 4 shows detection of recombinant protein (positive control). MW kDa indicates molecular weight marker in kDa.





**Figure 4** *PvARP* sub-cellular localization in mature schizonts. (A) Shows the detection of the protein on free merozoite surface. (B) *PvARP* labelling on mature schizonts. The nuclei are labelled with DAPI (blue). An amplified image of a merozoite (indicated by an arrow) is shown in small boxes.

anomalous migration on SDS-PAGE gel. The antibodies had specific reactivity to a ~40 kDa band; such reactivity was eliminated by using serum which had been pre-incubated with peptide 38582 (Figure 3C; lane 2).

A strong fluorescence signal, having an apparent concentration towards the apical pole, was found on free merozoites' surface and in mature schizonts when using the serum as primary antibody in the parasitized reticulocyte sample (Figure 4). The results led to the suggestion that *PvARP* could be expressed in apical organelles and then become relocated to the surface. However, other confocal or electron microscope assays are needed to determine the protein's exact localization pattern.

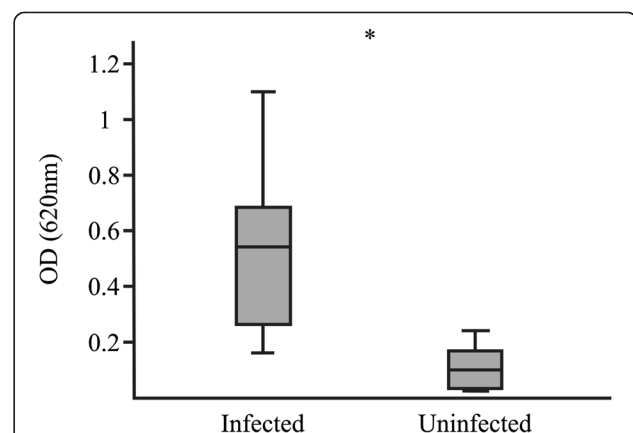
#### Antigenicity in humans

*PvARP* antigenic ability was evaluated by ELISA, using the sera from 38 patients who had suffered *P. vivax* malaria and 15 serum samples from people who had never suffered from the disease. The statistical test revealed a statistically significant difference between the medians ( $m$ ) of the groups (Wilcoxon rank-sum test.  $Z = 5.1$ ,  $p = 0.000$ ); it gave  $m = 0.5$  for the group of infected patients and  $m = 0.1$  for the control group (Figure 5), thereby corroborating the fact that the protein was able to trigger an antibody response in the host during natural *P. vivax* malaria infection, most sera being able to recognize native and recombinant protein, as demonstrated by IFA and Western blot, respectively. The results supported the idea of analyzing this protein's potential as a candidate for an anti-*P. vivax* vaccine.

#### Conclusions

This study has described how the *P. vivax* asparagine-rich protein was characterized. As demonstrated, *PvARP*

was conserved among different species belonging to the *Plasmodium* genus and shared some features of well-characterized surface and/or apical proteins being studied as candidates for a vaccine, such as prominent transcription and expression towards the end of the intra-erythrocyte life cycle and broad recognition by sera from patients infected with *P. vivax* malaria. The results supported the notion that this antigen could be a promising candidate for inclusion when developing an anti-malarial vaccine. Further immunogenicity assays and studies of the ability to induce protection in the experimental *Aotus* model are required.



**Figure 5** *rPvARP* antigenicity. The box diagram shows OD distribution (Y axis) for detecting *rPvARP* by sera from non-infected and infected individuals (X axis). \*: Infected individuals ( $n = 38$ ;  $\bar{X} \pm S = 0.5 \pm 0.2$ ; 95%CI = 0.16-1.1) and control ( $n = 15$ ;  $\bar{X} \pm S = 0.1 \pm 0.07$ ; 95%CI = 0.03-0.24).  $p$  value = 0.000.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

DAMP designed experiments, analyzed data and wrote the initial manuscript. AS carried out molecular biology and immunochemical assays. MAP designed, evaluated and coordinated the assays and corrected the final manuscript. All authors read and approved the final manuscript.

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# Characterising PvRBSA: an exclusive protein from *Plasmodium* species infecting reticulocytes

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## Abstract

**Background:** *Plasmodium vivax* uses multiple ligand-receptor interactions for preferential invasion of human reticulocytes. Several of these ligands have been identified by *in silico* approaches based on the role displayed by their orthologs in other *Plasmodium* species during initial adhesion or invasion. However, the cell adhesion role of proteins that are exclusive to species that specifically invade reticulocytes (as *P. vivax* and *P. cynomolgi*) has not been evaluated to date. This study aimed to characterise an antigen shared between *Plasmodium* species that preferentially infect reticulocytes with a focus on assessing its binding activity to target cells.

**Results:** An *in silico* analysis was performed using *P. vivax* proteome data to identify and characterise one antigen shared between *P. vivax* and *P. cynomolgi*. This led to identification of the *pvrbsa* gene present in the *P. vivax* VCG-I strain genome. This gene is transcribed in mature schizonts and encodes a protein located on the parasite surface. rPvRBSA was antigenic and capable of binding to a population of reticulocytes with a different Duffy phenotype. Interestingly, the molecule showed a higher percentage of binding to immature human reticulocytes (CD71<sup>hi</sup>).

**Conclusions:** This study describes for the first time, a molecule involved in host cell binding that is exclusive in reticulocyte-infecting *Plasmodium* species. This suggests that PvRBSA is an antigenic adhesin that plays a role in parasite binding to target cells.

**Keywords:** *Plasmodium vivax*, Antigenic protein, Adhesin, Reticulocyte

## Background

Basic research in *P. vivax* has been delayed, mainly due to difficulties associated with its *in vitro* propagation, resulting from the predilection of this species for invading immature erythrocyte cells (reticulocytes) [1, 2]. Consequently, bioinformatics approaches represent a good solution for identifying *in silico* vaccine candidates in *P. vivax* by comparative analysis, bearing in mind that many invasion-associated proteins from other *Plasmodium* species have already been described. Information derived from omics studies of *P. vivax* (genome [3], transcriptome [4] and proteome [5–8]) has been useful for large-scale

analysis of gene composition, transcripts and parasite proteins and, importantly, facilitate *in silico* predictions on the function of many *P. vivax* proteins.

Furthermore, *in silico* tools have been instrumental in characterising some *P. vivax* molecules interacting with reticulocytes, such as the Duffy binding protein (DBP) [9], reticulocyte binding proteins (RBP) [10–12], merozoite surface protein-1 (MSP-1) [13], rhoptry neck protein-5 (RON5) [14] and, recently, the *P. vivax* GPI-anchored micronemal antigen (GAMA) protein (manuscript in press). However, the number of *P. vivax* target cell binding proteins identified to date is low compared to available information on *P. falciparum*, suggesting that further studies are required to supplement the current set of *P. vivax* adhesin data, to improve our understanding of the molecular basis of parasite invasion.

Identifying *P. vivax* molecules with a role in host cell invasion by their similarity with proteins in *P. falciparum* has been a very promising approach. However, this has

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limitations when identifying those molecules involved in parasite recognition and invasion of reticulocytes. This study aimed to characterise a specific molecule from species infecting reticulocytes (e.g. *P. vivax* and *P. cynomolgi*) by determining its target cell binding profile.

## Methods

### Bioinformatics analysis, primer design and peptide synthesis

The currently available information published in *P. vivax* proteome studies [5–8] was used as the source for analysing *in silico* proteins which might be vaccine candidates. The criteria for selecting proteins included: a prominent expression of the codifying genes > 35 h post-invasion (required) according to transcriptome study of the *P. vivax* intra-erythrocyte life-cycle [4]; a positive prediction by SignalP 4.1 [15] and BaCellLo [16] of a secretion signal sequence and extracellular localisation, respectively; the presence (or not) of a GPI anchor sequence using FragAnchor software [17], as well as the presence of repeats having 90% similarity in amino acid (aa) sequences using T-REKS algorithm [18]. The Phobius [19], HMMTOP [20] and TMHMM [21] servers were used to predict transmembrane regions. The selected genes were analysed to identify orthologs in other *Plasmodium* species according to the PlasmoDB [22] and the Kyoto Encyclopedia of Genes and Genomes ortholog clusters (KEGG OC) [23] databases. The sequence of any gene selected for being characterised was scanned in the PlasmoDB database and used for manually designing specific primers (using Generunner software, version 3.05), the same as for B-cell linear epitopes all along their encoding sequence, predicting the highest average values for hydrophilicity, solvent accessibility and Parker's antigenicity using ANTHEPROT software [24].

### Propagating VCG-I strain parasites and isolating schizonts

Vivax Colombia Guaviare-I (VCG-I) strain parasites were propagated six years ago and used as the source of biologic material, as previously described in detail [25]. The blood sample containing parasite-infected cells was collected in heparin tubes and passed through a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden), according to an already-established protocol [26]. The schizont-stage enriched parasites were isolated from cells by incubating them for 5 min in 0.02 mM saponin buffer containing 7 mM  $K_2HPO_4$ , 1 mM  $NaH_2PO_4$ , 11 mM  $NaHCO_3$ , 58 mM KCl, 56 mM NaCl, 1 mM  $MgCl_2$  and 14 mM glucose, pH 7.5 and then washed extensively with PBS, pH 7.0.

### Extracting biological material

Isolated parasites were used as RNA, genomic DNA (gDNA) and total protein source. Total RNA was

extracted from the sample using the Trizol method and treated with *RQ1* (RNA-qualified) RNase-free DNase (Promega, Madison, USA) according to the manufacturer's recommendations. SuperScript III enzyme (RT+) (Invitrogen, Carlsbad, USA) was used for synthesising complementary DNA (cDNA) in the following conditions: 65 °C for 5 min, 50 °C for 1 h and 70 °C for 15 min. An additional reaction without the SuperScript III enzyme (RT-) was used as negative control, following 15 min incubation at 37 °C with RNase (Promega). A Wizard Genomic purification kit (Promega) was used for obtaining the gDNA. Regarding protein extraction, the parasites were homogenised in lysis buffer containing 5% SDS, 10 mM PMSE, 10 mM iodoacetamide, 1 mM EDTA and then spun at 16,000× *g* for 5 min. The proteins were recovered from the supernatant and quantified using a BCA protein assay kit (Thermo Scientific, Rockford, USA). RNA, cDNA, gDNA and total protein were stored at -70 °C until later use.

### Gene cloning and sequencing

The gDNA and cDNA (RT+ and RT-) samples were used as template in 25 µl PCR reactions containing 1× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA, USA), 0.3 µM primers and DNase-free water for completing the reaction volume. Specific primers were designed for amplifying the entire *P. vivax* reticulocyte binding surface antigen (*pvrbsa*) gene (Forward 5'-ATG AAA GGA ATA ATG AAT GG TT-3' and Reverse 5'-ATA ACC ATC CAA ATC GTC AAA-3') or for producing the recombinant protein excluding the signal peptide and the transmembrane region (Forward 5'-ATG ATA TTG TAC AGC GAC GAC TC-3' and Reverse 5'-GCT ATC TTT CTT CAC ATT ATA C-3').

The PCR began with a denaturing step at 98 °C for 3 min, followed by 35 cycles at 98 °C for 20 s, 56 °C for 15 s and 72 °C for 30 s. A Wizard PCR preps kit (Promega) was used for purifying gene amplicons obtained from three independent PCRs done with the RT+ and gDNA samples, once quality had been evaluated on agarose gel. Purified products were ligated to the pEXP5 CT/TOPO expression vector or in a new *in house* designed vector (pELMO) [27] for the gene obtained from gDNA and transformed in *E. coli* TOP10 chemically competent cells (Invitrogen). Several clones were grown for purifying the plasmid using an UltraClean mini plasmid prep purification kit (MO BIO Laboratories, California, USA). The insert integrity and correct orientation were then confirmed by sequencing, using an ABI-3730 XL sequencer (MACROGEN, Seoul, South Korea). ClustalW (NPS) software [28] was used for comparing manually the gene sequences from the Sal-I reference strain [3] and the primate-adapted VCG-I strain.

### Recombinant protein expression and extraction

*E. coli* BL21-DE3 (Invitrogen) cells which had been previously transformed with the recombinant plasmids were grown in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin, overnight at 37 °C using a Lab-line Incubator Shaker. The initial inoculum was seeded in 1 l LB and handled in the aforementioned conditions until reaching 0.5 OD<sub>600</sub>. After the culture was incubated on ice for 30 min, IPTG 1 mM was then used to induce expression for 16 h at room temperature (RT) at ~200 rpm. The cells were harvested by spinning at 2,400× *g* for 20 min and used for native extraction procedures. A new protocol for extracting proteins in a soluble form was used. Briefly, cellular pellet obtained from *E. coli* expressing PvRBSA was freeze/thawed for 3 cycles and then homogenised in native extraction buffer (NEB) (50 mM Tris-Cl, 300 mM NaCl, 25 mM imidazole, 0.1 mM EGTA and 0.25% Tween-20, pH 8.0). The mixture was then incubated for 1 h at 4 °C at 10 rpm using a tube rotator (Fisher Scientific, Waltham, USA) and the supernatant was collected by spinning at 16,000× *g* for 1 h.

### Protein purification

Solid-phase affinity chromatography was used for protein purification. The Ni<sup>2+</sup>-NTA resin (Qiagen, Valencia, CA, USA) was pre-equilibrated with NEB buffer, incubated with *E. coli* lysate overnight at 4 °C and the protein-resin mixture was then placed on a column. The unbound proteins were eluted by washing with 20 ml NEB buffer containing 0.1% Triton X-114 followed by 50 ml of the same buffer without detergent. Bound proteins were eluted with PBS containing imidazole at increasing concentrations (50 mM to 500 mM) in 3 ml fractions. The purification was confirmed by Coomassie blue staining and the fractions pooled and dialysed extensively in PBS, pH 7.2. The protein was quantified using a micro BCA protein assay kit (Thermo Scientific) and bovine serum albumin (BSA) as reference curve.

### Obtaining polyclonal antibodies

The VCG-I strain PvRBSA sequence was used for designing two 20 aa-long peptides (CG-KRNSSVSSLSDMGS YKNKS-GC (peptide 39478) and CG-VFGKGRKKPMK VKKGGGKIS-GC (peptide 39480)) which were then synthesised, according to a previously-established methodology [29], polymerised, lyophilised and characterised by RP-HPLC and MALDI-TOF MS. New Zealand rabbits were immunised with a 500 µg dose of each synthetic peptide emulsified in Freund's complete adjuvant (FCA) (Sigma, Missouri, USA) on day 0, whilst the same emulsified mixture in Freund's incomplete adjuvant (FIA) was inoculated on days 21 and 42. The pre-immune sera were collected before the first immunisation and hyper-immune sera were collected 20 days after the last dose.

Specific antibodies were purified by affinity chromatography using CNBr-activated Sepharose 4B (Amersham, Uppsala, Sweden). Briefly, 5 µmol of peptide were diluted in coupling buffer (0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl pH 8.3) and then incubated for 16 h at 4 °C with Sepharose resin. After washing ligand excess with 5 volumes of coupling buffer, the resin-free groups were blocked with 0.1 M buffer Tris-HCl for 2 h at quiescence at RT, followed by washing the resin 3 times with alternate pH solutions (0.1 M acetate buffer with 0.5 M NaCl, pH 4.0 and 0.1 M Tris-HCl with 0.5 M NaCl, pH 8.0). Five ml of each rabbit hyper-immune serum (diluted at 1:1 ratio with buffer coupling) were passed through the resin after being homogenised with PBS. Unbound antibodies were washed with 10 ml buffer coupling while strongly bound antibodies were eluted with 1 ml elution buffer (0.1 M glycine pH 7, 6, 5, 3.9 and 2.9) at descendant pH and neutralised with 1 M Tris pH 8.0 in a 1:9 ratio (elution buffer:neutralisation buffer). The antibodies were incubated with 45% ammonium sulphate for 1 h on ice with constant stirring and then for 16 h at 4 °C without shaking. After spinning at 16,000× *g* for 15 min, the pellet was homogenised in 100 µl PBS and the sample was extensively dialysed and stored at -20 °C until use.

### Protein localisation by indirect immunofluorescence (IFI)

Slides containing *Aotus* monkey infected reticulocytes were previously prepared, as described in previous work [30]. The samples were fixed and permeabilised by incubating them for 5 min at RT with PBS containing 4% paraformaldehyde (v/v) and then with PBS with 0.1% Triton X-100 (v/v). After blocking with 1% BSA-PBS solution (v/v) for 1 h at RT, each sample was incubated with anti-PvRBSA rabbit antibodies (1:30) or anti-PvRON2 mouse antibodies (1:20) in the same conditions. FITC-conjugated anti-rabbit IgG antibody (Sigma) at 1:30 dilution and Rhodamine-conjugated anti-mouse IgG antibody (1:200) monoclonal secondary antibodies were used for 1 h in darkness at RT. DAPI (0.5 µg/ml) was used for staining parasite nuclei for 10 min at RT and then was washed several times with PBS to remove excess reagent. The slides were examined under a fluorescence microscope (Olympus BX51) using 100× oil immersion objective.

### Western blot analysis of recombinant and parasite proteins

Total parasite and recombinant proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes which were blocked with 5% skimmed milk in TBS-0.05% Tween for 1 h. The membrane was cut into strips to be incubated for 1 h at RT with rabbit anti-PvRBSA purified antibodies (1:100 dilution) and then with the phosphatase-coupled goat anti-rabbit IgG

monoclonal secondary antibody (1:5,000) (Catalogue 9503 F, ICN) in the same conditions. The positive control for rPvRBSA Western blotting was a strip incubated with peroxidase-coupled mouse anti-histidine monoclonal antibody (1:4,500) (Catalogue A7058, Sigma). The blots were revealed with a BCIP/NBT colour development substrate kit (Promega) or VIP peroxidase substrate kit (Vector Laboratories, Burlingame, Canada) according to the manufacturers' indications. Each band's expected weight was determined by linear regression using XL-OptiProtein (Applied Biological Materials Inc, Richmond, BC, Canada) weight marker as reference.

#### Enzyme-linked immunosorbent assay (ELISA)

The recombinant protein was used for evaluating the presence of anti-rPvRBSA antibodies in samples taken from *P. vivax*-exposed individuals (who had suffered at least one episode of infection) in the municipality of Tierra Alta, Córdoba. The negative controls used here came from sera from healthy individuals who had never been affected by the disease. The ELISA was performed as described previously [30].

#### Cell binding assay

Cord blood samples were typed for determining the Duffy phenotype (Fya<sup>+</sup>/Fyb<sup>-</sup>; Fya<sup>-</sup>/Fyb<sup>+</sup>; Fya<sup>+</sup>/Fyb<sup>+</sup>) by standard blood banking methods using anti-Fya and Fyb sera. Five  $\mu$ L of cells were then incubated with 25  $\mu$ g rPvRBSA for 16 h at 4 °C at 4 rpm. DBP region II and III/IV were used as positive and negative controls, respectively [9]. After washing with 1% BSA-PBS solution (v/v), the sample was incubated with mouse anti-His-PE monoclonal antibody (1:40 dilution) (MACSmolecular-Miltenyi Biotec, San Diego, CA, USA) for 30 min in darkness. Reticulocytes and white cells were stained by incubating with anti-CD71 APC-H7 Clone M-A712 (1:80 dilution) (Becton Dickinson, Franklin Lakes, NJ, USA) and anti-CD45 APC clone 2D1 (1:80 dilution) (Becton Dickinson) monoclonal antibodies for 20 min at RT. A FACSCanto II cytometer (BD, San Diego, CA, USA) was then used for quantifying erythrocyte binding and FlowjoV10 software for analysing 1 million events. PE signal intensity was evaluated as a function of CD71 signal to determine CD71 low (CD71<sup>lo</sup>) and high (CD71<sup>hi</sup>) cells.

#### Statistical analysis

Statistical significance was assessed by comparing means, using a 0.05 significance level. Mann-Whitney U-test analysis was used for comparing the mean of the experimental group with the control in ELISA. Differences between means were compared by Tukey's range test when comparing multiple groups or *t*-test for comparing two groups for binding assays. GraphPad Software (San Diego, CA) was used for all statistical

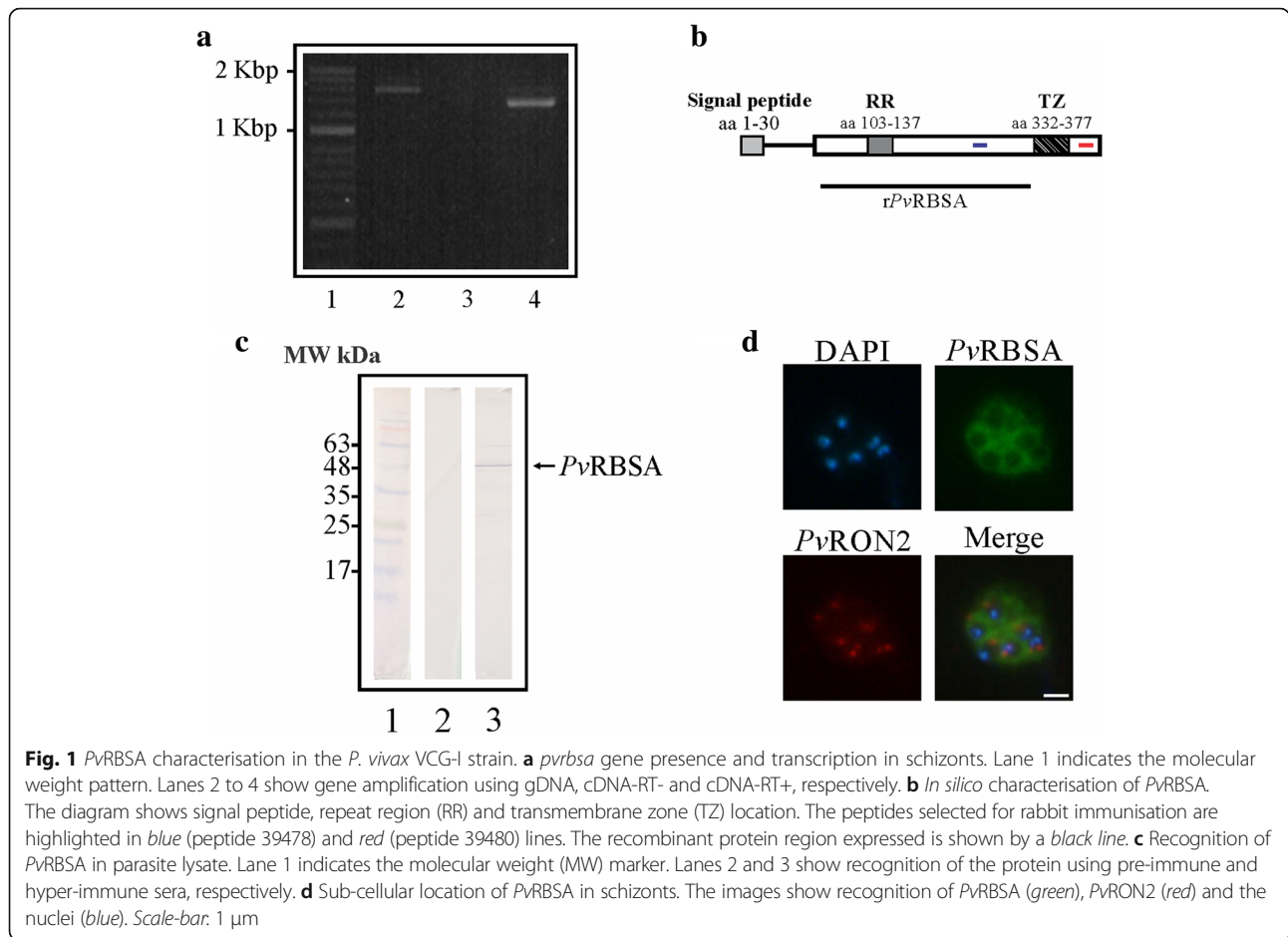
analysis. Mean values and standard deviations (SD) were calculated from the measurements of three independent experiments.

## Results

### Predicting *P. vivax* invasion-related proteins

The criteria established in the methodology led to identifying several genes encoding *P. vivax* molecules which play a role in cell binding (as previously reported), such as the RBPs [31], some RONS [14] and GAMA. Interestingly, one gene encoding a 48 kDa protein (PlasmoDB database ID: PVX\_096055) was identified which, apart from *P. vivax*, was also present in *Plasmodium cynomolgi* (one species infecting reticulocytes). This gene was named the *P. vivax* reticulocyte binding surface antigen (PvRBSA) according to the results showed in this study.

Regarding the *pvrbsa* gene, its presence and transcription in the *P. vivax* VCG-I strain was confirmed by PCR using specific primers (designed using the Sal-I strain gene sequence) and schizont gDNA and cDNA as template. Fig. 1a shows a 1.4 to 1.6 kbp amplification product using gDNA (Lane 2) corresponding to the complete gene whilst a 1.2 to 1.4 kbp product was obtained using cDNA as template (Lane 4). No product was amplified in the control sample, thereby indicating that the synthesised cDNA had not become contaminated with gDNA (Fig.1a, Lane 3). Aligning the gene sequences from the *Aotus* monkey-adapted VCG-I strain with those from the Sal-I reference strain led to one synonymous, 9 non-synonymous mutations and one deletion being identified (Table 1). Comparing the sequences obtained from cDNA (1,269 bp) (deposited in the NCBI under GenBank access KY349105) and gDNA (1,485 bp) led to observing that the *pvrbsa* gene was encoded by two exons, the first covering the signal peptide according to *in silico* prediction (D-cutoff = 0.450) (Fig. 1b). The *pvrbsa* gene encoded a 423 aa long protein having a molecular weight of around ~47.09 kDa including a signal peptide and being 7 residues shorter than that for the Sal-I strain. PvRBSA had 2 transmembrane regions located between residues 332 to 377 according to prediction by TMHMM, Phobius and HMMTOP servers and one repeat region (RR) located in aa 103 to 137, consisting of residues LT(G/E)S(N/R)ES as predicted by T-REKS (Fig. 1b); these have been numbered according to the VCG-I strain PvRBSA amino acid sequence. Amplifying *pvrbsa* from the synthesised cDNA sample confirmed that the gene was transcribed in schizonts, coinciding with transcriptional analysis of 3 *P. vivax* clinical isolates where *pvrbsa* had a prominent expression profile during TP7-TP9 times corresponding to parasite development during the mature stage (early and late schizonts) of the intra-reticulocyte life-cycle [4].



**Table 1** *PvRBSA* mutations found by comparing the nucleotide and amino acid sequences of *P. vivax* VCG-I and Sal-1 strains

Changes in <i>PvRBSA</i> nucleotide sequence in Sal-I and VCG-I <i>P. vivax</i> strains <sup>a</sup>	Changes in <i>PvRBSA</i> aa sequences in Sal-I and VCG-I <i>P. vivax</i> strains <sup>a</sup>	Mutation
c.29A > G	p.Tyr10Cys	Non-synonymous
c.391_411delCTAACAGGAAGTAATGAATCC	p.Leu131_Ser137del <sup>b</sup>	Deletion
c.533 T > G	p.Phe178Cys	Non-synonymous
c.796A > G	p.Lys266Gly	Non-synonymous
c.797A > G	p.Lys266Gly	Non-synonymous
c.801 T > A	p.Ser267Arg	Non-synonymous
c.803A > C	p.Tyr268Ser	Non-synonymous
c.808C > G	p.His270Asp	Non-synonymous
c.845C > T	p.Pro282Leu	Non-synonymous
c.1029 T > C	p.Lys343Lys	Synonymous
c.1091G > C	p.Trp364Ser	Non-synonymous

<sup>a</sup>Nucleotide and amino acid positions are numbered according to the Sal-I reference strain sequence alignment with the VCG-I strain

<sup>b</sup>Relative location for a region having 4 identical tandem repeats from amino acid position 103 to 130 in the VCG-I strain

### *PvRBSA* characterisation by molecular biology tools

Antibodies directed against 39478 and 39480 synthetic peptides were purified and used for evaluating the protein's presence and location in mature parasite forms (schizonts). Specific anti-*PvRBSA* antibodies detected one band in *P. vivax* VCG-I strain lysate treated in reduced conditions above the expected size by *in silico* analysis (43.8 kDa without the signal peptide) (Fig. 1c). Such discrepancy can be explained by anomalous migration caused by several acidic residues in the protein sequence (aspartic and glutamic acids). The antibodies also led to a surface fluorescence signal being visualised in mature schizonts like a "bunch of grapes", this being characteristic of proteins expressed on merozoite surface (Fig. 1d). There was no signal overlap for one apical marker (*PvRON2*). These findings led to suggesting that the *pvrbsa* transcript gave a protein product in *P. vivax* VCG-I strain schizonts, as shown in an earlier study by mass spectrometry analysis [6].

According to the classic approach, antigenic proteins should be considered for vaccine development given that a response against them could inhibit interaction with cells. Hence *rPvRBSA* was expressed, purified and



successfully obtained in soluble form (Additional file 1: Figure S1) to evaluate its antigenicity using sera from patients suffering *P. vivax* malaria and sera from people who had never suffered the disease. The screening gave 61% seropositivity in the patients group. The statistical test gave a significant difference between the means for recognition by the sera from the infected patients group ( $\bar{X} \pm SD = 0.38 \pm 0.24$ ) and the control group ( $\bar{X} \pm SD = 0.12 \pm 0.05$ ) (Mann-Whitney U-test:  $U = 52$ ,  $Z = -3.66$ ,  $P = 0.0001$ ) (Fig. 2), thereby highlighting that the protein was able to induce an immune response during natural infection.

#### PvRBSA interaction with human reticulocytes

Flow cytometry was used for quantifying rPvRBSA ability to bind cord blood reticulocytes using a gating strategy to exclude cell debris and select the CD71 + CD45<sup>-</sup> cell population (Fig. 3). The recombinant protein had a curve shift when PE signals from rPvRBSA binding assay and control (using CD71 + CD45<sup>-</sup> cells) were compared in a histogram. rPvRBSA bound to mature erythrocytes to a much lesser extent compared with reticulocytes (t-test:  $t_{(4)} = 13.74$ ,  $P = 0.0001$ ) (Fig. 4a, Table 2). The protein had similar binding activity to cells having a different Duffy phenotype ( $\bar{X} \pm SD = 9.17 \pm 1.4$ ) and to positive control ( $\bar{X} \pm SD = 23.8 \pm 9.8$ ) (ANOVA-Tukey:  $F_{(3,5)} = 2.43$ ,  $P = 0.181$ ), whilst there was a statistically significant difference in rPvRBSA binding activity compared to negative control ( $\bar{X} \pm SD = 2.0 \pm 0.34$ ) (Fig. 4b) (ANOVA-Tukey:  $F_{(3,5)} = 49.53$ ,  $P = 0.0001$ ). Interestingly, rPvRBSA had higher interaction with CD71<sup>hi</sup> than CD71<sup>lo</sup> cells

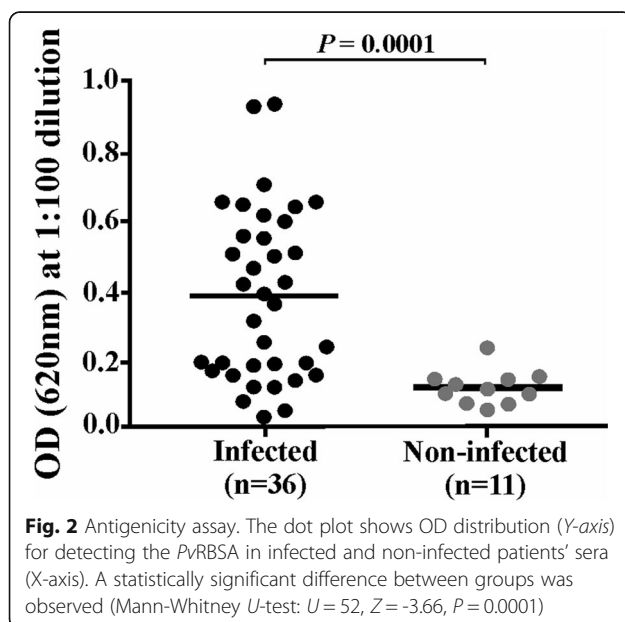
(Fig. 4c) (t-test:  $t_{(4)} = 16.44$ ,  $P = 0.0001$ ), suggesting that this molecule binds better to the more immature reticulocyte stages.

#### Discussion

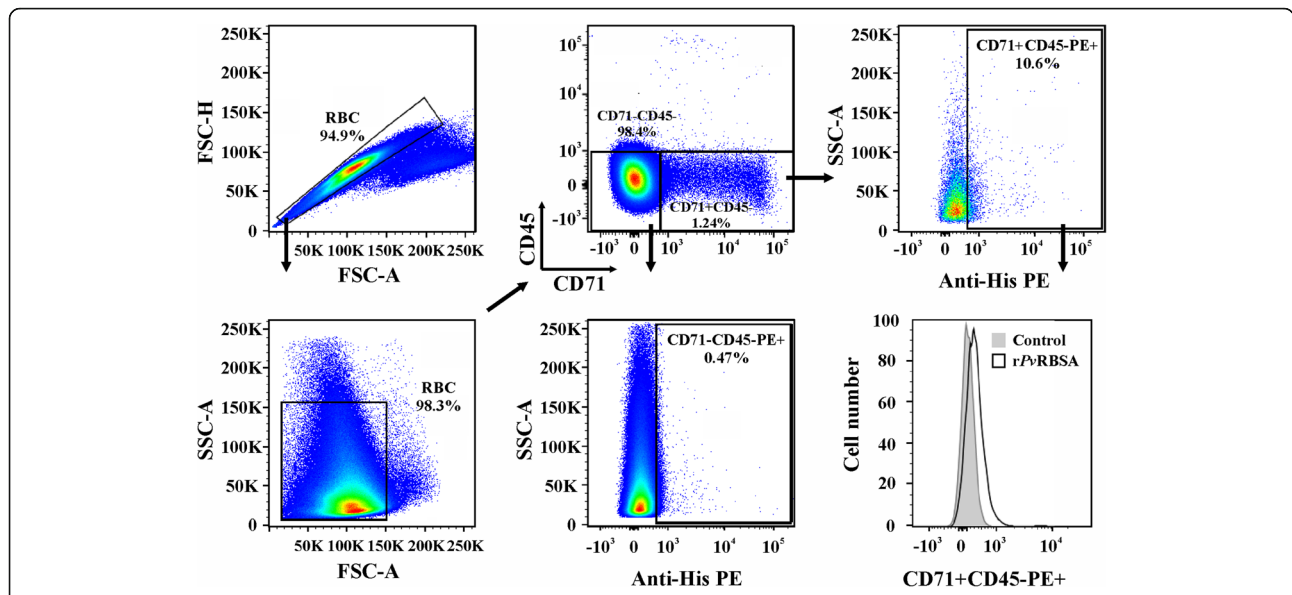
*Plasmodium vivax* has several proteins with essential functions in target cell binding and invasion. An important amount of such proteins has recently been identified in *P. vivax* by proteomics analysis which, combined with *in silico* analysis, has led to partly understanding the complex protein machinery used by the parasite and predicting the functions which some parasite proteins may have [5–8]. Exploiting the information available in proteome studies of *P. vivax*, a large-scale analysis was made for predicting protein vaccine candidates, taking into account the parameters described in the methodology. The screening identified PvRBSA, a molecule whose unique homologue is in *P. cynomolgi*, a species which invades reticulocytes and which is taxonomically very close to *P. vivax* [32].

The *in silico* analysis showed that PvRBSA has the characteristics of a good vaccine candidate, as reported for other parasite proteins. Two transmembrane regions were predicted. Transmembrane helices are usually 20 amino acids long, suggesting that the two helices identified for PvRBSA require a very tight loop to both fit into the membrane. Given these findings (predicted by several programmes), it was considered that the region spanning amino acids 332 to 377 is a transmembrane zone, though future investigation is necessary to ascertain their architecture.

In spite of the difficulty involved in basic research regarding *P. vivax*, given the intrinsic characteristics of its biology [1], the PvRBSA was characterised due to adapting the *P. vivax* VCG-I strain in primates [25], which led to sufficient biological material being obtained for developing the experimental assays. The methods used here showed that the *pvrbsa* gene was transcribed and translated for a surface protein in *P. vivax* VCG-I strain mature schizonts (Fig. 1a, d), thereby coinciding with the finding of PvRBSA peptides being detected in the first proteomic study in Colombia of a primate model-adapted *P. vivax* strain [6]. It has been found that parasite transcripts are strictly controlled during the development of the intra-erythrocyte life-cycle [4, 33] and that their codifying products correlate with having a specialised function. For example, more than 50 different *P. falciparum* transcripts having maximum expression during mature stages (>35 h post-invasion) encode proteins that play an important role during cell invasion [34]. The previous statement, added to the results concerning *pvrbsa* presence and expression in *P. vivax* schizonts, suggested that the molecule could have a function during reticulocyte adhesion.



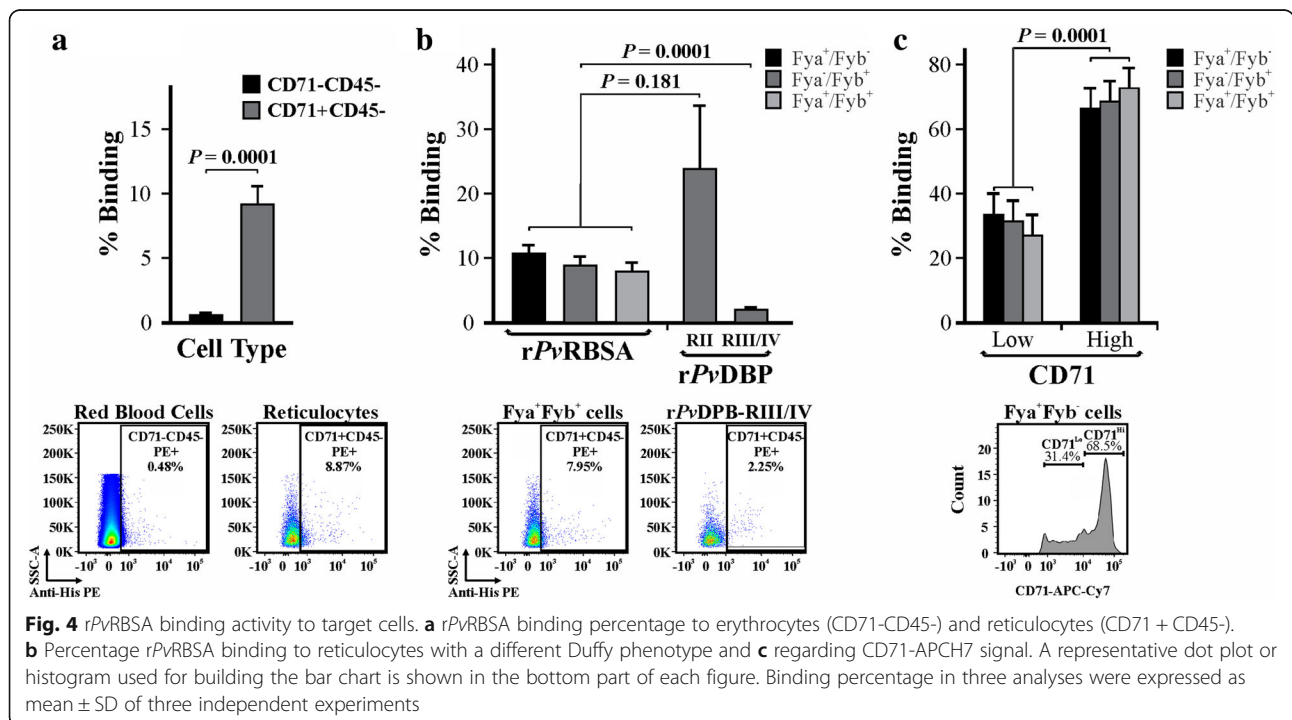
**Fig. 2** Antigenicity assay. The dot plot shows OD distribution (Y-axis) for detecting the PvRBSA in infected and non-infected patients' sera (X-axis). A statistically significant difference between groups was observed (Mann-Whitney U-test:  $U = 52$ ,  $Z = -3.66$ ,  $P = 0.0001$ )



**Fig. 3** Gating strategy for selecting reticulocytes and mature erythrocyte cell population. Doublets were excluded comparing FSC-H to FSC-A cytogram. Cells were then selected by their granularity by plotting SSC-A against FSC-A. The CD45 and CD71 signals were plotted for selecting reticulocyte (CD71 + CD45-) and mature erythrocyte (CD71-CD45-) populations. The cell percentage having bound protein was calculated using the PE signal against SSC-A. A representative histogram from three independent experiments analysing the PE signal for the rPvRBSA binding assay compared with the control is shown

Another important characteristic regarding proteins to be included in a vaccine is that they should be antigenic since it has been seen that an immune response induced during infection is related to naturally-acquired immunity [35, 36]. It was found that PvRBSA could trigger an immune response during natural *P. vivax* malaria

infection (Fig. 2), as described for other surface antigens in the *P. vivax* VCG-I strain, such as PvMSP-10 [37], Pv12 [38] and PvARP [30]. Once PvRBSA localisation pattern and ability to trigger an immune response had been determined, it was ascertained whether the protein could bind to the most immature human reticulocytes using anti-



**Fig. 4** rPvRBSA binding activity to target cells. **a** rPvRBSA binding percentage to erythrocytes (CD71-CD45-) and reticulocytes (CD71 + CD45-). **b** Percentage rPvRBSA binding to reticulocytes with a different Duffy phenotype and **c** regarding CD71-APCH7 signal. A representative dot plot or histogram used for building the bar chart is shown in the bottom part of each figure. Binding percentage in three analyses were expressed as mean  $\pm$  SD of three independent experiments

**Table 2** *rPvRBSA* binding percentage to mature and immature erythrocytes. The mean and standard deviation of three independent experiments is shown for each assay

Molecule	Phenotype	% Binding to mature erythrocytes	% Binding to reticulocytes
<i>rPvRBSA</i>	Fya <sup>+</sup> /Fyb <sup>-</sup>	0.47 ± 0.01	10.7 ± 1.29
	Fya <sup>-</sup> /Fyb <sup>+</sup>	0.48 ± 0.22	8.87 ± 0.65
	Fya <sup>+</sup> /Fyb <sup>+</sup>	0.79 ± 0.23	7.95 ± 1.94

CD71 monoclonal antibody (a specific marker for the cells [39]). *rPvRBSA* was able to interact with the youngest reticulocyte population (CD71<sup>hi</sup>) having different Duffy phenotypes in similar percentages (Fig. 4b). This binding pattern to cells with different Duffy phenotypes has also been reported for DBP [40].

On the other hand, although *rPvRBSA* was able to bind to mature erythrocytes, its interaction was much greater with reticulocytes (Fig. 4a, Table 2). Such preferential binding to this type of cells has also been observed in other *P. vivax* proteins such as DBP [9], MSP-1 [13], the erythrocyte binding protein (EBP) [12] and some RBPs [11, 41]. In the case of MSP-1, it was initially thought that target cell selection occurred at a later stage when RBPs were secreted. However, further receptor-ligand studies using *PvMSP-1*-derived 20-mer long peptides have shown that several peptides bind more strongly to reticulocytes than to erythrocytes, suggesting that this protein participates in the pre-selection of *P. vivax* target cells [13]. Furthermore, it has been shown that *Aotus* monkeys vaccinated with MSP-1 recombinant fragments containing reticulocyte-binding peptides have developed protective immunity against *P. vivax* challenge [42].

A recent study assessing five RBPs' target cell preference has shown the preferential binding to reticulocytes of just one of them (RBP2b). Interestingly, antibodies against RBP2b, acquired during natural *P. vivax* infection, have shown a strong protective effect [41]. These studies highlight the significant role for this type of molecule in interaction with *P. vivax* target cells. According to the results shown here, *PvRBSA* was localised on parasite surface and displayed a preferential binding profile for the more immature reticulocyte stages. It can thus be suggested that RBPs are not only participating in *P. vivax* preferential binding to reticulocytes (as was initially thought) but that other ligands are also pre-selecting this cell population, such as *PvMSP-1*, EBP, DBP and now, *rPvRBSA*.

## Conclusions

This study has described for the first time, an exclusive reticulocyte-infecting *Plasmodium* species molecule's characterisation and role in binding. The findings

highlight that *PvRBSA* is present in the *P. vivax* VCG-I strain genome, produces a transcript and encodes a protein having a surface location pattern. *PvRBSA* is antigenic and is an adhesin protein able to bind preferentially to human reticulocytes. Future studies should be undertaken aimed at assessing the protective efficacy induced when immunising with *PvRBSA* in the *Aotus* monkey experimental model.

## Additional file

**Additional file 1:** Recombinant *PvRBSA* purification. Lane 1: the proteins' molecular marker; Lanes 2–6: eluted protein using buffer with increasing concentration of imidazole (50 mM, 100 mM, 200 mM, 300 mM and 500 mM) stained with Coomassie blue; Lane 7: recognition of *rPvRBSA* by Western blot using anti-polyhistidine antibodies. (TIF 1539 kb)

## Abbreviations

ANOVA: Analysis of variance; CD71<sup>hi</sup>: CD71 high; CD71<sup>lo</sup>: CD71 low; cDNA: Complementary DNA; DBP: Duffy binding protein; EBP: Erythrocyte binding protein; ELISA: Enzyme-linked immunosorbent assay; GAMA: GPI-anchored micronemal antigen; gDNA: Genomic DNA; LB: Luria-Bertani; MSP-1: Merozoite surface protein-1; NEB: Native extraction buffer; OD: Optical density; *PvRBSA*: *P. vivax* reticulocyte binding surface antigen; RBP: Reticulocyte binding protein; RON5: Rhoptry neck protein-5; RT: Room temperature; SD: Standard deviation; VCG-I: Vivax Colombia Guaviare I

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## Availability of data and materials

All data generated or analysed during this study are included within this article and its additional file.

## Authors' contributions

DAMP devised and designed the study; DAMP, LAB and DMCA performed the experiments; DAMP, LAB and MAP analysed the results; DAMP and MAP wrote the manuscript. All authors read and approved the final manuscript.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Ethics approval and consent to participate

New Zealand rabbits were handled in strict accordance with Colombian Law 84/1989 and resolution 504/1996 and EU Directive 2010/63/EU for animal experiments, following established guidelines for the care and use of laboratory animals (National Institute of Health, USA). All efforts were made to minimise animal suffering. Sera were collected from 36 patients who had suffered episodes of *P. vivax* infection as well as from 11 healthy individuals who had never been affected by the disease. The newborn umbilical cord blood samples used in this research were collected by the Hemocentro Distrital (Bogotá). All individuals (progenitors regarding umbilical cord samples) signed an informed consent form after having received detailed information regarding the study's goals. All procedures were approved by FIDIC's ethics committee.

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RESEARCH

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# PvGAMA reticulocyte binding activity: predicting conserved functional regions by natural selection analysis

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## Abstract

**Background:** Adhesin proteins are used by *Plasmodium* parasites to bind and invade target cells. Hence, characterising molecules that participate in reticulocyte interaction is key to understanding the molecular basis of *Plasmodium vivax* invasion. This study focused on predicting functionally restricted regions of the *P. vivax* GPI-anchored micronemal antigen (PvGAMA) and characterising their reticulocyte binding activity.

**Results:** The *pvgama* gene was initially found in *P. vivax* VCG-I strain schizonts. According to the genetic diversity analysis, PvGAMA displayed a size polymorphism very common for antigenic *P. vivax* proteins. Two regions along the antigen sequence were highly conserved among species, having a negative natural selection signal. Interestingly, these regions revealed a functional role regarding preferential target cell adhesion.

**Conclusions:** To our knowledge, this study describes PvGAMA reticulocyte binding properties for the first time. Conserved functional regions were predicted according to natural selection analysis and their binding ability was confirmed. These findings support the notion that PvGAMA may have an important role in *P. vivax* merozoite adhesion to its target cells.

**Keywords:** Adhesin protein, *Plasmodium vivax*, Genetic diversity, Conserved functional region, Reticulocyte binding activity

## Background

*Plasmodium vivax* is a human malaria-causing parasite whose eradication is a priority on the international health agenda [1]. As a strategy for eradicating this species, several research groups have focused their efforts on developing a vaccine, as vaccination has been successful at controlling and eradicating other infectious diseases [2].

It has been suggested that vaccines should consist of key proteins or their fragments used by infectious agents to bind to the target cells [3, 4]. Hence, knowledge of proteins expressed by the parasite at the end of its intra-erythrocyte life-cycle, especially those interacting with

red blood cells (RBC), should prove most suitable as candidate vaccine components.

Current efforts to develop an anti-malarial vaccine have mainly focused on *P. falciparum*, given the availability of robust in vitro culturing techniques for this parasite (currently unavailable for *P. vivax*) which has led to a large-scale identification of genes [5], transcripts [6] and proteins [7]. This information has led to an improved understanding of the molecules involved in *P. falciparum* merozoite invasion of erythrocytes. For example, several adhesin molecules have been described in the apical organelles (rhoptries and micronemes), that facilitate interaction with cell receptors and promote parasite internalisation within the target cell [8]. Several of these proteins are immunogenic and are being evaluated as vaccine candidates in clinical studies [9]. The GPI-anchored micronemal antigen (GAMA) represents one apical protein that has an adhesive role in *Plasmodium* and *Toxoplasma*. *Plasmodium falciparum* GAMA (PfGAMA) binds to human erythrocytes, an interaction

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mediated by its binding region which is located in the amino terminal sequence, and is involved in the sialic acid-independent invasion pathway [10]. On the other hand, GAMA knockouts of *T. gondii* (*TgGAMA*) show a reduction in the ability of tachyzoites to attach to the host cell during invasion as well as a delay in the time to death in an in vivo model, suggesting a function during parasite adhesion and invasion [11].

Unfortunately, basic *P. vivax* research has been delayed mainly due to the parasite's preference for invading reticulocytes which are difficult to obtain in the high percentages needed for propagating *P. vivax* in vitro [12, 13]. However, it has been possible to characterise several molecules forming part of the parasite's selective human reticulocyte invasion route, such as reticulocyte binding proteins (RBPs) [14, 15], merozoite surface protein 1 (MSP-1) [16], some proteins from the tryptophan-rich antigen (TRAg) family [17] and the recently described rhoptry neck protein 5 (RON5) [18]. Some of these contain specific binding regions that have been identified using several strategies, such as mapping using peptides labelled with radioactive iodine, ELISA, flow cytometry or rosetting assays. However, these methodologies are laborious when large molecules must be analysed. Furthermore, sometimes it is not known whether these regions are polymorphic between isolates, which would be counterproductive for the development of a broadly protective vaccine.

A new strategy has recently been proposed for identifying selection signals and that enables the determination of conserved antigens or those having potential functional regions [19]. Cornejo et al. [20] and Garzón-Ospina et al. [19] identified natural selection signals in *P. vivax* genes when analysing the sequences of five genomes from different locations [21]. These results were supported by earlier studies, increasing the number of sequences analysed [22–24]. This type of analysis could therefore provide a viable approach for selecting conserved antigens that are subject to functional restrictions. However, no experimental evidence has been produced to support such approach.

Given the importance of conserved functional region prediction and the role of adhesin proteins during host-parasite interaction, and considering the interesting features displayed by GAMA in other apicomplexa, the present study aimed at characterising *P. vivax* VCG-I strain GAMA functional regions by selection signal prediction and then determine the role of such regions in binding to reticulocytes.

## Methods

### An approach to GAMA genetic diversity and evolutionary forces

Evolutionary methods compare the non-synonymous mutations rate ( $d_N$ , mutations altering protein sequences)

to the synonymous mutations rate ( $d_S$ , those encoding the same amino acid) in the search for natural selection signals. Deleterious mutations are usually removed from populations by negative natural selection ( $d_N < d_S$  or  $\omega < 1$ ). Regions displaying this kind of selection might have functional/structural importance, maintaining high sequence conservation between species [25]. On the other hand, mutations having an adaptive advantage (or a beneficial role) are fixed in a population by positive natural selection ( $d_N > d_S$  or  $\omega > 1$ ). Taking the above into account, functional regions could be predicted by evolutionary approaches [19]. *pvgama* gene DNA sequences from 6 *P. vivax* strains (VCG-I, Sal-I, Brazil-I, India-VII, Mauritania-I and North Korea [21]) and 5 phylogenetically-related species (*P. cynomolgi*, *P. inui*, *P. fragile*, *P. knowlesi* and *P. coatneyi*) [26] were obtained by tblastn (except for VCG-I) from the whole-genome shotgun contigs (wgs) NCBI database for assessing genetic diversity and evolutionary forces regarding GAMA. The MUSCLE algorithm [27] was used to align the sequences and the alignment was manually corrected. Nucleotide diversity per site ( $\pi$ ) was estimated from the *P. vivax* sequences and the modified Nei-Gojobori method [28] was used to assess natural selection signals by calculating the difference between synonymous and non-synonymous substitution rates ( $d_N-d_S$ ). Natural selection was also assessed by estimating the difference between synonymous and non-synonymous divergence rates ( $K_N-K_S$ ) using sequences from *P. vivax* and related species through the modified Nei-Gojobori method and Jukes-Cantor correction [29]. Specific codons under natural selection amongst species were identified using codon-based Bayesian or maximum likelihood approaches (SLAC, FEL, REL [30], MEME [31] and FUBAR [32]), following recombination by the GARD method [33]. Codon-based methods estimate the evolutionary rate ( $\omega$ ) at each codon using a statistical test to determine whether  $\omega$  is significantly different to 1 (neutral evolution). The Branch-site REL algorithm [34] was used to identify lineages under episodic positive selection (selection occasionally having transient periods of adaptive evolution masked by negative selection or neutral evolution). The Datamonkey web server was used to perform these analyses [35].

### Primer design, cloning and sequencing

The *Plasmodium vivax gama* (*pvgama*) gene sequence was taken from the PlasmoDB database [36] and scanned for PCR priming sites (Table 1) using Generunner software (version 3.05). Primers were designed to amplify either the entire *pvgama* gene or several smaller-sized fragments according to the natural selection analysis (Fig. 1). The gDNA (extracted using a Wizard Genomic purification kit; Promega, Madison, USA) and cDNA (synthesised with SuperScript III enzyme (RT+) (Invitrogen,

**Table 1** Primer designed for *pvgama* gene amplification

Target	Primer sequence (5' – 3') <sup>a</sup>	MT (°C)	Product size (bp)	aa position
<i>pvgama</i>	Fwd: ATGAAGTGCAACGCCTCC Rev: AAAAATGAATAGGAGCAACG	58	2313	1 to 771
<i>pvgama</i> -Nt	Fwd: ATACGGAATGGAACAACC Rev: AGTCGGTTCGTTATTCTCG		1284	22 to 449
<i>pvgama</i> -Ct	Fwd: CTGCTCAAGAACACGAAC Rev: GCTTCCACTCTGCAATTC		948	434 to 749
<i>pvgama</i> -CR1	Fwd: GACGATCATCTGTGTTCAAAAA Rev: GACCTCATTTTTGGACTTCTC	60	666	87 to 308
<i>pvgama</i> -VR1	Fwd: GGCGCCTTCTGCAGTC Rev: CATTAAATGGTGTGTCGCT		438	330 to 475
<i>pvgama</i> -CR2	Fwd: CAGGCGGCCATCTACTAA Rev: GCTCCCGTTGACGCCCTT		321	482 to 588
<i>pvgama</i> -VR2	Fwd: GCCGCAAACGCAGACGCC Rev: GTTTGCCGAGAAGCTTCCAC		384	626 to 753

Abbreviations: Nt and Ct amino and carboxyl terminal; CR conserved region, VR variable region; Fwd forward, Rev reverse, MT melting temperature, bp base pair, aa amino acid

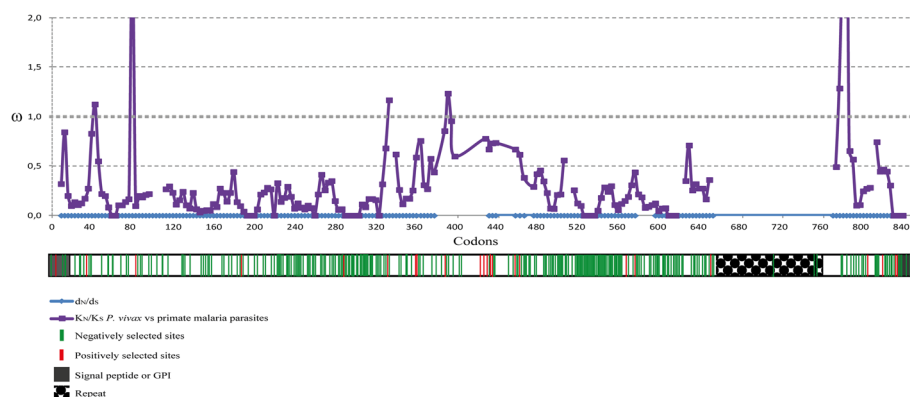
<sup>a</sup>Protein's expression start codon was included in forward primer's 5' end

Carlsbad, USA) samples from *P. vivax* VCG-I strain schizont-stage enriched parasites (propagated and obtained as previously described [37, 38]) were used as template in 25  $\mu$ l PCR reactions containing 1 $\times$  KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA, USA), 0.3  $\mu$ M primers and DNase-free water. Temperature cycling for PCR involved a denaturing step of 95 °C for 5 min, followed by 35 cycles of 98 °C for 20 s, T<sub>m</sub> °C (Table 1) for 15 s and 72 °C for 30 s or 1 min and 30 s depending on product size. A Wizard PCR preps kit (Promega) was used for purifying amplicons obtained from PCR with the RT+ and gDNA samples, once quality had been evaluated on agarose gel. Purified products were ligated to the pEXP5 CT/TOPO expression vector or pGEM (Promega) (for the gene obtained from gDNA) and transformed in TOP10 *E. coli* cells (Invitrogen). Several clones

obtained from independent PCR reactions were grown for purifying the plasmid using an UltraClean mini plasmid prep purification kit (MO BIO Laboratories, California, USA). Insert integrity and correct orientation were then confirmed by sequencing, using an ABI-3730 XL sequencer (MACROGEN, Seoul, South Korea). ClustalW (NPS@) software was used for comparing gene sequences from Sal-I reference strain and the primate-adapted VCG-I strain [39]. The *pvgama* gene sequence from *P. vivax* VCG-I strain was deposited in NCBI under accession number KT248546.

### Recombinant protein expression

The pEXP-*pvgama* recombinant plasmids were transformed in *E. coli* BL21-DE3 (Invitrogen), according to the manufacturer's recommendations. Cells were grown



**Fig. 1** Evolutionary rate ( $\omega$ ) sliding window. Intra-species  $\omega$  values ( $d_N/d_S$ ) are represented in blue whilst inter-species  $\omega$  values ( $K_w/K_s$  between *P. vivax* and malarial parasites infecting primates) are shown in purple. A  $\omega$  value equal 1 means neutral evolution,  $\omega < 1$  negative selection whilst  $\omega > 1$  means positive selection. A diagram of the gene can be observed below the sliding window. Negatively selected inter-species codons are shown in green whilst positively selected sites are shown in red. Numbering is based on the alignment in Additional file 1: Figure S1

overnight at 37 °C in 50 ml Luria Bertani (LB) medium containing 100 µg/ml ampicillin using a Lab-line Incubator Shaker. The initial inoculum was then seeded in 1 l of LB with ampicillin (100 µg/ml) and left to grow at 37 °C with shaking at ~300× rpm until reaching 0.5 OD<sub>600</sub>. The culture was incubated on ice for 30 min and then IPTG 1 mM was used to induce expression by incubation for 16 h at room temperature (RT) with shaking at ~200× rpm. The culture was then spun at 2400× g for 20 min and the pellet was collected for extraction of the recombinant protein.

#### Denaturing extraction

The cell pellet obtained from *E. coli* expressing PvGAMA-Nt and PvGAMA-Ct fragments was homogenised in denaturing extraction buffer (DEB) (6 M urea, 10 mM Tris, 100 mM NaH<sub>2</sub>PO<sub>4</sub> and 20 mM imidazole) containing the SIGMAFAST protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) and then lysed by incubating with 0.1 mg/ml lysozyme overnight at 4 °C at 10× rpm using a tube rotator (Fisher Scientific, Waltham, USA). The supernatant was collected by spinning at 16,000× g for 1 h.

#### Native extraction

PvGAMA-CR1, PvGAMA-VR1, PvGAMA-CR2 and PvGAMA-VR2 were extracted using a method for obtaining the molecules in native conditions with the respective positive and negative controls (region II and III/IV from the Duffy binding protein, DBP) (unpublished data). Briefly, the pellet was frozen/thawed for 3 cycles and then homogenised in native extraction buffer (NEB) (50 mM Tris, 300 mM NaCl, 25 mM imidazole, 0.1 mM EGTA and 0.25% Tween-20, pH 8.0). The mixture was incubated for 1 h at 4 °C at 10× rpm and the supernatant was collected by spinning at 16,000× g for 1 h.

#### Protein purification

Total lysate supernatant was incubated with Ni<sup>2+</sup>-NTA resin (Qiagen, Valencia, CA, USA) for purifying the proteins by solid-phase affinity chromatography, once protein expression had been verified by western blot. Briefly, the resin was pre-equilibrated with the respective buffer used for extracting proteins and then incubated with the *E. coli* lysate overnight at 4 °C. The protein-resin mixture was placed on a column and then weakly bound proteins were eluted by washing with 20 ml buffer containing 0.1% Triton X-114 followed by 50 ml of the same buffer without detergent. The proteins extracted in denaturing conditions were dialysed on the column by passing 20 ml DEB with urea in descending concentrations (6 M, 3 M, 1.5 M, 0.75 M and PBS). Bound proteins were then eluted with PBS containing imidazole at increasing concentrations (50 mM to

500 mM) in 3 ml fractions; those having a single band (confirmed on 12% SDS-PAGE by Coomassie blue staining and by western blot using anti-polyhistidine antibodies) were pooled and dialysed extensively in PBS, pH 7.2. A micro BCA protein assay kit (Thermo Scientific, Rockford, USA) was used for quantifying each protein, using the bovine serum albumin (BSA) curve as reference.

#### Peptide synthesis

One 6 histidine peptide was synthesised according to a previously-established methodology [40], polymerised, lyophilised and characterised by RP-HPLC and MALDI-TOF MS. The peptide was homogenised in PBS and then stored at -20 °C until use.

#### Blood sample collection and processing

Individuals with a clinical history of *P. vivax* (37 subjects) or *P. falciparum* (30 subjects) malaria, aged 18 to 50 year-old and living in malaria-endemic areas of Colombia (Chocó, Nariño, Córdoba, Vichada and Guaviare) were selected for this study. Sera from healthy individuals (16 adult subjects) who had never been affected by the disease and who were living in non-endemic areas were used as negative controls. The blood samples were collected in BD Vacutainer tubes without anticoagulant by personnel from the Fundación Instituto de Inmunología de Colombia (FIDIC) from October 2006 to March 2011 (for *P. vivax*) and June to October 1993 (for *P. falciparum*) and stored at 4 °C until transport. Samples were then transported to Bogotá for processing. Total blood was spun at 5000× g for 5 min and the serum was then recovered and stored at -80 °C in FIDIC serum bank (to date).

#### Enzyme-linked immunosorbent assay (ELISA)

PvGAMA antigenicity was evaluated in triplicate using serum from patients who had suffered episodes of *P. vivax* or *P. falciparum* infection. Briefly, 96-well polysorb plates were covered with 1 µg rPvGAMA-Nt, or rPvGAMA-Ct, overnight at 4 °C and then incubated at 37 °C for 1 h. The dishes were blocked with 200 µl 5% skimmed milk - PBS-0.05% Tween for 1 h at 37 °C. Antibody reactivity against the recombinant protein was evaluated by incubating the plates with 1:100 dilution of each human serum in 5% skimmed milk - PBS-0.05% Tween for 1 h at 37 °C. The dishes were incubated with peroxidase-coupled goat anti-human IgG monoclonal secondary antibody (1:10,000) (Catalogue 1222H, ICN) diluted in 5% skimmed milk - PBS-0.05% Tween for 1 h at 37 °C and then a peroxidase substrate solution (KPL Laboratories, Gaithersburg, MD, USA) was added to reveal the reaction, according to the manufacturer's recommendations. Optical density (OD) at 620 nm (detected by MJ ELISA Multiskan Reader) was



calculated by subtracting the OD value obtained from the control well value (no antigen). The cut-off value for evaluating the positivity threshold was determined by taking the average of the OD plus twice the standard deviation ( $\pm 2SD$ ) of healthy individuals' sera reactivity.

#### Cord blood sample processing

The newborn umbilical cord blood samples used in this research were collected by personnel from the Hemocentro Distrital (Bogotá) and then processed by SEPAX Cell Processing System (Biosafe, Eysins, Switzerland) to reduce nucleated cells, according to the manufacturer's recommendations. The samples were stored at 4 °C and Duffy antigen receptor for chemokines (DARC) presence was determined by agglutination assay using antibodies directed against the molecule's Fya or Fyb fraction. The percentage of nucleated cells was scored in 20 fields at 100 $\times$  magnification using Wright's stain before carrying out the binding assay.

#### Cell binding assay

Reticulocyte binding was tested in triplicate by flow cytometry and using the total cells from cord blood sample (Fya<sup>-</sup>Fyb<sup>+</sup> phenotype). Briefly, 5  $\mu$ l samples were incubated with 25  $\mu$ g of each recombinant protein (PvGAMA-CR1, PvGAMA-VR1, PvGAMA-CR2 and PvGAMA-VR2) for 16 h at 4 °C at 4 $\times$  rpm. Twenty-five  $\mu$ g of DBP region II and III/IV were used as positive and negative controls, respectively. The 6 histidine peptide was also used as control once the recombinant proteins contained a 6-histidine tag. A binding inhibition assay was also performed by incubating PvGAMA conserved recombinant proteins (CR1 and CR2) with a mixture of human sera (1:10 dilution) for 1 h at 4 °C before putting them in contact with cells. The samples were then incubated with mouse anti-His-PE monoclonal antibody (1:40 dilution) (MACSmolecular-Miltenyi Biotec, San Diego, CA, USA) for 30 min in the dark after washing with 1% BSA-PBS solution (v/v). White cells and reticulocytes were stained by incubating with anti-CD45 APC clone 2D1 (1:80 dilution) (Becton Dickinson, Franklin Lakes, NJ, USA) and anti-CD71 APC-H7 clone M-A712 (1:80 dilution) (Becton Dickinson) monoclonal antibodies for 20 min at RT. Subsequently, reticulocyte (CD71 + CD45-PE+) and mature erythrocyte (CD71-CD45-PE+) binding was quantified by analysing 1 million events using a FACSCanto II cytometer (BD, San Diego, CA, USA) and Flowjo V10 software. PE signal intensity in the reticulocyte population was evaluated regarding CD71 signal to determine CD71 low (CD71<sup>lo</sup>) and high (CD71<sup>hi</sup>) cells.

#### Statistical analysis

Mean values and standard deviations (SD) were calculated from the measurements of three independent experiments. Statistical significance was assessed by

comparing means using a 0.05 significance level for testing a stated hypothesis. Student's *t*-test and analysis of variance (ANOVA) were used for comparing the means of each experimental group to those for control. Tukey's multiple comparison test was used for multiple comparison of experimental group means to those for control. GraphPad Software (San Diego, CA) was used for all statistical analysis.

## Results

### PvGAMA genetic diversity and selection signals

*Pvgama* sequences were obtained from genomes of 5 different strains from different geographical regions (North Korea, Brazil, Mauritania and India). These were aligned with the VCG-I strain sequence and orthologous sequences from 5 phylogenetically-related species. The alignment revealed a size polymorphism in *pvgama* due to the [C/T]C[G/C]C[A/T]AA[C/T][C/G][A/G/C][G/A]AC[G/C/A] repeat which was not present in *P. cynomolgi*, *P. inui*, *P. fragile*, *P. knowlesi* or *P. coatneyi* (Additional file 1: Figure S1). Regarding *P. vivax*, 5 segregating sites and  $\pi = 0.0008$  were observed.

No significant values were found when evaluating synonymous and non-synonymous substitution rates ( $d_N-d_S = -0.001$  (0.001),  $P > 0.1$ ). However, synonymous divergence was greater than non-synonymous divergence ( $P < 0.0001$ ) when comparing *pvgama* sequences to each related species:  $K_N-K_S$  *P. vivax/P. cynomolgi* = -0.041 (0.006);  $K_N-K_S$  *P. vivax/P. inui* = -0.062 (0.008);  $K_N-K_S$  *P. vivax/P. fragile* = -0.030 (0.006);  $K_N-K_S$  *P. vivax/P. knowlesi* = -0.072 (0.009);  $K_N-K_S$  *P. vivax/P. coatneyi* = -0.049 (0.007). The evolutionary rate  $\omega$  ( $d_N/d_S$  and  $KN/KS$ ) sliding window showed that two highly conserved regions amongst species (codons 80–320 and 514–624) might be under negative selection ( $\omega < 0.5$ ). Furthermore, 308 negatively-selected codons were observed amongst species (Fig. 1); a lot of them were in the conserved regions. The Branch-site REL algorithm identified episodic positive selection signals in the lineages giving rise to *P. knowlesi* and *P. coatneyi* as well as the lineage formed by *P. cynomolgi* and *P. fragile* (Additional file 2: Figure S2). 22 sites showed evidence of positive selection amongst species (Fig. 1).

### Antigenic response was directed against the GAMA carboxyl fragment

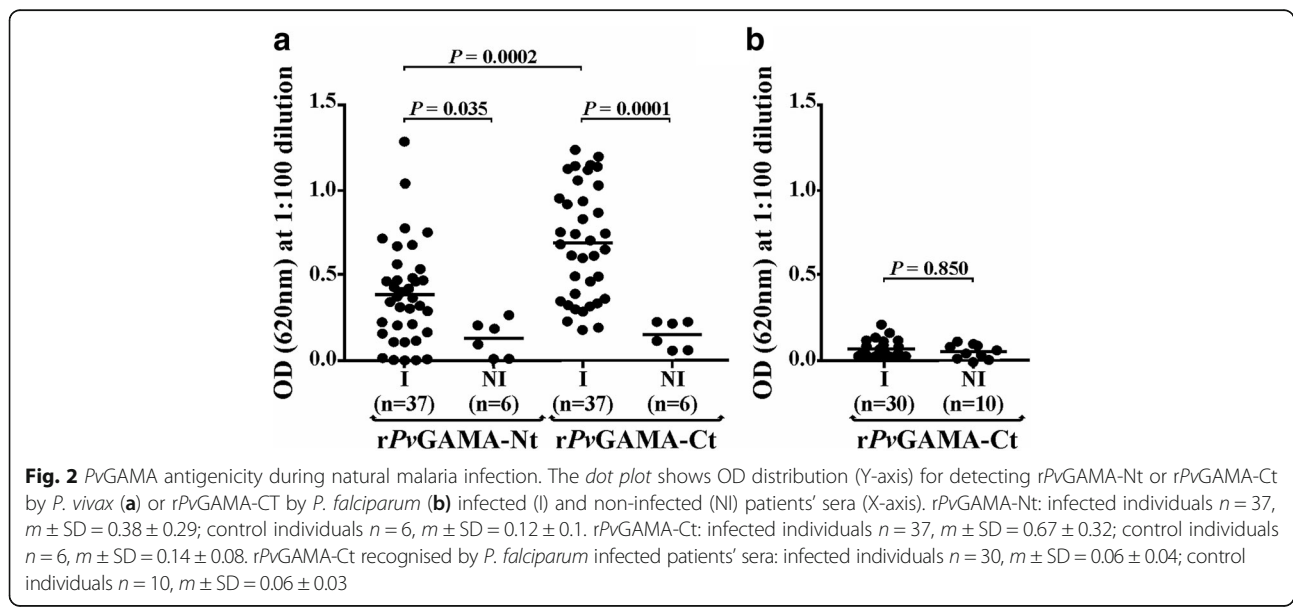
Based on the polymorphism analysis results, it was hypothesised that the carboxyl region was more antigenic than the amino one by the presence of the repetitive region. Hence, rPvGAMA-Nt and rPvGAMA-Ct antigenicity (obtained recombinantly; Additional file 3: Figure S3a, b) was evaluated using sera from 37 patients suffering of *P. vivax* malaria and sera from people who had never suffered the disease. rPvGAMA-Nt reacted

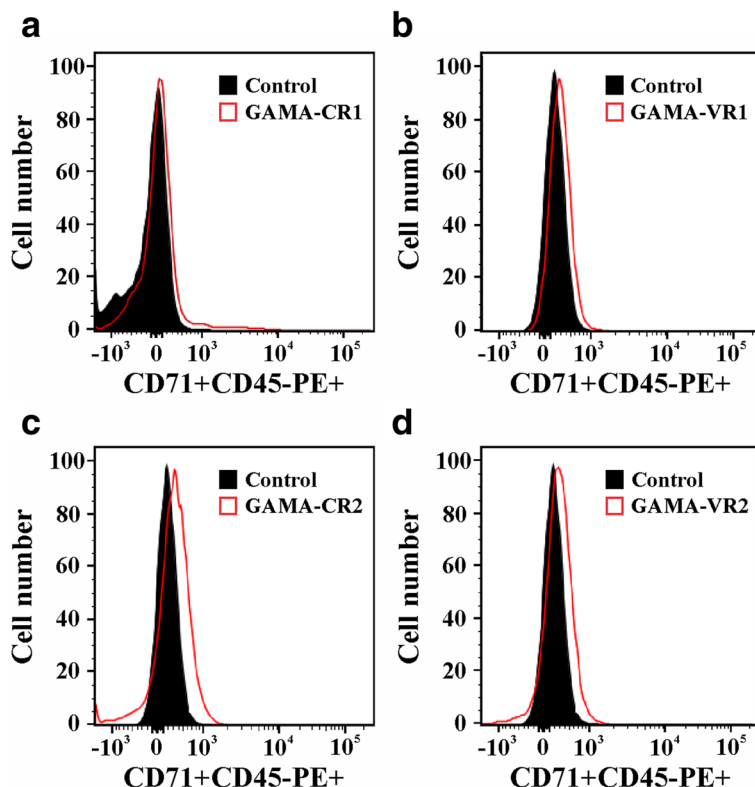
positively with 64.8% of the sera in screening (0.26 cut-off point) whilst 67.5% of them recognised rPvGAMA-Ct (0.47 cut-off point). These data agreed with a study of the profile of the humoral immune response for *P. vivax* in which rPvGAMA was recognised by 54.5% of the sera used in the array [41]. The statistical test for the assay with rPvGAMA-Nt gave a significant difference between the means (*m*) of the groups (ANOVA:  $F_{(1,41)} = 4.73$ ,  $P = 0.035$ ;  $m = 0.38$  for the group of infected patients and  $m = 0.12$  for the control group). Likewise, there was a significant difference between the means of the groups (ANOVA:  $F_{(1,41)} = 14.75$ ,  $P = 0.0001$ ;  $m = 0.67$  for the group of infected patients and  $m = 0.14$  for the control group) when rPvGAMA-Ct was detected by human sera (Fig. 2a). There was also a statistically significant difference when analysing the means of recognition for rPvGAMA-Nt and rPvGAMA-Ct (ANOVA:  $F_{(1,72)} = 16.01$ ,  $P = 0.0002$ ). Taking into account that the response was higher against PvGAMA-Ct, it was decided to confirm whether the antibodies generated during *P. falciparum* natural infection were able to detect this fragment. No significant difference (ANOVA:  $F_{(1,38)} = 0.036$ ,  $P = 0.850$ ) was seen for PvGAMA-Ct recognition by these sera (Fig. 2b). The significant reactivity against the recombinants by *P. vivax*-infected individuals' sera indicated that the protein could trigger an antigenic response during natural infection, this being higher and species-specific against the PvGAMA carboxyl region.

**PvGAMA bound to human reticulocytes**

Red blood cell samples having the Fya<sup>-</sup>Fyb<sup>+</sup> phenotype (Duffy +) taken from umbilical cord blood were incubated with conserved (CR1 and CR2) and variable (VR1 and VR2) regions extracted and purified in their

soluble form (Additional file 3: Figure S3c), predicted by natural selection analysis and then evaluated by flow cytometry to quantify the protein-cell interaction. The percentage of each recombinant binding to erythrocytes was calculated using the gating strategy described in Additional file 4: Figure S4, which enabled selecting the mature (CD71-CD45-) or immature (CD71 + CD45-) cell population to which a target protein was bound (labelled with anti-His PE antibody). All recombinant proteins had a curve shift when the PE signal was compared to control (cells not incubated with recombinant proteins) in the histogram (Fig. 3). Interestingly, the GAMA fragments bound to reticulocytes to a much higher percentage compared to mature erythrocytes (CR1: *t*-test:  $t_{(4)} = 24.9$ ,  $P = 0.0001$ ; VR1: *t*-test:  $t_{(4)} = 9.02$ ,  $P = 0.001$ ; CR2: *t*-test:  $t_{(4)} = 12.4$ ,  $P = 0.0001$ ; VR2: *t*-test:  $t_{(4)} = 24.8$ ,  $P = 0.0001$ ) (Fig. 4a). The conserved regions showed highest interaction with the reticulocytes compared to negative binding controls (ANOVA-Tukey:  $F_{(6, 12)} = 72.64$ ,  $P < 0.0001$ ). CR2 recombinant protein bound to 10.11% (SD = 1.33) of target cells, which was very similar to the positive control ( $m \pm SD = 11.8 \pm 1.15$ ) ( $P > 0.189$ ), whilst CR1 were able to bind to 6.36% (SD = 0.30) of the cells (Fig. 4a). Regarding PvGAMA variable regions, VR1 was able to bind to 3.08% (SD = 0.54) of the reticulocytes whilst VR2 bound 5.64% (SD = 0.37). CR1, CR2 and VR2 fragments had the highest interaction with CD71<sup>hi</sup> reticulocytes when binding percentages were analysed as a function of CD71 APC-H7 signal (CR1: *t*-test:  $t_{(4)} = 7.32$ ,  $P = 0.002$ ; CR2: *t*-test:  $t_{(4)} = 16.04$ ,  $P = 0.0001$ ; VR2: *t*-test:  $t_{(4)} = 3.71$ ,  $P = 0.021$ ), unlike VR1 and DBP-RII (VR1: *t*-test:  $t_{(4)} = 1.52$ ,  $P = 0.202$ ; DBP-RII: *t*-test:  $t_{(4)} = 0.19$ ,  $P = 0.853$ ) (as previously found [42])





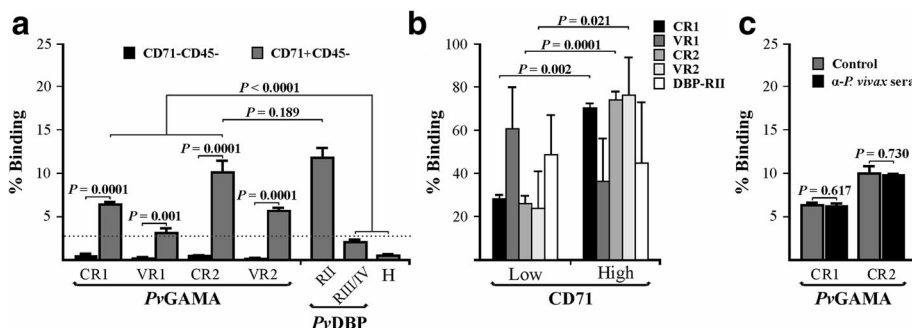
**Fig. 3** Flow cytometry analysis. Histograms of conserved (a and c) and variable (b and d) GAMA fragments compared to control (cells not incubated with the protein). Each figure is representative from three independent experiments

(Fig. 4b). These findings suggested that GAMA in *P. vivax* has a functional role in preferential interaction with human reticulocytes.

**Natural antibodies did not affect PvGAMA binding activity**

A cytometry adhesion inhibition assay was performed with sera from individuals suffering *P. vivax* malaria to determine whether the antibodies produced during natural infection could inhibit functional conserved regions (CR1

and CR2) interaction with reticulocytes. Figure 4c shows that conserved recombinant proteins pre-incubated with human sera were able to bind to target cells (CR1:  $m \pm SD = 6.21 \pm 0.27$ ; CR2:  $m \pm SD = 9.83 \pm 0.09$ ), giving a similar percentage to that for controls (CR1:  $m \pm SD = 6.5 \pm 0.08$ ; CR2:  $m \pm SD = 10.01 \pm 0.95$ ) (CR1:  $t$ -test:  $t_{(3)} = 0.55$ ,  $P = 0.617$ ; CR2:  $t$ -test:  $t_{(4)} = 0.37$ ,  $P = 0.730$ ), suggesting that the immune response was directed against regions which are not implicated in cell binding.



**Fig. 4** PvGAMA human reticulocyte binding activity. Flow cytometry analysis showing the recombinant binding percentage to CD71-CD45- and CD71 + CD45- cells (a) and regarding CD71-APCH7 signal (only for CD71 + CD45- cells) (b). Positive (DBP-RII) and negative (DBP-RIII/IV and H (6 histidine peptide)) binding controls are also shown. c CR1 and CR2 reticulocyte binding inhibition assay using human sera ( $\alpha$ -*P. vivax* sera). Binding percentage in both analyses were expressed as mean  $\pm$  SD of three independent experiments

## Discussion

Merozoite invasion of erythrocytes involves the participation of several parasite molecules expressed at the end of the intra-erythrocyte lifecycle, mainly those contained in the apical organelles, such as the rhoptries and micronemes [8]. Only a few of these molecules possessing a reticulocyte binding role in *P. vivax* have been identified and their binding domains mapped, suggesting an urgent need for performing further studies to supplement current knowledge on *P. vivax* adhesins. This will improve our understanding of the molecular basis of parasite invasion of reticulocytes. This study aimed at using natural selection analysis for identifying GAMA functional regions playing a potential role in reticulocyte binding.

According to the phylogenetic analysis, a repeat region (RR) localised between amino acids 591 and 695 consisting of residues [A/L]AN[A/G][N/D] was predicted. This RR was common in different *P. vivax* strains but not in phylogenetically-related species (Additional file 1: Figure S1). This characteristic has been found in several *P. vivax* antigens described in the *P. vivax* VCG-I strain located on the parasite surface (Pv12 [12], ARP [43]) or in the apical pole (Pv34 [44], RON1 [45], RON2 [46] and RON4 [47, 48]). DNA sequences from different *P. vivax* strains and phylogenetically-related species were thus compared to ascertain whether *gama* gene diversity has been modulated by immune pressure. Evidence of episodic positive selection was found in some parasite lineages (Additional file 2: Figure S2). As shown for other antigens [49–51], the episodic selection found in GAMA could be the outcome of adaptation to different hosts during malaria-primate evolution [50, 51]. Therefore, the insertions found in *P. vivax* could be an adaptation of the species to humans since the RR in malaria are associated with evasion of the host's immune response, making such response become directed against functionally unimportant regions [52, 53]. This hypothesis was supported by the fact that rPvGAMA-Ct (where the RR is located) can trigger a species-specific immune response (Fig. 2) which did not inhibit CR2 binding activity to reticulocytes (Fig. 4c).

Polymorphic regions induce high levels of strain-specific antibodies (allele specific) whilst conserved regions (directly implicated in interaction with cell receptors) are usually non-antigenic [54]. Therefore, the immune response must be directed against conserved regions to avoid different parasite strains evading immunity, thereby reducing vaccine efficacy. According to the selection signal identification strategy, low genetic diversity was found in the GAMA-encoding gene, comparable to that observed in *msp4* [55, 56], *msp7A/7 K/7 F/7 L* [57, 58], *msp8* [59], *msp10* [57, 59], *pv12*, *pv38* [22, 24], *pv41* [23, 24], *rap1/2* [60] and *ron4*

[48] which seem involved in host cell invasion. Despite the lack of statistically significant values for  $d_N-d_S$  difference,  $K_S$  divergence amongst species was greater than  $K_N$ , suggesting negative selection. Many codons were found to be experiencing negative selection which probably plays an important role in GAMA evolution. Two regions along the antigen were highly conserved amongst species, giving a  $< 0.5$  evolutionary rate ( $\omega$ ) (Fig. 1).

Given the polymorphism and selection analysis, it was decided to determine PvGAMA conserved and variable region interaction with reticulocytes to validate the *in silico* prediction of functional regions (Figs. 3 and 4) and elucidate the protein's function. A reticulocyte sample having a Duffy positive phenotype was used, given that PvGAMA reportedly has a binding role regardless of such antigen's expression [61]. Unlike Cheng and his group, the anti-CD71 monoclonal antibody was included for identifying GAMA regions' preference for immature reticulocyte binding as *P. vivax* merozoites have tropism for this cell type (characterised by the expression of the CD71 receptor [62]). Given that the CD71 marker is also present in activated lymphocytes, a nucleated cell depleted umbilical cord blood sample was used. The anti-CD45 was also included to totally exclude the lymphocytes from the analysis once the Wright staining revealed 0.4% of such cells (also confirmed by cytometry analysis) (Additional file 4: Figure S4). It was also confirmed that there was no difference in reticulocyte percentage by incubating the samples for 4 and 16 h at 4 °C (4 h:  $m \pm SD = 1.24 \pm 0.27$ ; 16 h:  $m \pm SD = 1.31 \pm 0.07$ ) ( $t$ -test:  $t_{(2)} = 0.32$ ,  $P > 0.777$ ). However, it was decided to use a prolonged incubation time to enable complete protein-cell interaction.

It was found that all PvGAMA fragments bound to mature erythrocytes (CD71-CD45-) though to a lesser extent compared to reticulocytes (CD71 + CD45-) (Fig. 4a), thereby supporting the fact that the protein preferentially interacts with the latter cell type. The conserved fragment located in the carboxyl region (CR2) had higher reticulocyte binding than the amino one (CR1) (Fig. 4a) coinciding with that shown recently for PvGAMA where this fragment [F2 (aa 345 to 589) or F7 (408 to 589) regions in that study] showed higher rosetting activity, unlike the F1 region (aa 22 to 344) (amino fragment) [61]. Interestingly, CR1 and CR2 had higher CD71<sup>hi</sup> reticulocyte binding percentages than to CD71<sup>lo</sup> (Fig. 4b), suggesting that GAMA mainly binds to such cell type's most immature stage. It has been reported that some reticulocytes' integral membrane components decrease as cells mature [63]. Therefore, the findings found here suggest that PvGAMA receptor is less abundant in CD71<sup>lo</sup> cells unlike CD71<sup>hi</sup>, as a consequence of cell maturation. The fact that more than 69% of the CD71 + CD45- cells were CD71<sup>lo</sup> ( $m \pm$

SD = 69.3 ± 3.3) can be the explanation of why PvGAMA fragment binding to 100% of the CD71+ reticulocytes was not found (Fig. 4a). It has been observed that several *P. vivax* proteins, such as DBP [64], MSP-1 [16], RBP1 [14], the erythrocyte binding protein (EBP) [42], RBP1a, RBP1b [65] and RBP2 [15], have preferential reticulocyte binding activity, being the RBPs particularly important in parasite cell selection. Taking the results obtained here into account, it can be suggested that *P. vivax* target cell selection is not only governed by the RBPs but other ligands are also taking place in this process, such as DBP, MSP-1, EBP and now PvGAMA.

Immunoreactive proteins are considered potential candidates for developing a vaccine as it has been seen that an immune response induced during infection is related to naturally-acquired immunity [66]. Antigenicity is thus one of the classical parameters for selecting molecules when developing a vaccine. Although there was an immune response against PvGAMA (Fig. 2), this was not sufficient to inhibit the conserved regions binding to reticulocytes (Fig. 4c). It has been observed that *P. falciparum* proteins' conserved regions (implicated in target cell binding) cannot trigger an immune response when used as vaccine candidates in the *Aotus* model whilst non-conserved ones trigger protective responses upon parasite challenge but those are strain-specific [54]. Accordingly, the PvGAMA antibodies produced/induced during natural *P. vivax* infection were directed against immunodominant epitopes which are unimportant in binding activity. Bearing in mind that functional regions usually evolve more slowly and that natural negative selection tends to keep these regions conserved amongst species [25], our experimental findings suggested that CR1 and CR2 located between residues 80–320 (40% of negatively selected sites) and 514–624 (64.5% of negatively selected sites) are functionally/structurally restricted and that vaccine design should thus be focused on them.

## Conclusions

To our knowledge, this study described PvGAMA reticulocyte binding properties for the first time. The PvGAMA antigenic response was principally directed against its carboxyl fragment which comprises by a repetitive region. On the other hand, it was shown that PvGAMA consists of two conserved binding fragments that bind preferentially to most immature human reticulocytes, which is consistent with the *P. vivax* invasion phenotype and highlights the fact that functional regions can be predicted by analysing natural selection. Further studies aimed at discerning the function of conserved regions as vaccine components are required.

## Additional files

**Additional file 1: Figure S1.** GAMA antigen alignment. *pvgama* sequences from 6 *P. vivax* strains were aligned with orthologous sequences from *P. cynomolgi*, *P. inui*, *P. fragile*, *P. coatneyi* and *P. knowlesi*. a DNA sequence alignment. b Deduced amino acid alignment. The sequences were obtained from GenBank: access numbers being India-VII AFBK01000586-AFBK01000587, North Korean AFNJ01000531, Brazil-I AFMK01000508-AFMK01000509, Mauritania-I AFNI01000333-AFNI01000334, *P. inui* NW\_0084818881, *P. fragile* NW\_012192586, *P. cynomolgi* BAEJ01000249, *P. coatneyi* CM0028561 and *P. knowlesi* NC\_0119061. (PDF 373 kb)

**Additional file 2: Figure S2.** Lineage-specific positive selection. Branches under positive episodic selection were identified by using the REL-site branch method. Episodic selection acts very quickly and involves a switch from negative to positive natural selection and back to negative and might enable adaptation to a new host. Phylogeny was inferred in MEGA v6 by the maximum likelihood method using the GTR + G evolutionary model.  $\omega^+$  model:  $\omega$  rate values. Pr ( $\omega = \omega^+$ ): percentage of sites evolving under positive selection. *P*-value corrected for multiple tests using the Holm-Bonferroni method. (TIF 470 kb)

**Additional file 3: Figure S3.** Obtaining recombinant proteins. a, b Recombinant GAMA protein expression and purification. Lanes 2–3 show non-induced and induced cell lysate, respectively. Lanes 4–5 show purified rPvGAMA-Nt and -Ct stained with Coomassie blue or analysed by western blot using anti-polyhistidine antibodies, respectively. c Purifying conserved (CR1 and CR2) and variable (VR1 and VR2) PvGAMA regions. Lanes 2, 4, 6 and 8 show purified recombinant proteins and lanes 3, 5, 7 and 9 show western blot detection. The proteins' molecular markers are indicated in Lane 1 on all figures. (TIF 5327 kb)

**Additional file 4: Figure S4.** Selection strategy for immature and mature erythrocyte populations. The doublets were excluded by plotting FSC-H against FSC-A. Cells were then selected by their granularity, using an SSC-A vs FSC-A cytogram. The CD45 vs CD71 signal was plotted for selecting reticulocyte (CD71 + CD45-) and mature erythrocyte (CD71-CD45-) populations and omitting activated lymphocytes (CD71 + CD45+). The percentage of cells having bound protein was calculated using the PE signal (CD71 + CD45-PE+). A representative histogram from three independent experiments analysing the PE signal for the CR2 binding assay compared to control is also shown. (TIF 10448 kb)

## Abbreviations

ANOVA: Analysis of variance; CD71<sup>hi</sup>: CD71 high; CD71<sup>lo</sup>: CD71 low; CR: Conserved region; DARC: Duffy antigen receptor for chemokines; DBP: Duffy binding protein; DEB: Denaturing extraction buffer; EBP: Erythrocyte binding protein; ELISA: Enzyme-linked immunosorbent assay; LB: Luria bertani; MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight; MS: Mass spectrometry; MSP-1: Merozoite surface protein 1; NEB: Native extraction buffer; OD: Optical density; PBS: Phosphate buffered saline; PvGAMA: *P. vivax* GPI-anchored micronemal antigen; *Pvgama*: *Plasmodium vivax gama*; RBP: Reticulocyte binding protein; RON5: Rhoptry neck protein 5; RP-HPLC: Reverse phase high-performance liquid chromatography; RR: Repeat region; RT: Room temperature; SD: Standard deviation; TRAg: Tryptophan-rich antigen; VCG-I: Vivax Colombia Guaviare 1; VR: Variable region

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**Availability of data and materials**

All data generated or analysed during this study are included within this article and its additional files. The *pvgama* sequence from *P. vivax* VCG-1 strain was deposited in the GenBank database under accession number KT248546.

**Authors' contributions**

LAB and DAMP devised and designed the study; LAB, DAMP, DGO, JFR and HDOS performed the experiments; LAB, DAMP, DGO and MAP analysed the results and wrote the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

All individuals who participated in this research (including progenitors regarding umbilical cord samples) signed an informed consent form after receiving detailed information regarding the study's goals. All procedures were approved by FIDIC's ethics committee.

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# CONCLUSIONES, RECOMENDACIONES Y PERSPECTIVAS GENERALES

## Conclusiones

En esta investigación, se analizó el proteoma de *P. vivax* y se describieron algunas de las proteínas expresadas en los estadios del ciclo de vida intraeritrocítico (anillo, trofozoito y esquizonte). Se identificaron 514 nuevas proteínas no reportadas en estudios anteriores, lo que permite aumentar el conocimiento de la composición proteica de *P. vivax*. Además, se encontraron moléculas involucradas en el metabolismo parasitario, en la variabilidad antigénica y en la capacidad de invadir los eritrocitos. El estudio del proteoma de la cepa VCG-1 de *P. vivax* presenta una importante fuente de información para la selección y el estudio de moléculas que pueden ser útiles en la prevención o el tratamiento de la infección causada por *P. vivax*.

Por otro lado, se realizó una primera aproximación para identificar el proteoma de eritrocitos del primate *A. nancymaae*. Los análisis comparativos realizados permiten conocer el perfil proteómico de los eritrocitos del primate y realizar análisis e interpretación de sus características moleculares; las proteínas identificadas serán útiles en la investigación biomédica futura. Además, los resultados validan el uso de primates de la especie *A. nancymaae* como modelo experimental para estudiar la malaria. Indudablemente, la predicción de componentes integrales de membrana proporciona una gran información para entender detalladamente el mecanismo de interacción entre los eritrocitos y los parásitos del género *Plasmodium*, en un modelo experimental con gran similitud al humano. Vale la pena destacar, que son los primeros estudios de proteómica realizados en Colombia donde se estudia, tanto el proteoma de una cepa de *P. vivax* adaptada a primates, como el de eritrocitos de *A. nancymaae*.

Adicionalmente, se propuso una estrategia racional para la selección de las moléculas potencialmente involucradas en el proceso de invasión celular. Con este enfoque, se identificaron *PvARP*, *PvRBSA* y *PvGAMA*, las cuales se caracterizaron en la cepa VCG-1 de *P. vivax* a nivel molecular. Además, se estandarizó por primera vez una técnica para obtener las moléculas de forma soluble, utilizando el sistema de expresión *E. coli*. Se validaron dos criterios clave para



considerar las proteínas como potenciales vacunas: la antigenicidad y capacidad de unión a las células hospederas. Así, *PvARP*, *PvRBSA* y *PvGAMA* son capaces de desencadenar una respuesta inmune durante la infección natural. De éstas, *PvRBSA* y *PvGAMA* presentaron actividad de unión a reticulocitos, al igual que se demostró en previos estudios para otras moléculas como *PvDBP*, *PvMSP-1*, *PvRBPs* y *PvRON5*. Los hallazgos destacan que *PvRBSA* y *PvGAMA* se componen de regiones que están implicadas en la interacción específica con las células diana, destacando su importancia para futuros estudios orientados a desarrollar estrategias de control contra la enfermedad.

Este estudio contribuye a aumentar el conocimiento básico sobre la biología de *P. vivax*, sobre todo en la caracterización de moléculas con actividad de unión celular. Se puede deducir de este estudio que la predicción *in silico* es una herramienta útil, de bajo costo y eficiente para identificar moléculas involucradas en la interacción de los merozoitos de *P. vivax* con los reticulocitos humanos. Además, estas herramientas también son útiles para predecir regiones funcionales de las moléculas mediante el análisis de selección natural, lo cual permitirá evitar respuestas inmunes alelo específicas, que reducen la eficiencia de las vacunas.

## **Recomendaciones**

Se debe realizar estudios de validación funcional orientados a confirmar la función de las moléculas que participan en procesos biológicos y metabólicos vitales de cada célula (parásitos de *P. vivax* y eritrocitos de *A. nancymaae*). En cuanto al proteoma de eritrocitos de *Aotus*, se sugiere relacionar los datos reportados con el estudio de las características genotípicas y transcriptómicas de *A. nancymaae* realizado por el Human Genome Sequencing Center of Baylor College of Medicine (BCM-HGSC).

Con el objetivo de correlacionar la parasitemia con los niveles de anticuerpos generados durante la infección natural, se recomienda hacer ensayos de antigenicidad utilizando sueros de pacientes con infección activa por *P. vivax*. Respecto a los ensayos de unión, se requiere el uso de eritrocitos con fenotipo Duffy negativo, para validar si *PvRBSA* utiliza la ruta alterna de invasión de los merozoitos a dichas células. Esto se basa en los recientes estudios de infección por *P. vivax* en individuos Duffy negativo.

Por otro lado, se recomienda realizar los estudios de predicción de regiones funcionales (mediante análisis de selección natural) con más moléculas de *P. vivax*, con el fin de poder incluir este paso dentro de la metodología para la búsqueda de regiones mínimas de unión conservadas y con restricción funcional. Adicionalmente, se requiere confirmar si la interacción de PvRBSA y PvGAMA es específica mediante ensayos de competición, lo cual permitiría tener resultados más consistentes. Se sugiere analizar las moléculas siguiendo la metodología propuesta por la FIDIC, con el fin de identificar las regiones mínimas de unión, como se reporta para otras proteínas descritas hasta la fecha, como DBP, MSP-1 y RBP-1.

## **Perspectivas Generales**

El estudio proteómico realizado por nuestro grupo ha tenido como objetivo principal la generación de bases de datos útiles para estudiar en el futuro las moléculas implicadas en la interacción parásito-hospedero. Por ende, sería importante estudiar las interacciones que pueden estar ocurriendo entre moléculas de *P. vivax* con las de eritrocitos de *A. nancymae* usando la tecnología de NAPPA.

Un aspecto interesante derivado de este estudio, es identificar por *docking* molecular los compuestos más afines a las dianas farmacológicas predichas en el estudio del proteoma de la cepa VCG-1 de *P. vivax*. Así mismo, sería interesante evaluar el efecto de algunos compuestos durante el desarrollo intraeritrocítico de *P. vivax* en ensayos *in vitro* controlados. Además, futuros ensayos irán orientados a evaluar los nuevos compuestos en el modelo experimental *Aotus* como una alternativa al tratamiento actual contra la malaria.

Por último, es imprescindible continuar el trabajo de selección de regiones funcionales para las 17 moléculas de *P. vivax* caracterizadas en el laboratorio de biología molecular de la FIDIC. Además, es importante utilizar otras metodologías ligando-receptor orientadas a estudiar las regiones mínimas de unión que puedan tener un papel importante en la actividad de invasión de los merozoitos de *P. vivax* a reticulocitos, siguiendo el enfoque empleado para *P. falciparum*.

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# **ANEXOS**

# **Anexo 1**

“Vacunas contra *P. vivax*: Desafío en la Investigación”

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# Vaccines against *Plasmodium vivax*: a research challenge

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Malaria caused by *Plasmodium vivax* continues being a public health problem in tropical and subtropical areas throughout the whole world. In spite of this species' epidemiological importance, its biological complexity has hampered advances being made in the field of vaccine development. Few antigens have been described and analyzed to date in preclinical and clinical studies, thereby highlighting the great challenge facing groups currently working on this parasite species. This review summarizes the most representative work done during the last few years and discusses the approaches adopted in making progress towards an anti-*Plasmodium vivax* vaccine.

**KEYWORDS:** antigens • malaria • *Plasmodium vivax* • preclinical and clinical studies • tropical and subtropical regions • vaccine

In spite of the progress made by government initiatives and the World Fund for the Control and Prevention of Malaria, this disease continues to be a public health problem all around the world. Approximately 216 million cases and an estimated 665,000 deaths occurred in 2011, mainly in children <5 years old, according to the latest data released by the WHO [1].

Human malaria can be caused by five parasite species from the *Plasmodium* genera (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*). *P. vivax* is predominantly distributed on the Asian and American continents and is responsible for 25–40% of the global malaria burden, causing between 132- and 391-million cases annually [2]. The search for an effective vaccine against *P. vivax* has become a great challenge given this species' biological complexity, its preference for invading reticulocytes, genetic variability mechanisms and the generation of latent forms (hypnozoites).

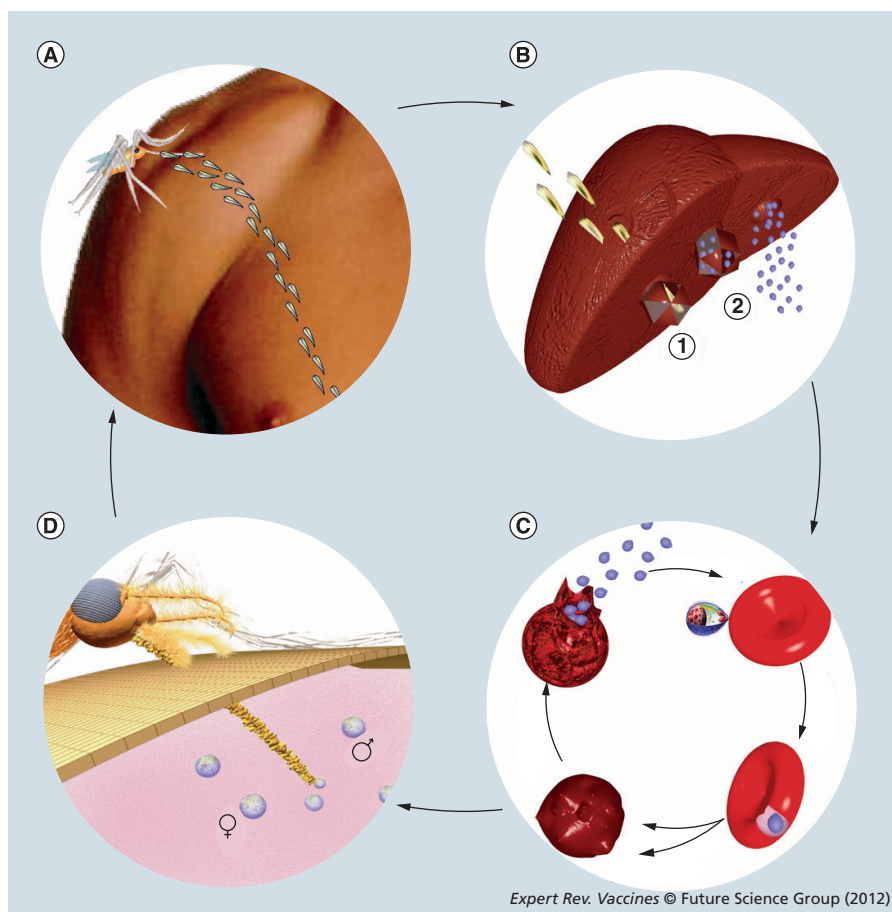
Although few *P. vivax* antigens have been identified and functionally characterized using traditional molecular biology, immunology and biochemistry approaches, most of the vaccine candidates being tested were found using the aforementioned methodologies. The currently available transcriptome [3], proteome [4] and comparative genomic analysis data [5]

for *P. vivax* could be extremely useful in the future to find new stage-specific proteins, similar to those described for other parasite species, which could be essential in developing a vaccine.

## The progress of an anti-*P. vivax* vaccine

Given that malaria represents one of the main public health problems around the world, several research groups have made great efforts to develop an effective vaccine against this parasitosis. Advances made to date with regard to the knowledge gained concerning the biology of *P. vivax* have not yet reached the same level as those regarding *P. falciparum*. However, research into the *Plasmodium* life-cycle and its mechanisms for invading red blood cells (RBCs) have led to the establishment of the most appropriate points of intervention for blocking the parasite's development, such as pre-erythrocyte, blood and sexual stages (FIGURE 1).

Immunization with irradiated sporozoites, recombinant expression of parasite proteins and production of antigens by peptide synthesis are among the methodologies used by research groups orientated towards developing an antimalarial vaccine. *P. vivax* studies to date have dealt with the limited number of candidates that have been identified, characterized (FIGURE 2) and evaluated in preclinical and clinical studies, compared with *P. falciparum*.



**Figure 1. *Plasmodium vivax* life-cycle and intervention points for developing vaccines against several development stages. (A)** Infection: sporozoite inoculation via female *Anopheles* mosquito bite. **(B)** Pre-erythrocyte stage: invasion of hepatic cells and generation of (1) hypnozoites or (2) hepatic schizonts. **(C)** Blood stage: beginning of the parasite's asexual development in reticulocytes. **(D)** Sexual stage: differentiation to gametocytes that are ingested by the mosquito.

### Vaccine candidates for which the greatest advances have been made to date

#### Pre-erythrocyte vaccine candidates

The main objective for vaccines directed against this stage is to prevent sporozoite invasion of hepatocytes, or impede the parasite's development within hepatic cells so as to avoid its proliferation in the blood stream. Early studies by Ronald Ross (1899) showed that infected mosquitoes transmitted avian malaria. Later, Sergeant *et al.*, in 1910 described that canaries immunized with irradiated *Plasmodium relictum* sporozoites developed partial immunity against exposure to the native parasite. In 1967, Nussenzweig *et al.* showed that immunizing rodents with 5,000–75,000 radiation-attenuated sporozoites induced total protection against challenge in studies orientated towards evaluating the immunological response to infection in preclinical assays, thereby leading to the concept of sterile protection [6]. Many years later, clinical trials reported by Clyde *et al.* in 1975 revealed vaccine safety and immunogenicity using *P. vivax* attenuated sporozoites in humans [7]. Individuals immunized with more than ~1,000 bites

from irradiated mosquitoes in such studies achieved short-term protection against the disease. *Aotus* monkeys have been intravenously inoculated with 2, 5 and 10 doses of 100,000 radiation-attenuated sporozoites in recent work; despite the low immune response, the primates became partially protected, as shown by the reduction in parasitemia [8].

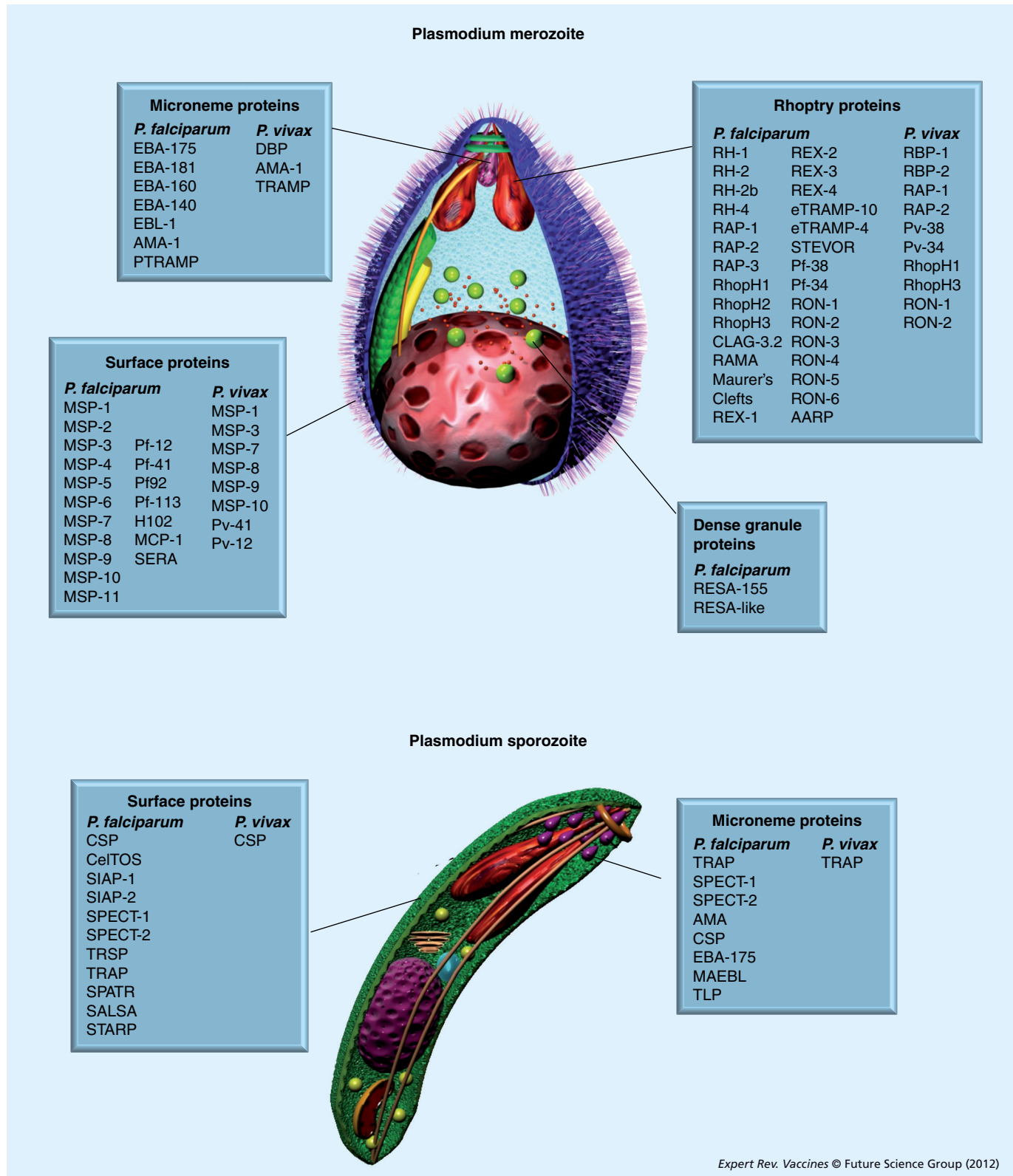
Although this pre-erythrocyte vaccine system continues to be considered the gold-standard, no new clinical studies have evaluated safety, reproducibility and protective efficacy in humans. This has been mainly due to technical difficulties presented when advancing *P. vivax* studies, such as not having an infective gametocyte-rich source that could be used as totally viable sporozoite precursors. In addition, it is difficult to estimate the required amount of irradiated sporozoites for creating a protective response in humans and the procedure involving using mosquito bites for immunizing humans is impractical (>1,000 mosquito bites). Using this methodology might be ineffective in individuals having a background of malaria given that even though invasion has been blocked in this first line of defence they could develop the infection as the result of relapses caused by hypnozoites (discussed later). This highlights the need for a multistage vaccine against this parasitosis.

Bearing the aforementioned in mind, efforts were initially centred on developing a subunit-based vaccine. The main candi-

dates have been proteins present on parasite surface or in apical organelles (FIGURE 2), namely the circumsporozoite protein (CSP) and the thrombospondin-related anonymous protein (TRAP).

CSP has been one of the most studied candidates in various human malaria species. Current preclinical trials with CSP have involved using chimerical recombinant proteins [9], multiple antigen constructs [10] or multiple antigen peptides [11]. These methods have led to the discovery that including CSP protein B- and T-cell epitopes in a vaccine induces high immunogenicity in rodents and primates. Yadava *et al.*, have immunized mice with the VMP001 chimeric antigen developed in 2007 [9] emulsified with Montanide ISA 720 [12]; this recombinant includes the chimeric repeat region representing the two major CSP alleles in *P. vivax* (VK210 and VK247), flanked by the N- and C-terminal regions. The VMP001 vaccine induced a cross-species immune response against *P. falciparum* and *Plasmodium berghei* and generated partial protection in the murine model as evidenced by the lower parasitemia developed in vaccinated mice compared with the control group, as well as the lower parasite burden in the liver





**Figure 2. Actual state of proteins identified in merozoite and sporozoite stages in *Plasmodium vivax* and *Plasmodium falciparum*, respectively.** Shows the proteins described to date with their respective localization in the parasite (surface, rhoptries, micronemes and dense granules).

*P. falciparum*: *Plasmodium falciparum*; *P. vivax*: *Plasmodium vivax*.

as evaluated by reverse transcription-PCR. Experiments in *Aotus* monkeys using N- and C-terminal region-derived long synthetic peptides (LSPs) and another based on CSP repeats combined with a tetanus toxin T-cell epitope, both emulsified in Montanide ISA 720 or Freund's complete adjuvant, have shown strong antibody responses that recognize the native protein [13]. Based on this, a Phase I clinical trial was carried out; this described that a vaccine based on individual LSPs or a mixture of these with Montanide ISA 720 was safe, tolerable and immunogenic [14]. A total of 40 individuals immunized with LSP formulated in Montanide ISA 720 or Montanide ISA 51 have recently shown seroconversion; however, LSPs formulated in Montanide ISA 51 produced greater antibody titers and IFN- $\gamma$  than those emulsified in Montanide ISA 720 [15]. The results suggested that a particular adjuvant could boost a vaccine's effect and this should be considered in future clinical trials.

A study showing the safety and reproducibility of infection caused by 3–9 bites by infected *Anopheles albimanus* mosquitoes in 18 malaria-naïve humans [16], in addition to the aforementioned study, evaluating patients' immune response to LSPs [15], have been carried out to promote Phase II trials. However, no reports describing Phase II trial results have been published to date.

Recent studies have focused on searching for a potent adjuvant able to trigger strong, long-lasting humoral immune responses when combined with the *P. vivax* CSP molecule. In particular, mice vaccinated with VMP001 emulsified with synthetic TLR4 (glucopyranosyl lipid adjuvant) in a stable emulsion [17] or conjugated to the lipid-enveloped polymeric PLGA nanoparticles (VMP001-NPs) with monophosphoryl lipid A [18] have shown significantly higher antibody titers than control groups, thus providing support for the use of these formulations in further clinical trials.

Parasite invasion is complex, involving many adhesion molecules [19]. Even though CSP is the major antigen, it is not the only one responsible for target cell invasion. A *P. vivax* study has led to the identification and characterization of a microneme protein called TRAP. Once it was demonstrated that TRAP induced high antibody levels in the Balb/c experimental model, its efficacy in the *Aotus* experimental model was evaluated; inoculating an LSP localized in TRAP's N-terminal region (containing the liver cell-binding domain, known as region 2) induced a good immune response. Nevertheless, protection results were not statistically significant when compared with the control group using this scheme [20].

### **Intra-erythrocyte stage candidates**

Considering that the clinical manifestations and severity of malaria infection coincide with the parasite's development within erythrocytes, antigens expressed during this stage represent a set of important vaccine candidates for blocking invasion. Even though studies have mainly been centred on the Duffy-binding protein (DBP) [21], merozoite surface protein-1 (MSP-1) [22] and apical merozoite antigen-1 (AMA-1) [23], other molecules such as reticulocyte-binding protein-1 (RBP-1) [24], rhoptry-associated protein-2 [25], MSP-9 [26] and -10 [27] have been identified, characterized and evaluated as vaccine candidates.

DBP is a microneme molecule participating in reticulocyte–merozoite tight junction formation. It has been established that this protein forms part of the obligatory reticulocyte invasion route involving interaction with the Duffy antigen receptor for chemokines (DARC), thus leading to wide-ranging research aimed at evaluating this antigen's function and potential as vaccine candidate.

Studies carried out by the Fundación Instituto de Inmunología de Colombia (FIDIC) have shown that DBP has 10 reticulocyte high-activity binding peptides (HABPs), which are mainly located in the protein's region 2 (RII) (FIGURE 3) [28]. Two HABPs able to bind to different HLA-DR molecules (called 1635 and 1638) have been found recently (FIGURE 3). These peptides induced a Th1/Th2 recall response (mainly IFN- $\gamma$  and IL-6) in 35 individuals who had suffered prior episodes of *P. vivax* malaria as shown by peripheral blood mononuclear cell (PBMC) lymphoproliferation assays, thus highlighting their potential use in a synthetic, subunit-based vaccine [29,30]. Future studies aimed at establishing these sequences' protection-inducing ability have been planned in the *Aotus* experimental model.

Preclinical trials with rodents and *Macaca* monkeys using recombinant PvDBP region 2 (PvDBP-RII) formulated with three adjuvants approved for human use (anhydrogel, Montanide ISA 720 and ASO2A) have shown a correlation between the level of antibodies produced and the potential for inhibiting domain binding to the DARC *in vitro* [21,31]; however, preclinical trials in the experimental *Aotus* model using the same region formulated in Montanide ISA 720 adjuvant have shown no protection [32]. Despite this, this antigen continues to be studied as it has been considered an important component in the obligatory route for invasion of reticulocytes, it has specific binding sequences, antibodies directed against PvDBP-RII are able to inhibit adhesion to target cells, it is highly antigenic and it has universal epitopes.

MSP-1 has been the most widely studied MSP identified to date. It has been suggested that PvMSP-1 probably undergoes a proteolytic processing similar to that of its *P. falciparum* ortholog, thus generating 83, 30, 38 and 42 kDa fragments, the latter being hydrolyzed again in 33 and 19 kDa products.

Several formulations have been evaluated in *P. vivax*. A study by the CDC (Atlanta, GA, USA) used a yeast-expressed recombinant consisting of the MSP-1 19 kDa C-terminal fragment (MSP-1<sub>19</sub>) and two tetanus toxoid T-helper epitopes for immunizing *Saimiri boliviensis* monkeys with aluminium hydroxide and block copolymer P1005 [33]. The group of monkeys immunized with MSP-1<sub>19</sub> emulsified in block copolymer P1005 had partial protection against challenge (three out of five monkeys) with a *P. vivax* Sal-1 homologous strain and generated a greater antibody response than the group immunized with MSP-1<sub>19</sub> only or in aluminium hydroxide. Immunization experiments were carried out on New World monkeys (*Callithrix jacchus*) vaccinated with MSP-1<sub>19</sub> plus a promiscuous T-cell epitope present in the 33 kDa region and a synthetic universal Pan allelic DR epitope emulsified in different adjuvants (Freund's, Quil A, CpG oligodeoxynucleotide (ODN) 2006 or MPL/trehalose dicorynomycolate). The results showed a better response when the recombinant was

administered with Freund's complete adjuvant and Quil A [34], suggesting that immunogenicity depended on the adjuvant used.

Other studies in the *Aotus* experimental model have been carried out by FIDIC; these included immunizing with two recombinant fragments (MSP-1<sub>14</sub> and MSP-1<sub>20</sub>) encompassing most of the MSP-1<sub>33</sub> proteolytic cleavage product. These recombinants are localized in a region presenting low variability and displayed high reticulocyte-binding ability [35], probably mediated by the HABPs comprised in them (FIGURE 3). A total of 50% [36] to 80% [22] of the primates immunized with the mixture of MSP-1<sub>14</sub> and MSP-1<sub>20</sub> recombinant proteins emulsified in Freund's adjuvant were partially protected, showing a relationship between antibody titers, IFN- $\gamma$  production levels and protection-inducing immunity.

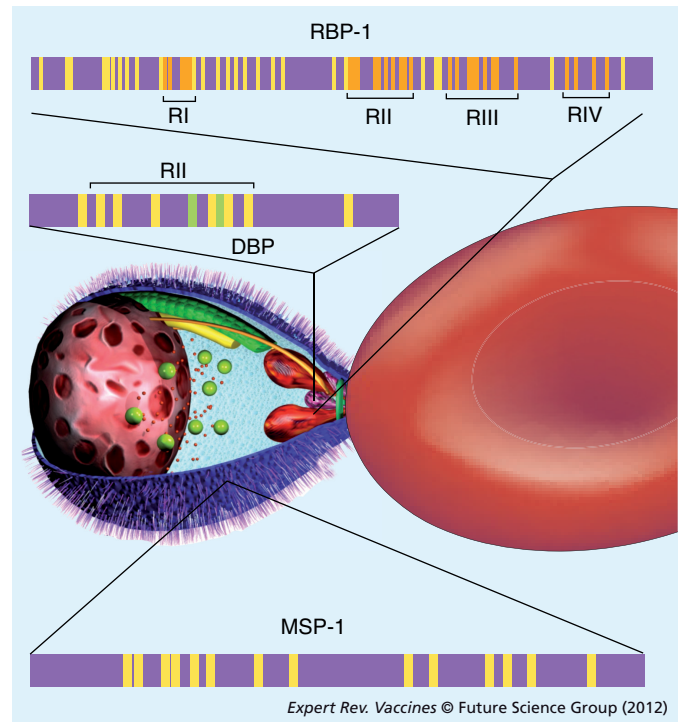
Additional studies have tested recombinant fusion proteins such as PvDBP-RII and MSP-1<sub>19</sub> obtaining greater immunogenicity in mice when using Montanide ISA 720 adjuvant as an immunobooster [31].

AMA-1 has also been considered to be one of the most important candidates given its participation in merozoites' apical reorientation and subsequent tight junction formation. It has also been found that the protein has an effect on dendritic cell maturation by upregulating CD1a and HLA-DR molecules [37]. The efficiency of Balb/c mice's humoral response has been recently proved regarding immunization with the AMA-1 recombinant protein (amino acids 43-487) formulated in Freund's complete adjuvant, aluminium hydroxide, Quil A, QS-21 saponin, CpG ODN 1826 or TiterMax<sup>®</sup> (CytRx, CA, USA). A strong immune response with any one of the adjuvants used was produced and thus it is planned to continue assays in nonhuman primates [23].

RBP-1 is a member of a cell-binding protein family identified in *P. vivax*; in the Sal-I strain, this family has 10 *rbp* genes including three partial genes (one *rbp-1* [PVX\_125738] and two *rbp-2* [PVX\_090330; PVX\_101590]), two pseudogenes (*rbp-2d* [PVX\_101585] and *rbp-3* [PVX\_101495]) and five full-length genes (*rbp-1a* [PVX\_098585], *rbp-1b* [PVX\_098582], *rbp-2a* [PVX\_121920], *rbp-2b* [PVX\_094255] and *rbp-2c* [PVX\_090325]) [5]. Mapping the whole of *P. vivax* Belem strain *rbp-1* (encoded by the *rbp-1a* gene) has led to the definition of four reticulocyte-binding regions (RI-RIV) housing several highly conserved high-binding peptides (FIGURE 3) [38]. A further study has shown that RBP-1 region I, recombinantly expressed in *Spodoptera frugiperda* (Sf9) insect cell line, intervenes in merozoites' specific binding to reticulocytes [39].

The only formulation that has been evaluated included the protein's region III (containing peptides with a greater binding ability) (FIGURE 3) recombinantly expressed in *Escherichia coli* and emulsified with Freund's adjuvant; although a significant increase in antibody production and T-lymphocyte stimulation have been observed, no protection was achieved when *Aotus* monkeys previously immunized with the recombinant were challenged with the *P. vivax* VCG-1 strain [24]. Therefore, plans have been made to carry out preclinical assays with protein regions I, II and IV for evaluating the formulation's immunogenic and protection-inducing ability.

Several rophtry proteins play an important role during invasion of RBC, some of them being classified within the high molecular



**Figure 3. High-activity binding peptides to reticulocytes identified in *Plasmodium vivax* MSP-1, RBP-1 and DBP proteins.** HABPs are shown in yellow. Green bars represent the universal peptides found in DBP protein region II (HABPs #1635 and 1638). Orange bars indicate regions where RBP-1 binds to reticulocytes (RI-RIV), which encompass those peptides showing greater binding ability. HABP: High-activity binding peptide.

weight or the low molecular weight complexes. The latter consists of three rophtry-associated proteins: RAP-1, -2 and -3, of which the first two have been described in *P. vivax* [40,41]. A study using the *P. vivax* RAP-2 recombinant protein formulated in Freund's adjuvant has shown partial protection in *Aotus* monkeys, given that significantly lower parasitemias in the immunized group were observed with respect to the control group [25].

Regarding MSP-9, two recombinants have been evaluated in rodents, one covering the N-terminal region (PvMSP-9-Nt) and another covering a block of tandem repeats in the protein (MSP-9-RepII). Both formulations were seen to be highly immunogenic and the N-terminal region stimulated IFN- $\gamma$  and IL-5 production in the PBMCs of immunized mice [26]. Regarding MSP-10, this protein was initially identified and characterized in 2005 by Pérez-Leal *et al.* [42], and has recently been shown to be antigenic during natural *P. vivax* malaria infection in humans. Its immunogenicity has also been proved in the experimental *Aotus* model using three types of adjuvants: Freund's, Montanide ISA720 or aluminium hydroxide. Two out of the three formulations triggered a strong antibody response, recognizing the protein on parasite surface (demonstrated by immunofluorescence assay); nevertheless, immunized monkeys did not become protected against challenge with the *P. vivax* VCG-1 strain [27].

### Transmission-blocking vaccine candidates

The antigens used by the parasite during fertilization and development within the mosquito vector have been used as a strategy for controlling malarial transmission. Pvs25 and Pvs28 are the main surface proteins presenting high expression levels during the ookinete stage; their characterization has led to antigenic and immunological evaluation in experimental models. Rodents immunized with recombinants produced in yeast and absorbed with aluminium hydroxide have been shown to generate a potent immune response capable of inhibiting oocyte development in mosquitoes [43]. In spite of this, more attention has been paid to Pvs25, since a greater yield is obtained when expressed in yeast, its polymorphism is limited and the immune response exerted does not seem to be genetically restricted in the experimental model used. Two clinical trials have been carried out by the Algerian Research Institute and the US National Institute of Allergy and Infectious Diseases (MD, USA). Individuals vaccinated with the Pvs25H recombinant protein formulated in alhydrogel tolerated the formulation and produced antibodies that were functionally active as significant transmission blocking was observed [44]. The other trial was made with the same recombinant but emulsified in Montanide ISA 51; this proved reactogenic in two patients who developed a severe local reaction and the study was thus stopped [45].

A new protein localized on the gamete surface has been identified (Pvs230). Immunizing mice with recombinant Pvs230 region I–IV emulsified in Vaxfectin® (Vical, CA, USA) led to the development of antibodies, and significantly reduced the number of oocysts formed within the mosquito's intestine and the vector infection rate, thereby suggesting that this candidate could be a good target for evaluation in preclinical assays [46].

### Promising candidates

While advances made in identifying *P. vivax* antigens have been limited, studying the transcriptome [3], the proteome's partial characterization [4] and immunoproteomic profile [47], as well as studies focused on comparing transcription profiles between different parasite species [5,48], have been of great benefit in obtaining information about proteins that may be important for vaccine development. Different vaccine candidates localized on the surface or apical organelles (rhoptries and micronemes) (FIGURE 4) have been identified by molecular biology methodologies.

It has been found that some *Plasmodium* proteins are associated with detergent-resistant membranes through glycosylphosphatidylinositol (GPI) anchors; six of them have been identified in *P. vivax* by means of bioinformatics tools and evaluated by molecular biology assays: PvMSP-8 [49], -10 [42], Pv12 [50], Pv34 [51], Pv38 [52] and apical sushi protein (PvASP) [53]. The first three antigens are localized on merozoite surface while Pv34, Pv38 and PvASP are located in the rhoptries. All of them have characteristics relevant to proteins considered good vaccine candidates, such as having a signal peptide, being anchored to the membrane through transmembrane helices or GPI anchors, having binding domains and being able to generate an immune response in natural and/or experimental infections. It has been particularly interesting that Pv34 induced a proliferative response of PBMCs

isolated from individuals having a background of *P. vivax* infection, thereby highlighting the importance of carrying out pre-clinical studies in experimental models. Other surface/rhoptry molecules have been described, such as PvMSP-7 [54], Pv41 [55], PvTRAMP [56], PvClag7 [57], PvRON2 [58] and PvRhopH3 [59]. Some of them have been shown to be immune response targets during a natural *P. vivax* infection (PvTRAMP, PvClag7) thereby supporting the idea of continuing with studies to evaluate their immunogenic activity in the *Aotus* experimental model.

Two new alanine- and tryptophan-rich proteins have been characterized (PvTARAg55 [60] and PvATRAg74 [61]); a proliferative response is induced by these antigens in PBMCs from infected individuals as well as a predominant Th2 response with high levels of IL-4 and IL-10. Another study characterizing and mapping antibody response to MSP-3 $\alpha$  has shown at least 15 antigenic determinants within this protein and antibody titers observed correlate with the time of exposure to infections [62].

Protein array or mass spectrometry techniques have been used for identifying new candidates; the first technique has led to 18 highly immunoreactive molecules being recognized by sera from *P. vivax*-infected patients [47] while the second allowed identification of four additional ones strongly recognized by immune sera [4]. Some of these match previously described antigens, suggesting that the approach used represents a good option for identifying new candidates.

### Problems & limitations regarding *P. vivax* research

Several obstacles inherent in the parasite's biology related to its cell tropism towards reticulocytes, high genetic variability, developing latent hepatic forms and its adaptation in a suitable experimental model have impeded advances being made in the search for a strategy for controlling the disease, such as vaccine development.

### In vitro culture & functional assays

Tremendous efforts have been made during the last 30 years for standardizing a methodology allowing a *P. vivax in vitro* continuous culture to be maintained. However, the preference of *P. vivax* for invading reticulocytes (representing approximately 0.5–1.5% of the total of RBCs circulating in adults) and the difficulty involved in obtaining a significant percentage of them are the main limitations regarding progress in this research area [63].

The first *in vitro* trials with *P. vivax* were reported during the 1980s; several groups managed to establish a preliminary *in vitro* culture from blood infected with early trophozoites and ring forms, using reticulocyte-rich samples obtained from cord blood. These assays led to DBP's functional characterization, demonstrated by inhibiting invasion by an antibody directed against the erythrocyte Duffy antigen binding region.

Several studies during the last few years have led to a significant advance in the area. The first of them used reticulocytes obtained by differentiating stem cells with specific factors [64]; nevertheless, it has still not been widely used given its complexity and high costs. The second assay managed to standardize a *P. vivax ex vivo* invasion assay protocol, which uses trypsin-treated enriched reticulocytes and a sample with *P. vivax* schizonts from different

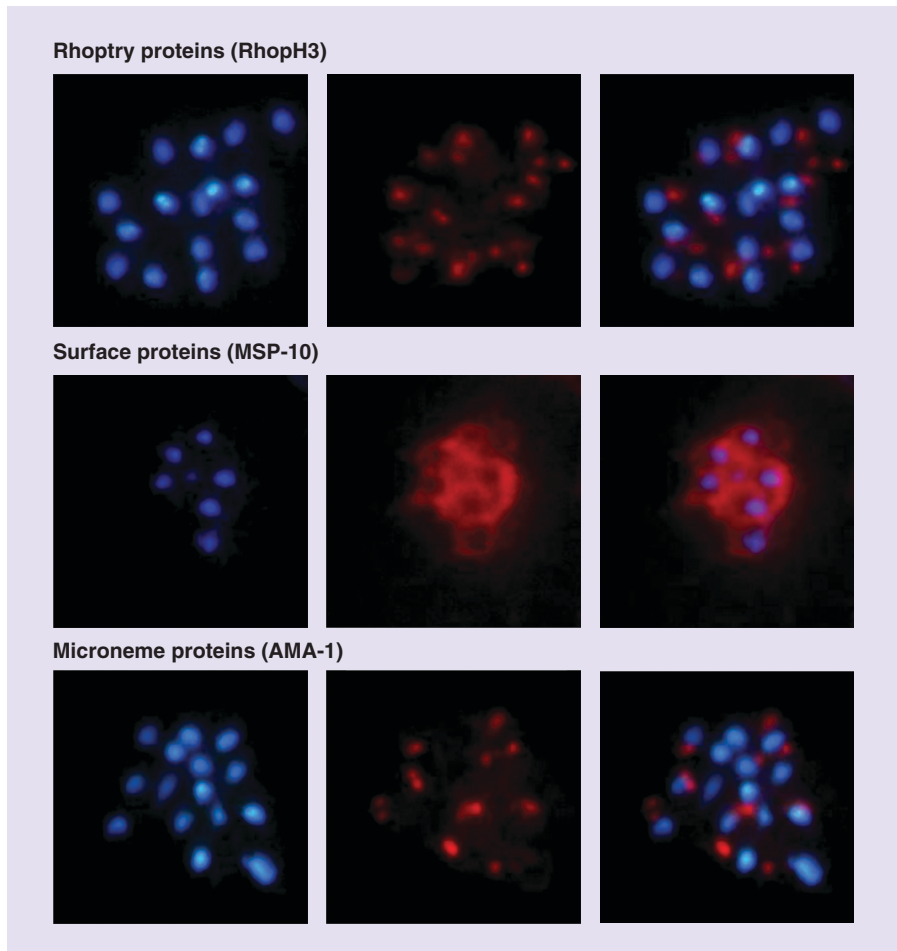
clinical isolates; this methodology has been shown to be practical, reliable and reproducible [65] and is currently being evaluated for propagation of the VCG-1 strain.

Based on the strength of FIDIC's studies involving the molecular characterization of 50 proteins implicated in *P. falciparum* invasion, advances have been made in screening three *P. vivax* molecules. These antigens' regions for specific binding to target cells have been determined by using enriched reticulocytes from samples from a patient suffering from  $\beta$ -thalassemia and synthetic peptides; DBP [28], RBP-1 [38] and MSP-1 [66] have thus been fully mapped (FIGURE 3). Such advances have provided valuable knowledge for developing novel strategies and methodologies providing in-depth knowledge about the importance of candidates and their inclusion in an epitope-based vaccine. However, technical difficulties associated with obtaining enough reticulocytes for binding studies are reflected in the number of candidates mapped for *P. vivax* compared with those from *P. falciparum* (three vs 50).

#### The parasite's genetic variability

The expression of *P. vivax* proteins with a high degree of polymorphism and the strain-specific immune responses induced by them represent formidable obstacles for developing an antimalarial vaccine. Several studies have shown great genetic variability in proteins such as DBP [67], MSP-1 [68], MSP-3 $\alpha$  [69], MSP-5 [70] and AMA-1 [71], which has mainly been attributed to selective pressure exercised by the host's immune system, thereby leading to the fixation of allele variants in the parasite population [71].

It has recently been shown that strong immune responses are directed against variable sequences that do not bind to target cells, whereas highly conserved sequences implicated in interaction with RBC molecules are not immunogenic [19,72]. PvDBP-RII contains both conserved as well as highly polymorphic residues; antibodies generated against the polymorphic ones are short-lived and strain-specific. Mutagenesis assays directed towards variable regions have shown that these are not functionally active for binding to erythrocytes [73]. A recent study involving the modification of a PvDBP-RII peptide (DEK<sup>null</sup>) in polar polymorphic residues (replaced by small hydrophobic residues), has shown that high immunogenicity is generated and induces the production of antibodies inhibiting binding to erythrocytes [74], thereby highlighting the fact that modifying polymorphic residues could lead to the production of more specific antibodies.



**Figure 4. Localization patterns for *Plasmodium vivax* rhoptry (RhopH3), surface (MSP-10) and microneme (AMA-1) proteins.** The merozoite nucleus is shown in blue (left panels); RhopH3, AMA-1 and MSP-10 localization is shown in red (central panels), the dotted pattern is characteristic of microneme and rhoptry proteins, whereas the 'bunch of grapes' pattern is characteristic of MSPs; right panels show the overlapping of the aforementioned images.

It is particularly interesting that the naturally infected *P. vivax* individuals have high specific antibody titers against DBP and MSP-1 variants, generating a selective effect in immune response regarding different strains [75,76]. These studies suggested that using conserved regions or the most frequently occurring alleles in a population must be born in mind when designing a vaccine.

#### *P. vivax* pathology

The parasite is characterized by its ability to generate a latent stage within a host's liver, called the hypnozoite (FIGURE 1), which is being characteristic of some species that infect primates (*P. simiovale*, *P. fieldi*, *P. cynomolgi* and *P. schwetzi*) and humans (*P. ovale* and *P. vivax*). This stage is generated after sporozoite invasion of liver cells. It has been shown that activating such latent forms propitiates a new wave of blood parasites that can occur in 3–5 months (short intervals) or 5–10 months (long intervals) [77,78]. Such patterns vary according to a vector's geographical area of origin since individuals from tropical areas have a pattern of frequent relapses within short intervals, whereas those from temperate areas tend to

have relapses in long intervals [77]. This parasite behavior hinders the undertaking of proper therapeutic measures, in particular when people suffer relapses when no longer living in malaria-endemic areas, which leads to delayed diagnosis and treatment.

Furthermore, studies by Chen *et al.* [79] and Imwong *et al.* 2012 [80] have shown that parasite strains found in patients suffering *P. vivax* relapses are genetically different from those found during the primary infection, thus suggesting that an effective pre-erythrocyte vaccine should include the different genotypes to completely block parasite development within the liver and prevent the generation of blood-stage parasites.

Many questions remain regarding hypnozoite activation mechanisms; further molecular studies dealing with the immune recognition of this particular stage should be undertaken considering that robust host–parasite model systems such as *P. cynomolgi*- and *P. simiovale*-infected *Rhesus* monkeys are now available [81].

Another feature of *P. vivax* is that this species can generate a rapid disease transmission, even before individuals become symptomatic. Different to *P. falciparum* behavior, *P. vivax* rapidly develops gametocytes, which can then be ingested by a mosquito vector thereby leading to perpetuation of the parasite's life-cycle even before the infected person is aware of having the disease [82].

### Experimental model

Several studies have focused on the search for an ideal animal model for malaria that could mimic the immune response induced in infected humans. The WHO recommended using *Aotus spp.* monkeys in 1988 as an appropriate experimental model for studying human malarial infections [83].

These primates have been widely used in research as their immune system molecules share a high degree of identity with their human counterparts (MHC, immunoglobulins, T-cell receptors and cytokines) [84]. Even though *Aotus spp.* monkeys do not naturally become infected by *Plasmodium* species causing malaria in humans, they have been shown to be highly susceptible to experimental infection. Research was thus advanced leading to adapting *P. vivax* strains (mainly Sal-1 [85], VCG-1 [86] and Chesson [87]) in New World primates such as *Aotus*, *Saimiri* and *Callithrix*, allowing the effectiveness of vaccine candidate antigens to be evaluated in preclinical studies.

The FIDIC Malaria Molecular Biology group adapted the *P. vivax* VCG-1 strain in *Aotus spp.* monkeys; this proved to be highly infective after 22 successive passes, reaching 7.88% parasitemia, as determined by Giemsa, acridine orange staining and real-time PCR [86]. This strain has been used in experimental infection studies involving *Aotus spp.* monkeys previously immunized with some of the previously described proteins (MSP-1 [35], RBP-1 [24], RAP-2 [25] and MSP-10 [27]) in which the immunogenicity, safety and protective efficacy of each of them has been evaluated. It has been found that unvaccinated nonsplenectomized monkeys were not reliably infected, suggesting that the strain cannot generate reproducible courses of the infection in animals with their spleen intact. These results agreed with previous studies using the Sal-1 [88] and Chesson strains [87]. In light of this, the search for new strains able to cause infection in the experimental model without

the need for surgical intervention is necessary for continuing to progress in such assays.

### Expert commentary & five-year view

Although few preclinical and clinical studies have been carried out during the last few years, there is no denying that a representative progress has been made in characterizing new antigens that are relevant for developing a vaccine against *P. vivax*. The aforementioned inherent difficulties when working with *P. vivax*, as well as the limited number of monkeys that can be used for experimentation (considering that they require to be splenectomized), have highlighted a bioinformatics approach as an interesting alternative for candidate screening. Comparative analysis of the transcriptional profile for malaria-causing parasite species has led to the identification of *P. vivax* genes having a counterpart in other *Plasmodium* species; in the *P. vivax* transcriptome analysis, Bozdech *et al.*, identified 3,566 *P. vivax* open reading frames showing shifts in mRNA abundance across the intra-erythrocyte stage, of which 2,923 have orthologs in *P. falciparum* [3]. *Plasmodium* parasite species share some invasion routes involving genes that are transcriptionally regulated and expressed. Recently, an *in silico* analysis using probabilistic profile hidden Markov models trained with proteins from other *Plasmodium* species for which the role in invasion and other biological parameters had been experimentally determined, allowing the identification of 45 *P. vivax* open reading frames with a potential role in invasion; 13 of them had already been described as vaccine candidates, thus validating the approach [89]. In a complementary study, Frech *et al.* analyzed the chromosome-internal regions of six published *Plasmodium* genomes including *P. vivax* [48]. In this study, 173 genes were identified as *P. vivax* species-specific with an unannotated function; interestingly, a nonsynthetic cluster of eight genes was found on chromosome 6 that might have a similar function in target cell invasion to that of the *P. falciparum* cluster [48]. These results suggest that studying *P. vivax* species-specific genes could lead to a better understanding of this species' unique biological mechanisms, in particular the preference for invading reticulocytes and the development of latent hepatic forms. It is thus expected in the near future that studies leading to the functional characterization as well as determining the antigenic properties of the protein products encoded by the aforementioned genes are undertaken.

The classical approach that has been followed by most groups working in malaria for selecting candidates for a subunit vaccine involves screening for those proteins that had been shown to be more antigenic. Unfortunately, the most antigenic regions usually tend to be the most polymorphic ones, thus making it difficult to avoid strain-specific immune responses. A different approach, reviewed in a recently published paper, has been followed by our group for establishing a logical and rational methodology for designing a vaccine against *P. falciparum* [90]. Rather than targeting the antigenic regions, this approach focused first on identifying conserved HABPs to the respective target cells (in this case RBCs). Taking into account that these conserved HABPs displayed low antigenicity and immunogenicity, they were then

modified to allow a better fit into MHC Class II, thus inducing strong immunogenicity and protection-inducing ability in the *Aotus* experimental model.

Given the urgent need to move forward in the search for a *P. vivax* vaccine, following a similar approach to that undertaken for *P. falciparum* could be a promising strategy to control the malaria burden caused by this parasite species. Some novel advances might allow us to overcome the barriers that exist when working with *P. vivax*; the identification and selection of molecules functionally relevant in target cell binding could be partially solved by comparative genomics studies. Also, reticulocyte purification from different sources enriched in this type of cells will facilitate identifying new HABPs to target cells and their role in invasion inhibition. HABPs need to be modified to allow a better fit into the MHC Class II binding groove, which requires us to carry out 3D-structural analyses for both native and modified peptides by nuclear magnetic resonance. Although strain-specific immune responses can be avoided by selecting HABPs conserved between the different parasite isolates only, the MHC Class II allelic diversity displayed by the host cannot be ruled out; different modified HABPs with a preferential binding to the various HLA-DR families are thus required to provide proper protective coverage to the vaccinated population.

So far, several binding regions have been identified in three *P. vivax* merozoite proteins: DBP, MSP-1 and RBP. HABPs found in such molecules have been synthesized in native and modified forms following the aforementioned methodology and *in vitro* MHC Class II binding assays, as well as immunization experiments in the *Aotus* monkey model are underway. These and novel molecules will continue to be analyzed with the goal of designing a potentially effective multiantigen and multiepitope vaccine against *P. vivax*.

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#### Key issues

- *Plasmodium vivax* malaria is a prevalent public health problem in tropical and subtropical regions that requires immediate attention.
- The search for a vaccine against *P. vivax* has become an ongoing challenge given the species' biological complexity regarding its cellular tropism, its high genetic variability and the formation of latent forms.
- Immunization with irradiated *P. vivax* sporozoites was the first representative advance providing evidence of blocking the parasite's infection of host hepatocytes.
- Advances made during the last few years evaluating the transcriptome, proteome and *in silico* comparative analysis of different parasite species have been essential to identify antigens that could be included in a vaccine.
- Immune response mechanisms associated with protection have been determined in preclinical studies carried out when immunizing with the attenuated parasite or recombinant proteins.
- Identifying specific reticulocyte-binding sequences opens up a new research field for developing a chemically made, multiepitope and multiantigen vaccine.
- The development of chemically synthesized vaccines represents one of the most promising rationale and logical strategies to be followed for producing a first-generation vaccine against the second most prevalent malarial parasite species around the world: *P. vivax*.

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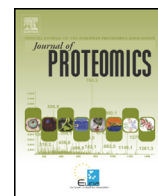
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## **Anexo 2**

“Proteoma de los Eritrocitos de *A. nancymaae*”



## The *Aotus nancymae* erythrocyte proteome and its importance for biomedical research



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### ABSTRACT

The *Aotus nancymae* species has been of great importance in researching the biology and pathogenesis of malaria, particularly for studying *Plasmodium* molecules for including them in effective vaccines against such microorganism. In spite of the forgoing, there has been no report to date describing the biology of parasite target cells in primates or their biomedical importance. This study was thus designed to analyse *A. nancymae* erythrocyte protein composition using MS data collected during a previous study aimed at characterising the *Plasmodium vivax* proteome and published in the pertinent literature. Most peptides identified were similar to those belonging to 1189 *Homo sapiens* molecules; >95% of them had orthologues in New World primates. GO terms revealed a correlation between categories having the greatest amount of proteins and vital cell function. Integral membrane molecules were also identified which could be possible receptors facilitating interaction with *Plasmodium* species. The *A. nancymae* erythrocyte proteome is described here for the first time, as a starting point for more in-depth/extensive studies. The data reported represents a source of invaluable information for laboratories interested in carrying out basic and applied biomedical investigation studies which involve using this primate. **Significance:** An understanding of the proteomics characteristics of *A. nancymae* erythrocytes represents a fascinating area for research regarding the study of the pathogenesis of malaria since these are the main target for *Plasmodium* invasion. However, and even though *Aotus* is one of the non-human primate models considered most appropriate for biomedical research, knowledge of its proteome, particularly its erythrocytes, remains unknown. According to the above and bearing in mind the lack of information about the *A. nancymae* species genome and transcriptome, this study involved a search for primate proteins for comparing their MS/MS spectra with the available information for *Homo sapiens*. The great similarity found between the primate's molecules and those for humans supported the use of the monkeys or their cells for continuing assays involved in studying malaria. Integral membrane receptors used by *Plasmodium* for invading cells were also found; this required timely characterisation for evaluating their therapeutic role. The list of erythrocyte protein composition reported here represents a useful source of basic knowledge for advancing biomedical investigation in this field.

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### 1. Introduction

Animal research has been essential for understanding and studying some human diseases, particularly those having the greatest impact around the world, such as malaria. For example, using rodents (BALB/c, C57BL/6, NOD/SCID or humanised strains) has led to obtaining valuable information about this parasite pathogenesis [1,2]. Rodent parasite species (*Plasmodium chabaudi*, *Plasmodium vinckei*, *Plasmodium berghei*

and *Plasmodium yoelii*) are different to those infecting humans (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*), therefore having differences regarding their biology and immune response [3]; this means that extrapolating such studies in humans is not always reliable.

Non-human primates represent another model; they have been shown to be the most suitable for studying pathogenesis, immunology and anti-malarial vaccine development, given that they are genetically and immunologically more similar to humans [1]. It is worth noting that some of these primates (mainly *Saimiri* sp. [4,5] and *Aotus* sp. [6,7]) have been widely used in basic and applied biomedical research.

*Aotus* spp. has been used since its susceptibility to experimental infection by parasites from the genus *Plasmodium* was shown in the

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1960s [8]; several parasite strains have been adapted since then in this model for studying malaria and developing possible pharmacological treatments or vaccines [9]. Within the genus, the *Aotus nancymaae* species has been infected with different *Plasmodium* strains (*P. falciparum*: Santa Lucia, Indochina I/CDC and Uganda Palo Alto strains; *P. vivax*: Chesson, ONG, Vietnam Palo Alto, Salvador I and Honduran I/CDC; *P. malariae*: Uganda I/CDC), as reported by Collins and his group several years ago [10].

The *Aotus* species has led to an enormous advance regarding pre-clinical studies highlighting the immunological and protective role of various molecules or parts of them from the *P. falciparum* FVO strain; taking into account that the complex machinery involved in erythrocyte invasion used by this parasite for infecting cells is partly known today, the *Aotus* model has been essential for describing the fundamental basis when identifying vaccine components against this parasite species [11,12]. On the other hand, these primates develop very reproducible infection following experimental infection with the *P. vivax* VCG-1 (Vivax Colombia Guaviare-1) strain, having high levels of parasitaemia (>5%) after 22 passages [13]; this has been of great importance for advancing molecular (MSP-7, Pv38, RAP-1 and RBP-1 between others) [14–17] and immunological (MSP-1<sub>20</sub> and MSP-1<sub>14</sub> from MSP-1<sub>33</sub> fragment) [18,19] characterisation studies of some molecules from the *P. vivax* species and evaluating their usefulness in developing an effective vaccine. These findings highlight the fact that using *A. nancymaae* in combination with the *P. falciparum* FVO or *P. vivax* VCG-1 strains is valuable for screening suitable vaccine candidates for later testing in humans.

In spite of *A. nancymaae* species having led to a promising advance in developing an anti-malarial vaccine, the biology of its erythrocytes still remains unknown (these being vital hosts for *Plasmodium*). Most studies have focused on establishing the similarity between primate and human genes encoding proteins related to the immune response [20–22]. The revolution in omic sciences represented by Baylor College of Medicine's Human Genome Sequencing Centre (BCM-HGSC) has led to the genome and transcriptome of species being studied through the Owl Monkey Genome Project. However, no study describing primate protein composition has been carried out to date. Taking the importance of studying *A. nancymaae* erythrocytes into account, our group was thus interested in obtaining the greatest amount of information possible about the proteome of these cells using data obtained from a previous study by our research group [23] and evaluating it in terms of protein composition and function.

## 2. Material and methods

### 2.1. Reanalysing proteome data

Tandem mass spectrometry (MS/MS) data came from a sample consisting of a mixture of mature erythrocytes and reticulocytes (*P. vivax* infected and non-infected ones); two samples had a 50:1 ratio (mature erythrocytes:reticulocytes) whilst the other had a 1.11:1 ratio as it had previously been subjected to a Percoll gradient to enrich infected reticulocytes (preferential invasion target for *P. vivax*) [23]. Data were used for searching for similar peptides, using the human proteome reported in the UniProt database [24]. Mascot [25] and SEQUEST algorithms [26] and Thermo Scientific Proteome Discoverer software were used with astringent search parameters. In brief, the most recent UniProt *H. sapiens* (AUP000005640), *P. vivax* (AUP000008333) and *P. falciparum* (AUP000001450) proteomes were used for compiling a FASTA file containing common non-human contaminants (trypsin, Lys-C and BSA). Thermo's Proteome Discoverer (version 1.4.0.288) was then used for analysing each file in batch and in MudPit [27] for replicates from the same sample; the latter led to identifying low quantity proteins. Results having a <0.01 (high confidence) q-value were filtered using a Mascot Score threshold above 20 and 1.5, 2.0, 2.25, 2.5, 2.75, 3, 3.2, 3.4 for SEQUEST HT (XCorr) for charge states from 1 to 7 and from

3.4 for values >7. An Excel file was generated for each filter showing protein identification details (accession code, description and coverage), including all scores and identified peptides. Redundant UniProt access codes were manually eliminated so that the total list of molecules identified here could be reported.

### 2.2. Searching orthologous genes in New World monkeys

The search strategy for *H. sapiens* orthologous molecules with New World primates involved using the biological DataBase network (bioDBnet) [28], an online web resource enabling the search for orthologue identifiers in different species. UniProt accession codes from *A. nancymaae*-*H. sapiens* analysis were used for searching for orthologous molecules in *Callithrix jacchus*, the only species from the primate family phylogenetically related to *Aotus* for which proteome data is available to date. Molecules identified as non-orthologous were analysed again using the OrthoDB database [29].

### 2.3. Identifying erythrocyte proteins

The proteins identified here were compared to the most extensive profiling of human erythrocyte RNAs published to date [30]. UniProt accession codes were converted into Ensembl gene ID codes with bioDBnet [28] and then compared to 8092 genes expressed as a >0.5 threshold according to an erythrocyte transcriptome study [30].

### 2.4. Protein annotation according to gene ontology terms

Gene ontology (GO) annotations available in the UniProt database were analysed using the Software Tool for Rapid Annotation of Proteins (STRAP, version 1.5) [31], developed by Boston University School of Medicine's Cardiovascular Proteomics Centre (Boston, MA). The National Institute of Allergy and Infectious Diseases (NIAID) Database for Annotation, Visualization, and Integrated Discovery (DAVID) [32,33] was also used for categorising molecules according to GO terms; stringent parameters were used to ensure statistical significance (thresholds: EASE value = 0.001 and Count = 2).

### 2.5. Predicting cell membrane molecules

The Red Blood Cell Collection (RBCC) database was used for predicting Surface molecules; RBCC integrates the proteome of human RBC proteins identified to date [34]. The search parameters involved being a highly confident match in both hRBCD and BSc\_CH, a blood group or CD marker, experimentally tested in the Sarkadi-lab. The UniProt accession codes for each protein so identified were then manually downloaded for compiling the supplementary table.

## 3. Results

### 3.1. *Aotus* protein prediction

The flow chart in Fig. 1 shows how *A. nancymaae* proteins were identified and analysed. *A. nancymaae* mass spectra were initially imported in Proteome Discoverer software and compared to the information available for the *H. sapiens* proteome by data Mascot and SEQUEST search algorithms; 1084 fully-tryptic and 1052 semi-tryptic molecules were identified using digestion search parameters; 901 were recognised by both parameters, whilst 183 fully-tryptic and 151 semi-tryptic ones were only recognised by one parameter (Supplementary data 1). Primate peptides had great similarity with those from humans, representing 1189 molecules (SD 2). The bioDBnet and OrthoDB tools were used for confirming *A. nancymaae* proteins by comparing the orthology of molecules found in this study with the available information regarding New World monkey proteomes in the UniProt database (Fig. 1). It was found that 95.7% (1138 proteins) of the proteins

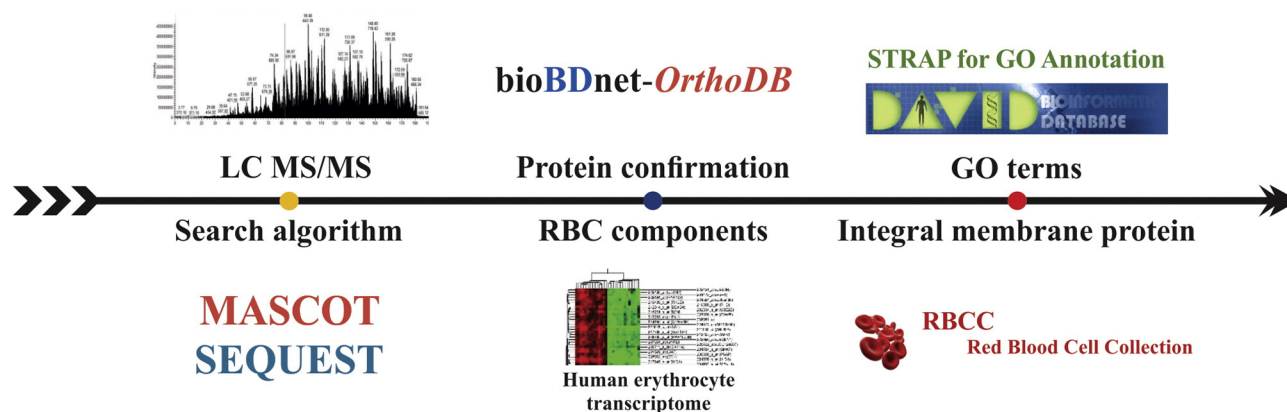


Fig. 1. A flow chart showing the analysis for identifying and confirming *A. nancymae* erythrocyte proteins.

identified had orthologues in New World primates whilst 4.3% (51 molecules) of them did not (SD3); they were therefore excluded from further analysis.

### 3.2. Specific erythrocyte proteins

*Aotus* RBC proteins were discriminated by comparing the transcriptome data reported for human erythrocytes (Fig. 1) [30]. According to such analysis, 811 *A. nancymae* proteins were typical of erythrocytes, given the evidence of transcription found (SD4). There was no evidence of transcription for 327 proteins which might have been due to most encoding genes (222) not complying with transcription inclusion criteria used in this study (RPKM > 0.5). Furthermore, some proteins may have had reduced transcription, this being typical during erythrocyte maturation. Other proteins were associated with cell receptors (i.e. immune components (C3b (P01024)/C4b (P04003)) or proteins from serum (serum albumin (A0A087WWT3)) (SD4, no transcription evidence sheet) which are non-typical for RBC and hence do not contain an encoding gene. Transcriptome analysis of *A. nancymae* erythrocytes is required to validate such hypothesis.

As expected, key proteins in gas exchange were identified (SD4), such as carbonic anhydrase 1 (E5RII2) and 2 (P00918), aquaporin-1 (P29972) and  $\beta$ -globin (P68871) as well as other subunits from the major cytoplasmic components (haemoglobin alpha (P69905), gamma (E9PBW4), delta (P02042), zeta (P02008), theta (P09105) and mu (Q6B0K9) chains). Proteins comprising the ankyrin (ankyrin (P16157), band3 (P02730), Rh (P18577) and 4.2 (P16452) proteins) or 4.1R (band 4.1R (P11171), glycophorin C (P04921), the Kx protein (P51811), dematin (Q08495), adducin (alpha (P35611) beta (P35612) and gamma (Q9UEY8) chains), tropomodulin-1 (P28289) and several tropomyosin proteins) complexes, together with other molecules which are essential for maintaining erythrocyte structure and function (such as spectrin alpha (P02549) and beta (P11277) chains) were also detected (SD4, marked by asterisks).

### 3.3. Analysis of functional distribution

STRAP was used for scanning the subcellular localisation and molecular and biological functions for the 811 proteins for which there was evidence of transcription (Fig. 1) [31]. Exploring the known functions reported in UniProt revealed that the proteins identified in this study participated in the following biological processes: cellular, developmental, immune system, interaction with cells and organisms, localisation, metabolic, regulation and response to biological stimuli (Table 1). Regarding molecular function terms, most were implicated in binding function (439 molecules) or were associated with catalytic activity

(326 molecules) whilst fewer were known for having a role in antioxidant activity (10 molecules), molecular transduction (9 molecules) and molecular structure (36 molecules) (Fig. 2A). Regarding cell localisation, some were extracellular, cytoplasm, nucleus and/or membrane components (Fig. 2B).

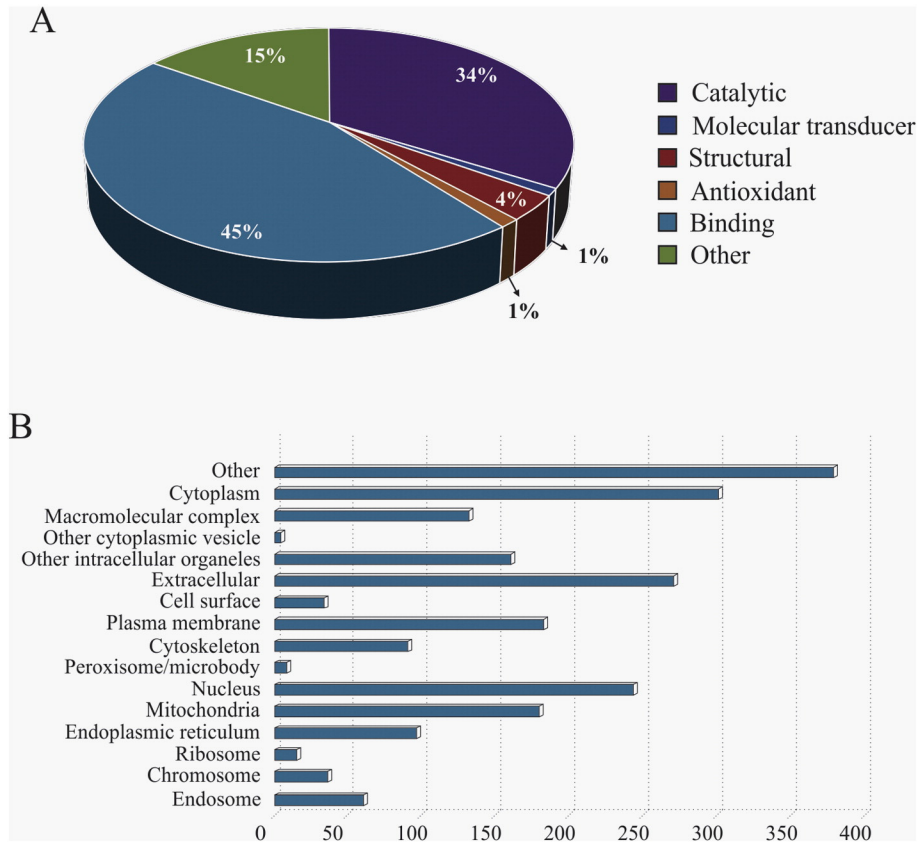
DAVID software was used for analysing GO term enrichment [32,33] in the search for the most relevant groups having statistical significance (Fig. 1). Regarding the 477 molecules identified by the software, the functional annotation chart showed that the commonest biological process (BP) terms determined were as follows: establishing localisation (175 molecules,  $p = 1.8E^{-25}$ ), transport (173 molecules,  $p = 4.9E^{-25}$ ), localisation (180 molecules,  $p = 1.1E^{-21}$ ), generating precursor metabolites and energy (48 molecules,  $p = 1E^{-19}$ ) and ATP metabolic processes (27 molecules,  $p = 7.9E^{-17}$ ) (SD5). Regarding molecular function (MF), 77 were related to nucleoside-triphosphatase, pyrophosphatase or hydrolase activity ( $p = 1.3E^{-22}$  to  $1.8E^{-21}$ ), 38 to GTPase activity ( $p = 1.1E^{-18}$ ), 325 to protein binding ( $p = 2.1E^{-16}$ ) and 237 to catalytic activity ( $p = 2, 9E^{-16}$ ) (SD5). Most proteins in the cell component (CC) category were intracellular, the most common ones being cytoplasm, mitochondria or organelle envelop (SD5). In spite of similarity between some terms stated by STRAP and DAVID software (e.g. BP: localisation, transport and metabolic processes; MF: binding and catalytic activity; CC: cytoplasm and mitochondria), there was discrepancy regarding the number of molecules comprising them, probably due to the different training sets and statistical analysis used by each tool. Hence, and because of the databases' dynamic nature, the analysis reported here cannot be considered as absolute.

### 3.4. Predicting integral membrane or surface-associated proteins

The RBCC database was used for predicting cell surface protein localisation, aimed at determining cellular receptors which could

Table 1  
Proteins involved in biological processes.

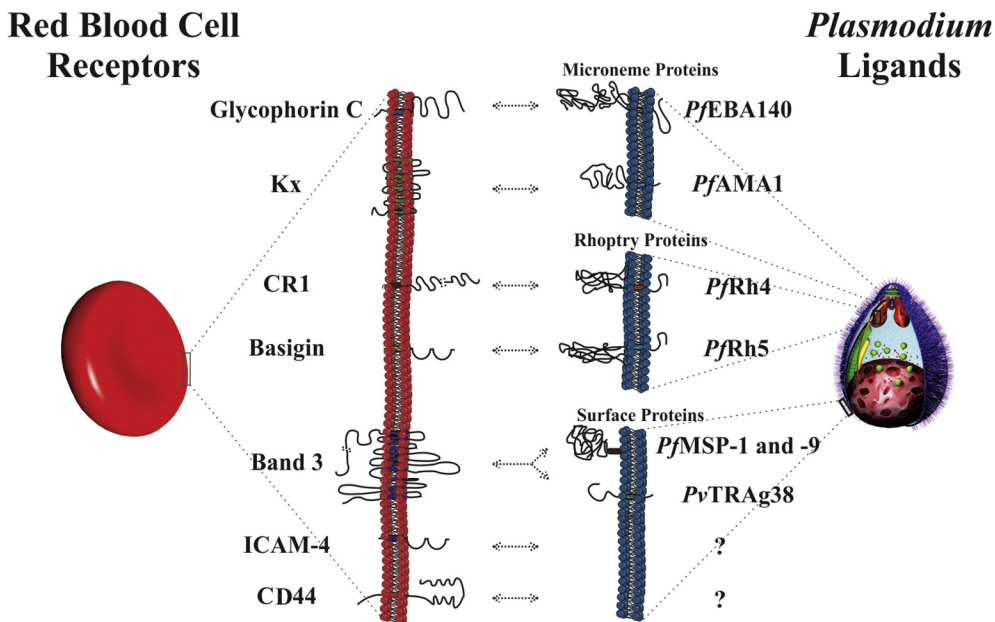
Biological function	Number of proteins
Cellular	429
Developmental	104
Immune system	51
Interaction with cells and organisms	91
Localisation	190
Metabolic	77
Regulation	354
Response to stimulus	110
Other function	234



**Fig. 2.** In silico prediction of biological processes for *Aotus* proteins identified here. A) Distribution of proteins involved in molecular function. B) A bar chart showing protein distribution according to cell location.

determine infection by *Plasmodium* species (Fig. 1) [34]; 155 molecules were identified, 51 of which were annotated as integral membrane proteins and 54 as being membrane-associated (SD6). Receptors having a known *P. falciparum* and *P. vivax* binding pattern were found, such as glycophorin C (*PfEBA140* receptor [35]), band 3 (*PfMSP-1* [36], *PfMSP-*

9 [37] and *PvTRAg38* receptor [38]) and Kx (*PfAMA1* receptor [39]), and one having an unknown binding pattern (as its absence reduced parasite invasion of cells) such as that for ICAM-4 [40] (Fig. 3). Other receptors used by Plasmodia, such as the CR1 (*PfRh4* receptor [41]), basigin (*PfRh5* receptor [36]) and CD44 [42], were found manually, as



**Fig. 3.** A schematic representation showing integral membrane proteins identified here which have been experimentally validated (in humans) as erythrocyte receptors (glycophorin C, band3, Kx, CR1, basigin, ICAM-4 and CD44) and their respective ligands in *Plasmodium* (*PfEBA140*, *PfMSP-1*, *PfMSP-9*, *PvTRAg38*, *PfAMA1-II*, *PfRh4* and -5).

their prediction by the RBCC database was not accurate due to the codes used by such databases (e.g. CR1 was P17927, basigin was P35613, CD44 was P16070).

#### 4. Discussion

Given *A. nancymaae* species' experimental importance, this study was focused on an analysis based on the similarity of MS/MS spectra with those for *H. sapiens*; the aim was a comparative identification of new proteins, taking advantage of the additional data available, collected during a previous study [23]. Such comparison revealed similarity between both species for various peptides; this was reasonable due to phylogenetic closeness between primates and humans (SD1 and SD2). Such finding supported the fact that the primates' proteome being acquired from the search in human databases is an acceptable search strategy, as shown recently [43]. On the other hand, the relatively low amount of identified *Aotus* proteins (1189 molecules) could have resulted from using *P. vivax* infected samples as a source for the analysis. We must highlight that our original goal was to characterise the *P. vivax* proteome [23] and for such purpose, samples were processed to remove most biological "contaminants" from RBC; this resulted in a greater abundance of parasite proteins masking *Aotus* peptide detection.

The search for orthologues in New World primates using bioDBnet and OrthoDB databases led to confirming 1138 proteins (95%) (SD3); this approach was used as *A. nancymaae* genome or transcriptome data had not been released. Some of them were just identified by one database, possibly due to differences between their mapping algorithms. Unlike bioDBnet, which allowed comparison with *C. jacchus* (the only species from the family having its proteome available at the time), OrthoDB has recently been shown to have the advantage of making predictions using some *A. nancymaae* data. However, it is not practical to analyse a whole data set one-by-one because this requires a tremendous amount of time for processing it.

A filter based on transcriptome analysis was applied for determining the *Aotus* erythrocyte proteome, once the sample used for the initial MS/MS analysis consisted of erythrocytes and reticulocytes [23]. It was found that 811 molecules were mature erythrocyte components, as determined by transcript evidence for each of them (SD4). The attempt to identify genuine reticulocyte components proved unsuccessful as this cell, just like mature RBC, transcribed all genes encoding the proteins identified here (data not shown). The forgoing has been supported by a recent study showing that mature erythrocytes and reticulocytes consist of the same molecules but differ in abundance [44]. Considering the 811 molecules as erythrocyte components, these were compared to a list of the 4135 *H. sapiens* RBC molecules identified to date by MS/MS [44–56]; evidence was found regarding 403 of them (SD4, accession codes shown in bold).

Various molecules were identified here (SD4) which are abundantly expressed in reticulocytes and then become reduced (integrin  $\beta$ 1 (P05556), some ribosome subunits, sodium/potassium-dependent ATPase and tubulin subunits) or completely lost as cells matured (such as transferrin receptor 1 (P02786)). This was to be expected given that the sample was heterogeneous and such cell types were not eliminated for initial analysis [23]. Likewise, even though the erythrocytes were devoid of organelles, some of their components were found, possibly because they are cell remains which should have become removed as they matured [57]. Complement receptor 1 (CR1) was found, possibly playing the same role in immune-adherence clearance as described in a previous study [58]. The forgoing was justified, since MS/MS spectra for immune components such as C3b and C4b were also found in the initial list of molecules (SD2).

Interestingly, several cytoplasm and cytoskeleton molecules which are very important for maintaining cell integrity [59] were also found (SD4, marked by asterisks). Given that Plasmodium infection affects cell morphology during invasion and growth [60,61] and several of such molecules could be affected during parasite infection and host

cell remodelling, it would be interesting to compare primate proteomes of uninfected versus infected red blood cells in future studies.

Analysis of GO term enrichment revealed a correlation between biological processes formed by most proteins and vital cell functions (SD5). For example, molecule localisation and transport are essential for correct cell structure organisation; various proteins participate in this as they are implicated in macromolecular complex formation, such as ankyrin and 4.1R, others forming the cytoskeleton and some responsible for phospholipid transport (such as flippases and scramblases) whose function is to maintain membrane stability and integrity [59]. Important proteins were also found regarding precursor metabolite and energy generation which is very essential for maintaining a number of vital cell functions, such as metabolism [62]. Likewise, there was clear correlation between molecular function and cell viability; proteins implicated in nucleoside-triphosphatase, hydrolase and GTPase activity could be participating in ubiquitin-dependent cell degradation which is essential for a cell's correct maturation, as already established [46]. The amount of molecules grouped in terms of cell localisation was quite significant, coinciding with proteins grouped in terms of major significance in biological processes and molecular function.

It is well-known that RBC membrane proteins have functional heterogeneity since they act as transport molecules and also as cell adhesion and interaction proteins [59]. Such proteins are of interest in the biomedical field, given that some pathogens such as those from the genus *Plasmodium* use them as binding receptors to interact with cells [63,64]. For example, proteins having sialic acid (SA), such as glycoporphin A, B or C, are receptors for proteins from the *P. falciparum* erythrocyte binding ligand (EBL) family whilst those lacking SA, such as the CR1, basigin, semaphorin, band 3 and Kx, facilitate the adhesion of proteins from the reticulocyte-binding-like protein homologue (RH) family. This gives rise to the observation that *P. falciparum* can bind to RBC using SA-dependent or -independent pathways [65,66]. Regarding *P. vivax* infection, it has been described that the parasite only invades reticulocytes and one of the few receptors described so far is the Duffy antigen receptor for chemokines (DARC) [67].

Concerning the 51 integral membrane proteins identified by the RBCC database, 5 receptors previously reported for *P. falciparum* were found [63]: one receptor from the SA-dependent pathway (GPC) and four from the SA-independent route (CR1, basigin, band3 and Kx). Band3 has recently been identified as a receptor for a *P. vivax* molecule belonging to the tryptophan-rich protein family [38]. DARC was not identified in this study, possibly due to it being a protein whose peptides generated by digestion with trypsin are long and are thus not detected by MS [68]. The fact that these integral membrane components are Plasmodium receptors (as experimentally validated on humans) suggested that the remaining 46 identified here might also be essential for or be implicated in parasite entry to cells. Further research should thus involve assays aimed at confirming which of them are determinants regarding the pathogenesis of malaria. Identifying other receptors and a complete understanding of erythrocyte-parasite interactions represents a key area for research and, in the future, developing a rational therapeutic method allowing malarial infection to be controlled.

#### 4.1. Concluding remarks and perspectives

The present study has reported the characterisation of the *A. nancymaae* erythrocyte proteome for the first time. The in silico approaches to the large datasets led to ascertaining this primate's erythrocyte proteomic profile and to interpreting its biological and molecular characteristics. Combining the data gathered here with the early release of *A. nancymaae* genomics and transcriptomics data provided by the Baylor College of Medicine's Human Genome Sequencing Centre (BCM-HGSC) will be enormously useful for strengthening bioinformatics analyses.

Integral membrane proteins were amongst the most important receptors described here, some of which have been shown to be involved



in *P. falciparum* merozoite invasion of human erythrocytes (e.g. glycophorin C, Kx, CR1, basigin, Band3, ICAM-4 and CD44). The *P. vivax* invasion pathway depends on Duffy antigen interaction with the DBP ligand [67]. Earlier studies have described the presence of this pathway in the *A. nancymae* primate [69], suggesting that some integral membrane proteins identified here may also be involved in such host-parasite interactions; further receptor-ligand interaction studies are thus needed to validate and fully describe the complex invasion process led by *P. vivax* parasites in invading monkey erythrocytes. These findings will further highlight *Aotus* primates as an appropriate model for basic and applied biomedical investigation.

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2016.10.018.

### Transparency document

The Transparency document, associated with this article can be found, in online version.

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## **Anexo 3**

“Caracterización de *PvGAMA* en la cepa VCG-1 de *P. vivax*”

## Caracterización de PvGAMA en la Cepa VCG-1 de *P. vivax*

### Identificación del gen *pvgama* y caracterización *in silico*

Una de las aproximaciones que se utiliza para identificar cientos de proteínas en *P. vivax* es la proteómica, la cual, en combinación con el análisis *in silico*, permite conocer la compleja maquinaria proteica que utiliza el parásito y predecir la función de algunas de sus proteínas (35-37). Por ende, se hizo un análisis a gran escala de los datos del proteoma de *P. vivax* reportados hasta el año 2014, para encontrar moléculas con características idóneas que un candidato a vacuna debe tener, como ser codificadas por genes con un perfil de transcripción mayor a 35 horas del ciclo intraeritrocítico y tener señales de secreción. El tamizaje permitió identificar GAMA cuyo homólogo en *P. falciparum* es descrito como un potencial candidato a vacuna (106, 107).

El gen *pvgama* codifica para una proteína de 771 aa de longitud con un peso molecular de ~82,7 kDa, siendo 33 residuos más larga que su homólogo en la especie *P. falciparum* (*PfGAMA*: 738 aa) (106). Posee una secuencia señal de secreción con un sitio probable de hidrólisis entre los aa 20 y 21, y una secuencia de anclaje GPI localizada entre los residuos 750-771, según la predicción obtenida con los programas SignalP 4.0 y FragAnchor, respectivamente (Figura 1A) (108, 109). El análisis de la secuencia en la base de datos Interpro reveló la presencia de una secuencia repetida compuesta de 21 copias del pentapéptido (A/L)AN(A/G)(N/D), localizada entre los residuos 591 y 695.

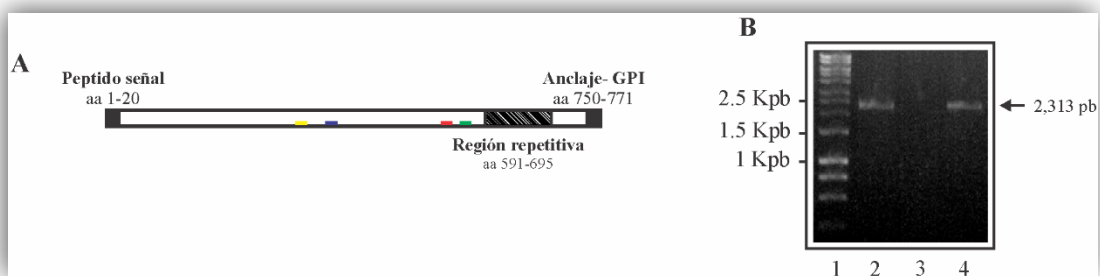


Figura 1: Identificación de GAMA en la cepa VCG-1 de *P. vivax*. (A) Caracterización *in silico* de PvGAMA. El diagrama muestra la localización del péptido señal, la región repetitiva, la secuencia de anclaje GPI y los péptidos seleccionados para el ensayo de inmunización en conejos (líneas de color). (B) Transcripción del gen *pvgama* en esquizontes. Carril 1 indica el patrón de peso molecular. Carriles 2 y 4 corresponden a la amplificación del gen usando ADNg sintetizado con retrotranscriptasa (RT+) y ADNg, respectivamente. El control de una síntesis de ADNg sin retrotranscriptasa (RT-) se muestra en el carril 3.

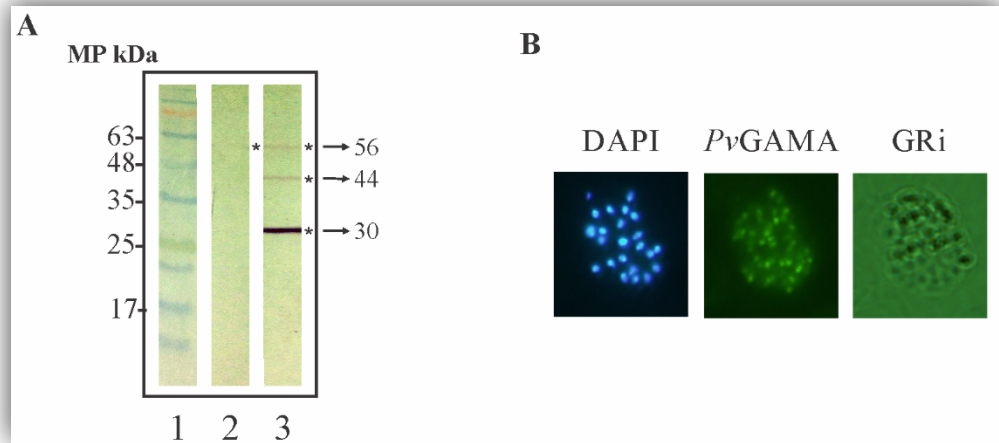
A pesar de la dificultad para realizar investigación básica en *P. vivax*, dadas las características intrínsecas de su biología (31), se caracterizó la proteína GAMA gracias a la adaptación de la cepa VCG-1 de *P. vivax* en primates (110). Según los resultados obtenidos, el gen *gama* está presente en el genoma de la cepa VCG-1 de *P. vivax* y se transcribe en el estadio esquizonte (Figura 1B), lo que coincide con el análisis transcripcional de 3 aislados clínicos de *P. vivax*, donde aumenta su transcripción de manera importante durante los tiempos TP7-TP9, lo que corresponde a estadios maduros del desarrollo intra-reticulocito (esquizontes tempranos y tardíos) (34). Al comparar las secuencias obtenidas a partir de ANDc (depositada en el NCBI con el número de acceso KT248546) y ADNg, se observó que el gen es codificado por un solo exón. El alineamiento de las secuencias de genes de la cepa VCG-1 adaptada a monos *Aotus* con la cepa de referencia Sal-1, permitió identificar 3 mutaciones: c.258 T>C (p.Thr86Thr), c.1926 T>A (p.Ala642Ala) y c.1929 T>C (p.Asn643Asn).

### **Expresión de PvGAMA en esquizontes**

Para determinar la presencia y localización de la proteína en las formas maduras de parásitos (esquizontes), se utilizaron anticuerpos de conejo dirigidos contra varios péptidos sintéticos diseñados sobre la secuencia de PvGAMA (Figura 2A). El suero pos-III detectó varias bandas de distinto peso molecular sobre el lisado de parásito tratado en condiciones reductoras (Figura 2A carril 3); éstas corresponden a dos bandas intensas (de 44 y 30 kDa) y otra de 56kDa de menor intensidad, la cual también se detectó utilizando el suero pre inmune (Figura 2A carril 2). Dicho procesamiento difiere del encontrado para GAMA de *P. falciparum*, la cual sufre dos procesamientos: el primario que corresponde a la formación de un dímero de 37 y 49 kDa a partir de la proteína completa (80 kDa), y el secundario, un fragmento de 42 kDa a partir del producto de 49 kDa (107).

No fue posible obtener anticuerpos dirigidos contra el fragmento teórico de 37 kDa del extremo amino-terminal de PvGAMA, lo que impidió verificar los productos de procesamiento proteolítico generados hacia dicho extremo. Lo anterior se debió a la dificultad para seleccionar péptidos de células B idóneos que pudieran ser utilizados en ensayos de inmunización, por sus bajos valores de antigenicidad de Parker e hidrofiliidad. La identificación de la proteína GAMA en el lisado de esquizontes de la cepa VCG-1 de *P. vivax*, coincide con el estudio del proteoma de

aislados de *P. vivax* provenientes de 10 pacientes sintomáticos de Tailandia, donde se identificó un péptido de GAMA (con un 99% de confianza) en la muestra de esquizontes por espectrometría de masas (36).



**Figura 2.** Detección de *PvGAMA* nativa en esquizontes. (A) Reconocimiento de *PvGAMA* usando anticuerpos generados en conejo. Carril 1 indica el marcador de peso molecular en kilodaltons (MP kDa). Carriles 2 y 3 muestran el reconocimiento de *PvGAMA* usando suero pre-inmune y pos-III, respectivamente. (B) Localización sub-celular de *PvGAMA* en esquizontes maduros. Las imágenes muestran el reconocimiento de la proteína (verde), el núcleo (azul) y los glóbulos rojos infectados (GRi) (luz blanca).

Al usar los anticuerpos anti-*PvGAMA* para localizar la proteína, se observó una señal de fluorescencia punteada en los merozoitos contenidos en esquizontes maduros (Figura 2B), la cual es característica de proteínas localizadas en organelos apicales (roptrias o micronemas) y coincide con el patrón de expresión apical de su homólogo en *P. falciparum* (106). Lo anterior, sumado a los resultados de transcripción y expresión de GAMA en estadios maduros de *P. vivax* (primordialmente esquizontes), sugiere que posiblemente la molécula pueda tener una función durante el proceso de invasión a reticulocitos. Así, se evaluó la capacidad de unión de *PvGAMA* a reticulocitos, cuyos resultados han sido descritos en el capítulo 2 de esta Tesis Doctoral.

## **Anexo 4**

“Reticulocitos: Células Diana de Invasión de *P. vivax*”

# Reticulocytes: *Plasmodium vivax* target cells

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Reticulocytes represent the main invasion target for *Plasmodium vivax*, the second most prevalent parasite species around the world causing malaria in humans. In spite of these cells' importance in research into malaria, biological knowledge related to the nature of the host has been limited, given the technical difficulties present in working with them in the laboratory. Poor reticulocyte recovery from total blood, by different techniques, has hampered continuous *in vitro* *P. vivax* cultures being developed, thereby delaying basic investigation in this parasite species. Intense research during the last few years has led to advances being made in developing methodologies orientated towards obtaining enriched reticulocytes from differing sources, thereby providing invaluable information for developing new strategies aimed at preventing infection caused by malaria. This review describes the most recent studies related to obtaining reticulocytes and discusses approaches which could contribute towards knowledge regarding molecular interactions between target cell proteins and their main infective agent, *P. vivax*.

## Introduction

Reticulocytes are erythroid cells which have not reached maturity, being characterised by presenting a reticular network formed by residual RNA (Orten, 1934). These cells represent around 1–2% of circulating human red blood cells (RBCs) and have a short life span (24 h). It has been demonstrated that varying the percentage of reticulocytes as well as defects in their messenger RNA are associated with some diseases having variable clinical relevance (Benz and Forget, 1971; Bessman, 1990; Suzuki et al., 1993).

Some parasite species belonging to the *Plasmodium* genera causing malaria in humans (*Plasmodium vivax*) and rodents (*Plasmodium berghei*) have shown a certain preference for invading reticulocytes (Butcher et al., 1973; Mons, 1990; Cromer et al., 2006). *P. vivax* is one such species which is characterised by being the most widely distributed throughout tropical and sub-tropical zones, causing the highest morbidity indexes on the Asiatic and American continents (Guerra et al., 2010). Obtaining a sufficient amount and concentration of reticulocytes is essential for establish-

ing *in vitro* *P. vivax* cultures, meaning that studying them has become one of the essential research topics for groups working on malaria caused by this parasite species.

Knowledge of reticulocyte biology regarding the type of cell receptors required for invasion by the parasite is limited. Studies of the human reticulocyte transcription profile (Goh et al., 2007) and analysing murine reticulocytes' partial proteome (Prenni et al., 2012), added to using methodologies for evaluating protein–protein molecular interactions, could be of great use for carrying out future research focussed on an elucidating interaction mechanisms between this target cell and its pathogen.

## Literature search

Literature included in this review was found querying the PubMed database from 1900 to date, using the following search terms: 'reticulocytes', 'reticulocyte purification', 'reticulocyte cryo-preservation', 'reticulocyte proteome', '*Plasmodium vivax in vitro* culture', '*Plasmodium vivax* vaccine' and '*Plasmodium vivax* and reticulocytes'. The literature was initially analysed according to titles and abstracts and then relevant studies were fully reviewed. The inclusion criteria were: (i) studies describing reticulocytes' biology focussing on receptor characterisation and proteomics,

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**Key words:** *In vitro* continuous culture, Molecular interaction, *P. vivax*, Prevention strategy, Reticulocyte.

**Abbreviations used:** DARC, Duffy antigen receptor for chemokines; HSC, haematopoietic stem cell; MSP, merozoite surface protein; RBCs, red blood cells; RBP, reticulocyte-binding protein.



or (ii) their use in *P. vivax* research and (iii) the results were restricted to studies written in English language.

## Reticulocytes and malaria

The first microscopic descriptions of reticulocytes were made by Wilhelm Erb in 1865; he demonstrated the presence of granular material in human RBC and different animal species. Twenty years later, Paul Ehrlich reported that some erythrocytes from patients suffering from anaemia, stained with methylene blue, had fine, dense, elegant blue networks; which is why they are called 'reticulated erythrocytes' (Orten, 1934). Gloria Gronowicz and her group defined the complex process of these cells maturing into erythrocytes from 1984 onwards (Gronowicz et al., 1984), thereby leading to their main characteristics being determined: from residual RNA content left behind after nuclear loss (formerly known as reticular network), the presence of ribosome, mitochondrial, lysosome and endocyte vesicular granules and so on. At clinical level, such cells' count is used for distinguishing the effectiveness of RBC production in bone marrow (Piva et al., 2010), or, on the contrary, for evaluating the type of cell disorder being presented: aplastic anaemia, haemolytic anaemia, haemoglobinopathy,  $\beta$ -thalassaemia or reticulocytopenia (Benz and Forget, 1971; Bessman, 1990; Suzuki et al., 1993).

Interest in studying reticulocytes among groups working on malaria has emerged as a result of research orientated towards standardising an *in vitro* *P. vivax* culture, due mainly to this parasite species' preference for invading immature RBC (Bass and Johns, 1912; Mons, 1990). It is well known that *in vitro* cultures require the constant addition of reticulocytes (given these cells' maturation rate, *i.e.* 24 h at 37°C) for perpetuating parasite growth; however, given these cells' reduced percentage in adult humans' blood, managing to enrich or finding a source having a high percentage of them has been one of the main research challenges. As a result of this issue, studies reporting *P. vivax* proteins' functionality and their potential as vaccine candidates are limited, compared with those described for *Plasmodium falciparum* (Patarroyo et al., 2012). As developing a vaccine against pathogenous agents involves ascertaining a particular microorganism's biology and its respective target cells (Patarroyo

et al., 2011), in the case of *P. vivax*, it is recommendable that a methodology be standardised for obtaining a good amount of reticulocytes from different biological sources, and also evaluating techniques for characterising receptor–ligand molecular interactions between such cells.

## Reticulocyte sources

Different blood sources have been used in the attempt to enrich reticulocytes, including animals (primates and mice), healthy people and people suffering from blood disorders such as haemochromatosis (Golenda et al., 1997) and  $\beta$ -thalassaemia (Ocampo et al., 2002) and cord blood from newborns (Table 1). Different protocols have also been used for obtaining viable reticulocytes supporting parasite infection aimed at improving basic knowledge about their biology. A discontinuous *in vitro* *P. vivax* culture has recently been partially standardised using enriched cord blood reticulocytes (Russell et al., 2011), representing an advantage for continuing studies in this area.

## Isolation from animal blood

Primates from the genera *Aotus nancymai* and BALB/c mice are frequently used as animal models for studying the pathogenesis of malaria. In attempts to standardise a culture for *P. vivax* and *Plasmodium yoelii*, these animals have been anemised by haemolytic drugs and consequently the percentage of reticulocytes in blood has increased considerably. The classical CF11 column filtration method (Sriprawat et al., 2009) has been used for eliminating leucocytes from primates' blood samples and obtaining completely pure reticulocytes (Mons et al., 1988). Even though the objective of obtaining enriched reticulocytes has been successful, these cells have shown certain fragility, given that infection could not be maintained. Mice anemised by Swardson-Olver's group had 58–72% reticulocytes which were recovered by cardiac puncture, biotinylated and enzymatically treated with trypsin and chymotrypsin. It was observed that cells pre-treated with trypsin were infected threefold more by *P. yoelii* 17X strain parasites than those pre-treated with chymotrypsin when injected in BALB/c mice, thereby suggesting that the recovered reticulocytes were viable hosts (Swardson-Olver et al., 2002). The methodology for enriching reticulocytes by inducing anaemia in animals has

**Table 1 | General panorama of sources and methods for enriching reticulocytes evaluated to date**

Reticulocyte source and sample collection	Enrichment method	Yield (%)	Viability in <i>in vitro</i> culture	Expenses/suitability based on source	References	
Blood obtained from animals by venipuncture or by cardiac puncture	Splenectomised monkeys were treated with phenylhydrazine (100 mg/kg body weight). Leucocytes were removed by using a CF-11 column or leucocyte filters	ND	No viability	Less expensive but reticulocyte source very difficult to obtain. Useful for research in <i>P. vivax</i>	Mons et al. (1988)	
	Mice were treated with phenylhydrazine (10–15 mg/kg body weight)	>30	Not tested	Less expensive and a good reticulocyte source to study mice malarial	Gronowicz et al. (1984)	
	Mice were treated with 1.5 mg of phenylhydrazine	58–72			Swardson-Oliver et al. (2002)	
	Mice were treated with an anaemia-inducing strain of Friend leukaemia virus (FVA). Reticulocytes were separated by using 1–2% deionised bovine albumin gradient	>95			Koury et al. (2005)	
	Mice were injected intraperitoneally with 2 ml of normal saline and then 700 $\mu$ l of blood were removed by retro-orbital puncture. A two-step isotonic Percoll gradient with density of 1.058 and 1.096 mg/ml was used for reticulocyte recovery	35–50			Liu et al. (2010)	
Peripheral blood of people obtained by venipuncture	Mice were treated with 1.5 mg of phenylhydrazine	ND			Wooden et al. (2011)	
		50			Prenni et al. (2012)	
	Blood extracted from healthy people was treated to enrich reticulocytes and/or remove leucocytes	62% Percoll gradient Magnetic beads loaded with anti-human transferrin receptor antibodies Percoll/renografin-60 gradient CF-11 column or Plasmodipur filter	ND 15–42	Yes Not tested	Not expensive but it is a poor reticulocyte source. Useful for research in <i>P. vivax</i>	Barnwell et al. (1989) Brun et al. (1990)
		8–15 and rarely 25	No viability		Lanners (1992)	
		ND	Not tested		Sripawat et al. (2009)	
	Blood extracted from people having haemochromatosis or $\beta$ -thalassaemia, respectively, was treated to enrich reticulocytes and/or remove leucocytes	Differential centrifugation and leucocyte separation filter CF-11 column	15–20 >85	Yes Not tested	Not expensive but patients suffering from such diseases are scarce in some latitudes of the world	Golenda et al. (1997)
					Ocampo et al. (2002); Rodriguez et al. (2002); Urquiza et al. (2002)	
Cord blood of newborns collected in heparin tubes	Cord blood was used to enrich reticulocytes and/or remove leucocytes	RCXL2 high-efficiency leucocyte reduction filters	ND	Not tested	Not expensive and recommended source Goh et al. (2007)	

(Continued)

Table 1 | Continued

Reticulocyte source and sample collection	Enrichment method	Yield (%)	Viability in <i>in vitro</i> culture	Expenses/suitability based on source	References
	CF-11 column	6.9–7.9	Yes		Udomsangpetch et al. (2007)
	70% isotonic Percoll gradient and a CF-11 column	57.8	Yes		Russell et al. (2011)
	Hypotonic sodium chloride solution and a CF-11 column	1–28	Yes		Grimberg et al. (2012)
	Reticulocytes obtained by maturing erythroid cells isolated from cord blood by using 30–60% Percoll discontinuous gradients	ND	Yes		Panichakul et al. (2007)
	Reticulocytes were obtained from cord blood by using 70% isotonic Percoll gradient. Leucocytes were removed using a CF-11 column. Sample was cryopreserved in liquid nitrogen with Glycerolyte 57	>40	Yes		Borlon et al. (2012)
	Reticulocytes were obtained by maturing HSCs. Glycerolyte, Glycerol + liquid Sorbitol or IMDM/10% DMSO/40% FCS were used for cryopreservation	ND	Yes	Expensive but recommended source	Noulin et al. (2012)

ND: Not determined.

been used in other studies orientated towards evaluating reticulocyte maturation (Koury et al., 2005; Liu et al., 2010), as well as studying the partial proteome (Prenni et al., 2012).

### Isolation from adult human blood

Even though the percentage of reticulocytes is low in adults, several enrichment techniques have been explored. Barnwell et al. (1989) obtained reticulocytes from human blood using a 62% Percoll gradient. The results showed that these cells supported *in vitro* parasite invasion and development (Barnwell et al., 1989). Norbert Lanners used the discontinuous Percoll/renografin-60 gradient technique in 1992 for separating reticulocytes from fresh human blood. Even though the amount of cells obtained was considerable (8–15%), they were fragile and did not support parasite development (Lanners, 1992). Once such disadvantage had been described, several research groups focussed their efforts on finding other sources and improving techniques for obtaining immature erythrocytes.

Reticulocytes, just like all cells, display a series of cell surface markers. It has been shown that transferrin receptors disappear during maturation, thereby suggesting that they are specific for immature red cells (Frazier et al., 1982). Bearing such difference in mind, a purification protocol using anti-transferrin antibodies coupled to magnetic beads was developed (Brun et al., 1990); the results revealed 15–42% reticulocyte enrichment. However, the difficulty of eluting cells coupled to beads has been the main factor limiting this method.

Blood from patients suffering haemochromatosis has a high percentage of reticulocytes (3–5%). Taking advantage of such proportion of cells, Golenda et al. (1997) used a differential centrifugation separation protocol. Leucocytes were removed by a leucocyte separation filter later on, achieving around 10–12 ml final volume having 15–20% reticulocytes per peripheral blood unit. These cells were able to support a short-term *P. vivax* culture.

Our group reported using blood from a patient suffering from  $\beta$ -thalassaemia in 2002, demonstrating

that such blood contained greater than 85% reticulocytes (Ocampo et al., 2002; Rodriguez et al., 2002; Urquiza et al., 2002). Samples obtained by venipuncture were washed and passed through a CF11 cellulose column to remove leucocytes and then used for identifying peptide sequences from *P. vivax* proteins binding with high affinity to target cells. Comparing reticulocyte and mature erythrocyte binding revealed that the former had specific receptors for the proteins being evaluated.

### Isolation from cord blood

The animal and/or adult human blood reticulocyte isolation studies had limited application, given the intensive labour involved in the procedure and a lack of available patients in some parts of the world suffering from such diseases; this led to exploring other areas. Udomsangpetch et al. (2007) were the first to report that umbilical cord blood, containing a high percentage of reticulocytes (3–8%), supported an *in vitro* *P. vivax* culture which was maintained for a short period (not more than 30 days). Russel et al. (2011) then used a procedure for enriching cord blood reticulocytes using a 70% isotonic Percoll gradient. Leucocytes and platelets were removed from twenty-eight 20 ml volume samples using two rounds of filtering with a CF11 column. After being separated by a density gradient, an average of 57.8% reticulocytes contained in 191  $\mu$ l blood was obtained; these were kept stable for 4 wk. This represented an advantage for culturing, as having a greater amount of reticulocytes in a small volume favours *P. vivax* invasion and growth.

Grimberg et al. (2012) developed a simple, rapid and easily used methodology for enriching reticulocytes from cord blood. The authors used selective enrichment on 10 cord samples using a hypotonic sodium chloride solution, taking advantage of the difference in reticulocytes' osmotic pressure compared with that of other types of cell. Reticulocytes increased by 1–28% after eliminating cells lysed by several washings and removing leucocytes by CF11. The cells maintained their integrity and were viable, demonstrated by analysing haemoglobin stability and *in vitro* *P. falciparum* culture assay. Even though this methodology is not suitable for use with normal adult blood, or for cryopreserving cells, it is clear that it represents an optimum procedure for obtaining cells for immediate use.

### Maturation of haematopoietic cells

Extensive research focussed on studying the umbilical cord has led to determining that this is an important source of mesenchymal, somatic and haematopoietic stem cells (HSCs), this being of great importance in the field of biomedical and clinical research (Pelosi et al., 2012). HSCs can be differentiated in any type of cell present in the blood by the activity of specific differentiation factors; such cells can also be found in bone marrow or other tissues. Thinking about this line as a potential source of erythrocytes, Panichakul et al. (2007) introduced a method for obtaining a large amount of reticulocytes from HSC maturation into erythroid cells. Their results revealed a large amount of reticulocytes on day 14 determined by brilliant Cresyl blue staining; given their morphology, cell surface markers and susceptibility to *P. vivax* infection, the authors considered them to be completely viable. Although this method has led to fresh erythroid cells being obtained in different stages of maturation, it is very complex to use and is costly, meaning that it may have limited application in research groups working on malaria.

### Reticulocyte cryopreservation

Although most reticulocytes are successfully recovered by using some of the methods mentioned in the 'Reticulocyte sources' section, their rapid maturation turns out to be a restrictive factor when using this type of cells in lengthy assays. Several studies focussed on reticulocyte preservation have been developed to overcome this problem. Borlon et al. (2012) analysed the possibility of cryopreserving reticulocytes obtained from cord blood; these cells were enriched according to the previously mentioned methodology (Russell et al., 2011), homogenised in 20% glycerol and then stored in liquid nitrogen. Once the cells had been unfrozen using the sodium chloride method, their ability to host infection was evaluated *in vitro*. The results showed that passing cells through liquid nitrogen did not affect their stability as they matured normally; cells were also viable and supported parasite invasion. A complementary study by Noulin et al. (2012) showed that erythrocytes obtained from HSC maturation could be cryopreserved for 1 year without damage to the receptors located on plasma membrane. Cryopreservation was successful using two media containing different preservative agents; the first consisted of 28%

glycerol, 3% sorbitol and 0.9% sodium chloride, whereas the other contained 10% DMSO/40% FCS (Noulin et al., 2012). The use of these methods represents an advantage concerning reticulocyte use in malaria research and in studies using analytical tools focussed on omic sciences, such as microarrays and/or proteomics.

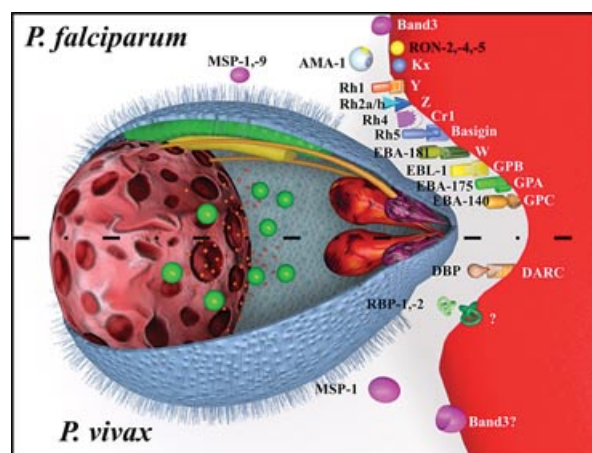
### Cell receptors characterised for *P. vivax*

Even though it is clear that some reticulocyte molecules are essential for the progress of *P. vivax* infection, their description, characterisation and the exact nature of molecular interactions with target cell proteins still remains unknown. Characterising cell surface receptors will provide valuable information for developing prevention strategies, mainly vaccines. In spite of the foregoing, advances in knowledge regarding reticulocyte receptors for *P. vivax* have notably lagged behind the body of knowledge concerning erythrocyte receptors for *P. falciparum* (Sim et al., 1994; Rayner et al., 2001; Goel et al., 2003; Maier et al., 2003; Li et al., 2004; Mayer et al., 2004; Kato et al., 2005; Mayer et al., 2009; Tham et al., 2010; Crosnier et al., 2011; Gunalan et al., 2011; Sahar et al., 2011; Triglia et al., 2011; Tham et al., 2012) (Figure 1) (Table 2).

The Duffy antigen receptor for chemokines (DARC) is the only reticulocyte receptor used by *P. vivax* during invasion which has been described and functionally evaluated to date (Figure 1). This molecule is a transmembrane glycoprotein which is expressed on the surface of various cells, including human erythrocytes and reticulocytes (Peiper et al., 1995; de Brevern et al., 2005). The DARC has 337 amino acids, having an estimated ~35 kDa molecular weight and crosses the RBC membrane seven times. Its identity as a *P. vivax* receptor became known in a study involving the genotyping of individuals proving resistant to the disease which determined that their RBC lacked DARC expression (Miller et al., 1976). It has also been shown that this receptor is required for infection caused by *P. knowlesi* (Haynes et al., 1988; Adams et al., 1990) and *P. yoelii* (Swardson-Oliver et al., 2002). The advent of 3D prediction and mutational studies has facilitated understanding the interaction between the DARC and its ligand, the *P. vivax* Duffy binding protein (VanBuskirk et al., 2004; Singh et al., 2006). Russel

**Figure 1 | Red blood cell and reticulocyte receptors described to date with their respective *P. falciparum* and *P. vivax* binding ligands**

Parasite ligands are shown in black and RBC receptors are shown in white letters, respectively. GPA, GPB and GPC, glycoporphin A, B and C; CR1, complement receptor 1; DARC, Duffy antigen receptor for chemokines; EBA, erythrocyte binding antigen; EBL-1, erythrocyte binding like 1; RON-1, -2, -5, Rhoptry Neck Proteins -1, -2 and -5 which participate in forming the *tight junction* complex with AMA-1, apical merozoite antigen 1; MSP-1, merozoite surface protein 1; DBP, Duffy binding protein; RBP-1, -2, reticulocyte binding proteins -1 and -2. Y, Z, W and ? represent receptors not yet identified.



et al. (2011) have shown this receptor's functional importance *in vitro*. Consistent with assays by Barnwell et al. (1989) years ago, DARC interaction between the parasite ligand became inhibited on incubating reticulocytes with murine Fy6 antibodies directed against the receptor's N-terminal region, leading to the suggestion that DARC is an obligatory route for *P. vivax* invasion of reticulocytes.

The strong inclination of *P. vivax* for invading reticulocytes and its inability to infect mature RBC (which also express the Duffy antigen) suggest that this cell type's specificity could be attributed to an interaction with other membrane receptors. Several reports referring to identifying infection in Duffy-negative individuals (Ryan et al., 2006; Cavasini et al., 2007a; 2007b; Mendes et al., 2011) have supported the hypothesis that DARC is not the only component required for invasion by *P. vivax*, as initially proposed. Moreover, parasite proteins participating in target cell recognition and having a preference for

**Table 2** | Red blood cell receptors required for *P. falciparum* and *P. vivax* invasion

Cell type	Receptor	Ligand	Ligand localisation	Reference
RBC	GPA	EBA-175	Micronemes	Sim et al. (1994)
	GPB	EBL-1	Micronemes	Mayer et al. (2009)
	GPC	EBA-140 (BAEBL)	Micronemes	Maier et al. (2003)
	W	EBA-181	Micronemes	Revised in Tham et al. (2012)
	Kx	AMA-1	Micronemes	Kato et al. (2005)
	Y	Rh1	Rhoptries	Rayner et al. (2001)
	Z	Rh2a/b	Rhoptries	Gunalan et al. (2011); Sahar et al. (2011); Triglia et al. (2011)
	CR1	Rh4	Rhoptries	Tham et al. (2010)
	Basigin (CD147)	Rh5/Ripr	Rhoptries	Crosnier et al. (2011)
	Band 3	MSP-1, -9	Surface	Goel et al. (2003); Li et al. (2004)
Reticulocyte	DARC	DBP	Micronemes	Peiper et al. (1995)
	Band3?	MSP-1	Surface	Rodriguez et al. (2002)
	?	RBP-1, -2	Rhoptries	Galinski et al. (1992); Cantor et al. (2001)

GPA, GPB and GPC, glycophorin A, B and C; CR1, complement receptor 1; EBA, erythrocyte binding antigen; EBL-1, erythrocyte binding like 1; AMA-1, apical merozoite antigen 1; MSP-1, merozoite surface protein 1. Y, Z, W and ? represent receptors not yet identified.

reticulocyte binding have been identified, including reticulocyte-binding proteins 1 and 2 (RBP-1, -2) (Galinski et al., 1992; Cantor et al., 2001) and merozoite surface protein 1 (MSP-1) (Rodriguez et al., 2002) (Figure 1). Previous studies have found that RBP-1 and MSP-1 contain peptides which bind to reticulocytes with high affinity; RBP-1 binds to 26 and 41 kDa receptors, whereas MSP-1 binds to 18 and 20 kDa molecular mass receptors (Rodriguez et al., 2002). These latter ones seem to be reticulocyte-specific because their molecular weight differs from that determined for Band 3, which has been considered as the MSP-1 receptor, according to experiments carried out with its *P. falciparum* homologue (Goel et al., 2003). Despite these studies, the receptors for MSP-1 in *P. vivax* as well as those for RBPs have not been experimentally characterised so far. The foregoing highlights the need for using different tools for characterising reticulocytes' surface molecules for understanding the molecular basis of their function as malaria parasite receptors. This will lead to proving the existence of an alternative *P. vivax* invasion route (Mons, 1990) and developing strategies for preventing the infection.

## Perspectives

Improving methods to obtain large reticulocyte amounts is required to solve one of the main challenges in *P. vivax* malaria research: the characterisation of antigens with potential role in invasion. Various methods have been tested for cell separa-

tion in suspension, such as the use of immunomagnetic beads or selective separation by flow cytometry (Davies, 2012); these methods could be used for reticulocyte purification to prevent the damage caused to these cells when passing them through an isotonic Percoll gradient, a CF-11 column or when they are enriched using a hypotonic solution. This will facilitate obtaining a greater number of viable cells to use in *P. vivax in vitro* culture assays or studies focussed on understanding *P. vivax* infection dynamics.

Additional techniques orientated towards evaluating the identity of the molecules involved in protein–protein interactions between *P. vivax* and reticulocytes are thus needed. Among existing methodologies, using affinity etiquettes for purifying complexes, co-immunoprecipitation, chemical crosslinking, the two-hybrid system and confocal microscopy (Phizicky and Fields, 1995; Berggard et al., 2007; Wu et al., 2007) have proved of great use when molecularly analysing target cell–parasite protein interactions. Some of the approaches here mentioned, together with mass spectrometry and structural analysis, open the way forward for ascertaining the molecular basis for invasion and also for resolving the deficiencies which have arisen when studying antigens for inclusion in a potentially effective vaccine against malaria caused by *P. vivax*.

The 'omic' sciences are an interesting alternative when evaluating cell receptors facilitating parasite adhesion. Oligonucleotide microarrays and mass spectrometry assays have been used for studying

reticulocyte genes and proteins which are being expressed. Goh et al. (2007) used the microarray technique; they identify 107 genes, 103 of which were differentially expressed in umbilical cord reticulocytes. This profile provides an interesting source of information for carrying out probabilistic studies aimed at identifying surface receptors for the *P. vivax* proteins described to date (Patarroyo et al., 2012). Two mass spectrometry studies of immature murine erythrocyte membrane fractions have been carried out; the first reported 45 proteins having increased expression in a reticulocyte-enriched sample (Wooden et al., 2011), whereas the second identified 587 (Prenni et al., 2012), some of them being characterised as plasmatic membrane proteins. Such results provide invaluable information about the possible receptors which could be involved in their selectivity as host cells.

Furthermore, an open research line is the study of exosomes. These are internal vesicles located in multi-vesicular bodies which are involved in selective removal of several proteins during reticulocyte maturation and differentiation process to erythrocytes. A recent study has revealed that exosomes derived from reticulocytes infected with the non-lethal *P. yoelii* 17X strain contained antigens which were able to modulate the immune response, suggesting that these vesicles can play roles beyond those initially described, intercellular communication and antigen presentation among them (Martin-Jaular et al., 2011). Further studies to evaluate exosomes' importance in malaria research are in need, such as their detailed proteomic characterisation, seeking for reticulocyte receptors that make the *P. vivax* parasite infection so selective.

## Conclusion

Although the study developed by Russell et al. (2011) has been the most promising for reticulocyte recovery since it has shown to be suitable for culturing *P. vivax* *in vitro*, further optimisation of target cell enrichment methods is needed to address several fundamental questions regarding both reticulocyte and parasite biology. Enrichment methods could be improved by avoiding the use of Percoll, CF-11 column or hypotonic solutions, and developing new approaches for recovering cells in suspension.

Advances in studying reticulocytes' biological aspects have provided valuable information for ascertaining these cells' genetic determinants regarding their susceptibility to different diseases, including infection caused by parasites. Further studies are required for understanding the precise role played by receptor–ligand interactions between cells and their main infectious agent, thereby also being useful for finding therapeutic targets and establishing other methods for preventing infection, including the development of chemically made vaccines.

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## Conflict of interest statement

The authors have declared no conflict of interest.

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