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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DEDICATORIA

A Dios, esencia de la vida.

A mis padres y hermanos, ejemplo de perseverancia.

A mi esposa e hijo, mi mayor tesoro.

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

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Abreviatura	Término
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*: *Pv*ARP, *Pv*RBSA y *Pv*GAMA, así como su antigenicidad. De particular interés, se encontró que *Pv*RBSA y *Pv*GAMA se unen en mayor proporción a reticulocitos que expresan el receptor CD71 de forma abundante ($CD71^{hi}$), 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 (*Pv*ARP, *Pv*RBSA and *Pv*GAMA) were identified. Particularly interesting was the finding that a higher percentage of *Pv*RBSA and *Pv*GAMA bound to reticulocytes abundantly expressing the CD71 receptor ($CD71^{hi}$), 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).

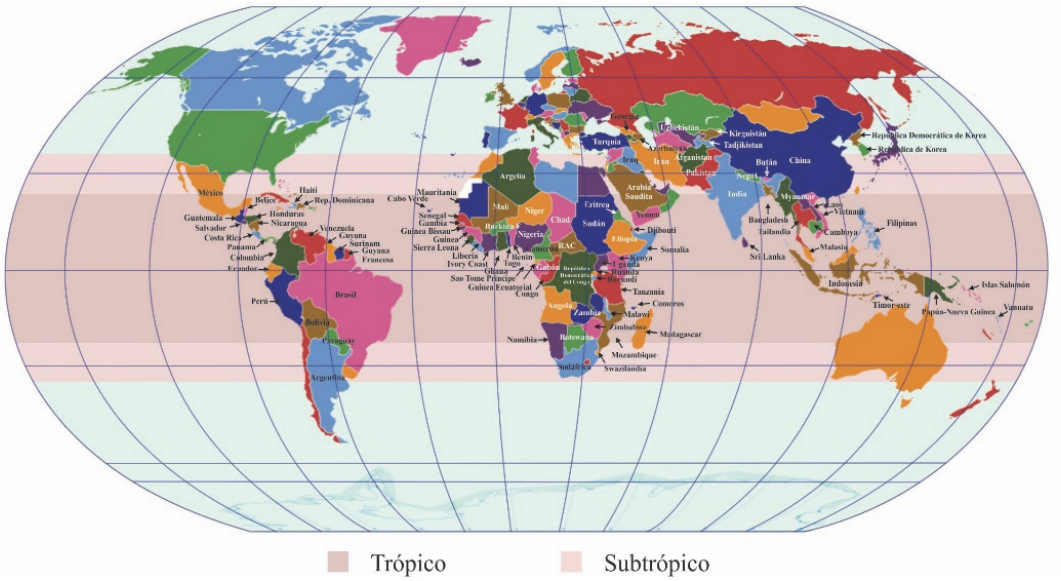

Figura 1. Países endémicos de malaria según la OMS.

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.

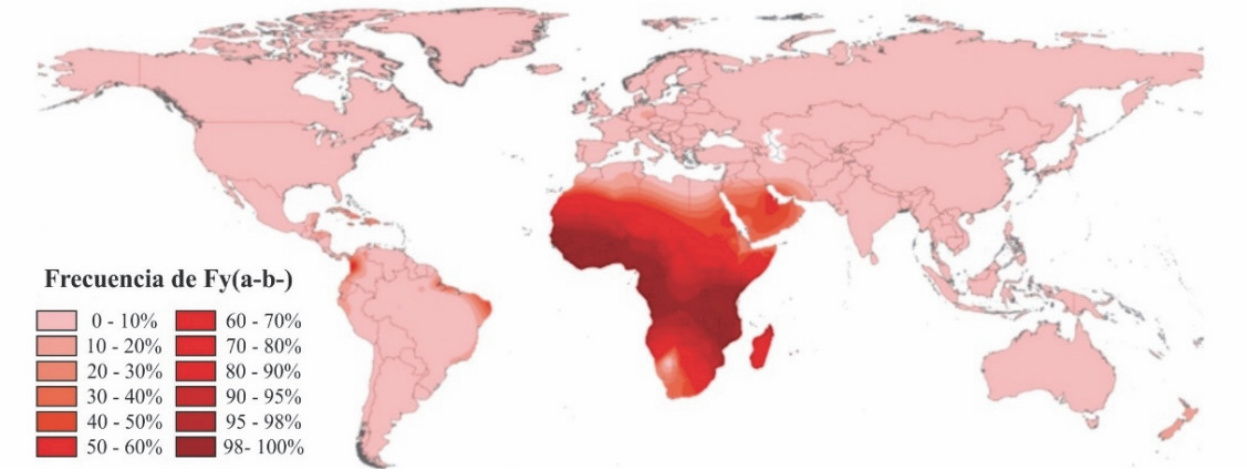

Figura 2. Distribución global del fenotipo Duffy negativo. Imagen modificada de Howes *et al*., 2011 (6).

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 genero *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 estadío 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.

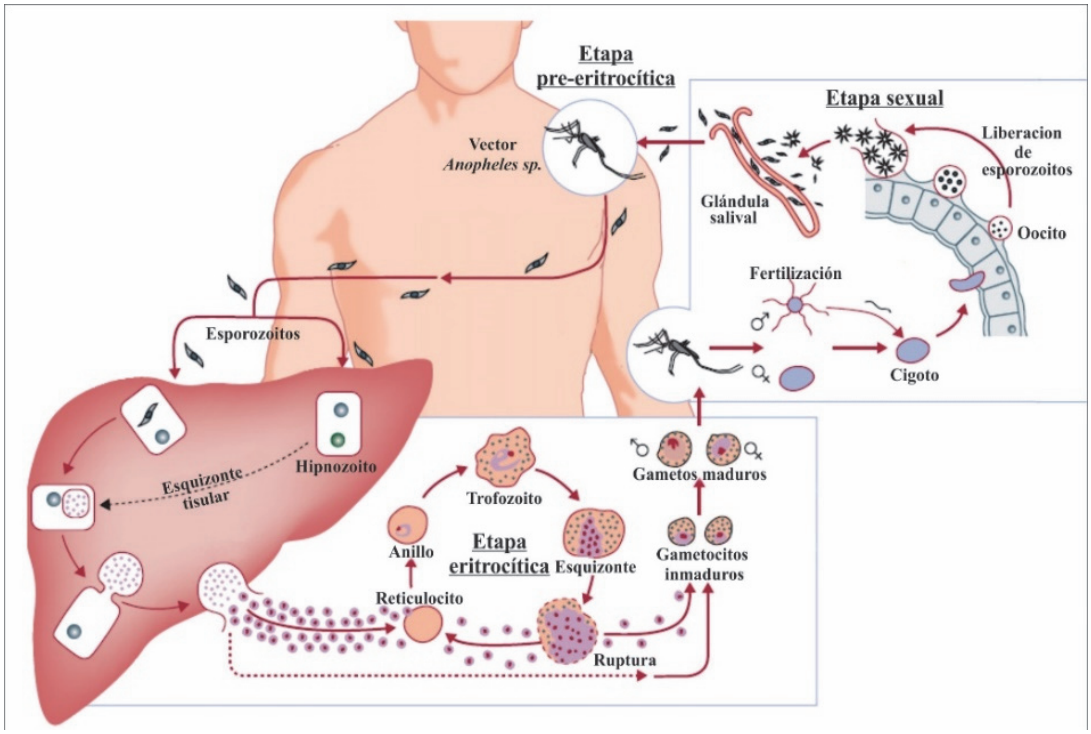

Figura 3. Ciclo de vida de *Plasmodium*. Imagen modificada de Mueller *el al*., 2016 (14).

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).

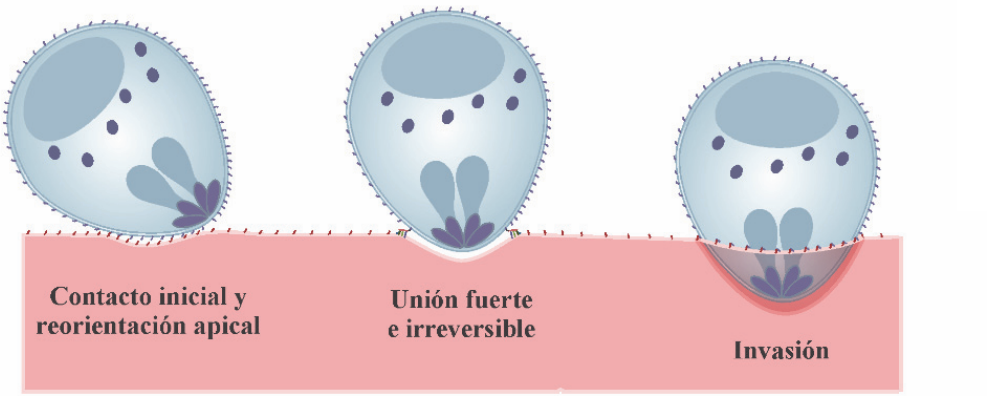

Figura 4. Fases de invasión de merozoitos de *Plasmodium* a eritrocitos humanos. Imagen modificada de Koch *et al*., 2016 (18).

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	*P. falciparum* - Año (Ref)	*P. vivax* - Año (Ref)
Genoma	2002 (26)	2008 (32)
Transcriptoma	2003 (27)	2005 (33), 2008 (34)
Proteoma	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 protein*s). 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.

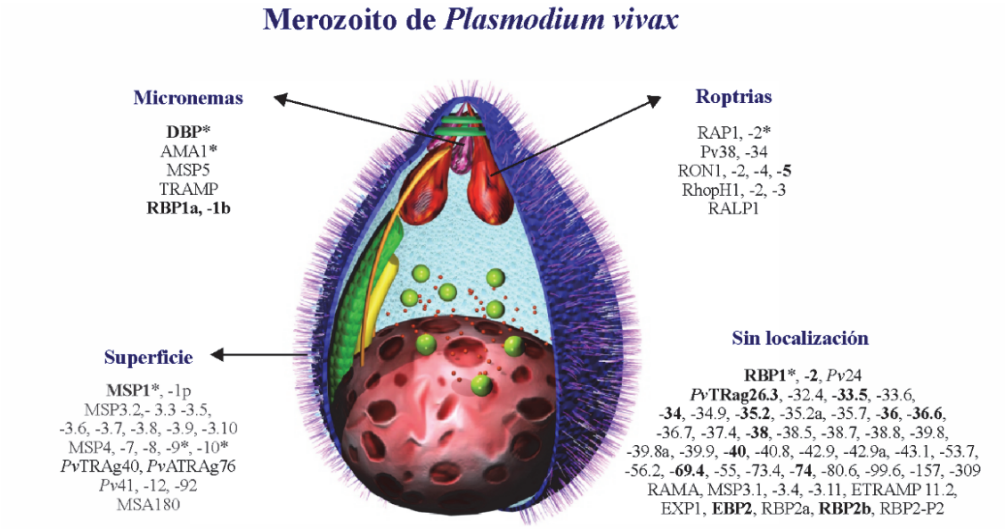

Figura 5. Proteínas del estadio intraeritrocítico de *P. vivax* caracterizadas a la fecha. Los asteriscos indican las moléculas evaluadas previamente a nivel pre-clínico. Los nombres en negrita corresponden a las moléculas con capacidad de unión a reticulocitos.

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 ^{36}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 β-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}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, inmunoflorescencia indirecta (IFI) o citometría de flujo. En el primer caso, un fragmento de 33 kDa de la proteína *Pv*RON5 expresado en *Escherichia coli* y purificado (r*Pv*RON5-$_{33kDa}$), se incubó con una muestra de reticulocitos enriquecidos mediante un gradiente de Percoll. Posteriormente, r*Pv*RON5-$_{33kDa}$ 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, r*Pv*RON5-$_{33kDa}$ 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 microscopia, revelaron que r*Pv*RON5-$_{33kDa}$ 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 r*Pv*RON5-$_{33kDa}$ 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 (*Pv*TRAg (TRAg40), *Pv*TRAg26.3, *Pv*TRAg33.5, *Pv*TRAg34, *Pv*TRAg35.2, *Pv*TRAg36, *Pv*TRAg36.6, *Pv*TRAg38, *Pv*TRAg69.4 y *Pv*TRAg74 (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 protectiva 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 protectiva, 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 protectiva (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 epítopos de DBP inducen una respuesta de tipo Th1/Th2 (con secreción de IFN-γ 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 protectiva 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-γ (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 protectiva 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 INFγ 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 protectiva 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 *Pv*RBSA 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 *Pv*ARP y *Pv*RBSA) o en los organelos

apicales (en el caso de *Pv*GAMA). A pesar de lo anterior, en este punto no hay suficiente evidencia para sugerir que *Pv*ARP, *Pv*RBSA y *Pv*GAMA 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 *Pv*ARP, *Pv*RBSA y *Pv*GAMA a las células diana permite dar respuesta a la segunda pregunta de investigación planteada en este trabajo.

Se han detectado anticuerpos anti-*Pv*ARP, *Pv*RBSA y *Pv*GAMA 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 *Pv*GAMA. Se observó una mayor reactividad de los sueros contra *Pv*GAMA-Ct que contiene la RR. Hay que poner de manifiesto que la respuesta de anticuerpos anti-*Pv*GAMA-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 *Pv*ARP y *Pv*GAMA, 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). *Pv*ARP 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, *Pv*RBSA, 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.

*Pv*GAMA 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, *Pv*GAMA 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 protectiva.

Como se demostró, *Pv*RBSA y los fragmentos de *Pv*GAMA 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 ($CD71^{hi}$) (105), se determinó si *Pv*RBSA y *Pv*GAMA 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 *Pv*RBSA y tres fragmentos de *Pv*GAMA se unen en mayor proporción a las células que expresan $CD71^{hi}$, 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 *Pv*RON5 (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 *Pv*RBSA y *Pv*GAMA 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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CAPÍTULO 2

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

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Characterizing *Pv*ARP, 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 (*Pv*ARP) 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 *Pv*ARP were used to confirm protein expression by Western blot as well as its subcellular localization by immunofluorescence. Recognition of recombinant *Pv*ARP by sera from *P. vivax*-infected individuals was evaluated by ELISA.

Results: VCG-1 strain *Pv*ARP 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 *Pv*ARP 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 antimalarial 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 (*Pf*AARP) [18]. The *Pf*AARP-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 (*Pf*AARP-N) obtained as a recombinant is recognized by antibodies from patients who have been naturally infected by *P. falciparum.* Rabbit antibodies directed against *Pf*AARP-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 *Pf*AARP 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

*Pv*ARP 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 AntheProt 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 $MgCl_2$, 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′- CATTT GATCAGAGACGAC -3′ and reverse 5′- TTGGCACTTT TGTCACGA -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′- AT CCCTAGCAATGCTTCG -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_{600}; 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_2PO_4 and 10 mg/mL lysozyme) supplemented with protease inhibitors (1 mM PMSF, 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^{+2}-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-$PvARP_{38582}$). 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$_{38582}$ 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$_{38582}$ 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$_{38582}$ 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 florescence 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 r*Pv*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 ± SD) of healthy individuals' sera reactivity.

Statistical analysis

Differences in average OD for r*Pv*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 *Pvarp* 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*

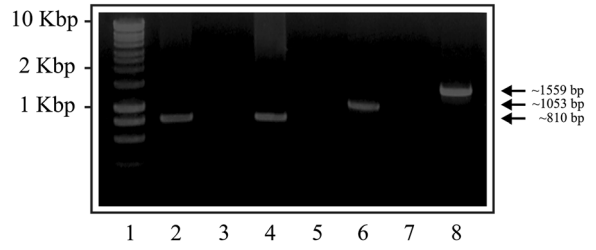

Figure 1 *Pvarp* gene transcription during blood stage. Lane 1 indicates the molecular weight marker. Lanes 2–4 show *Pvarp* gene amplification using cDNA RT+, RT- and gDNA, respectively. Lanes 6–8 show the amplification of the *Pvron1-a* region using cDNA RT+, RT- and gDNA as template, respectively. 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 *Pv*ARP *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 *Pv*ARP nucleotide and amino acid sequences regarding the reference strain (Sal-1)

Base pairs*	Amino acids*	Mutations/ Deletions	Changes in *Pvarp* nucleotide sequences in *P. vivax* strains		Changes in the *Pv*ARP amino acid sequences in *P. vivax* 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 *Pv*ARP in the Sal-I reference strain.

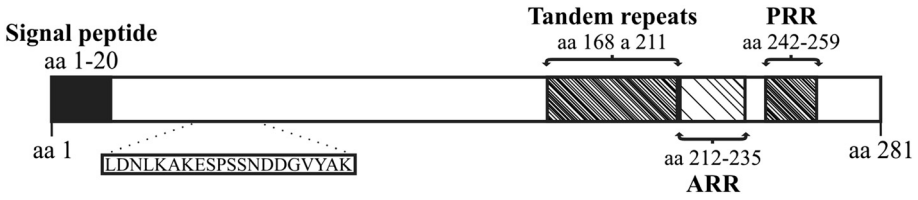

Figure 2 *In silico* **characterization of *Pv*ARP, showing signal peptide localization, tandem repeats (TR), asparagine (ARR) and proline (PRR) amino acid repeat regions and the peptide selected for the antibody purification assay (shown in the box).**

longer when compared to its homologue *Pf*AARP (217 aa) [18]. *Pv*ARP 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], *Pv*34 [35] and rhoptry neck proteins 1 and 2 [23,36]. Even though several studies have shown that the tandem repeats of *Pv*CSP 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.

*Pv*ARP expression in schizonts and subcellular localization

Specific human antibodies against an N-terminal *Pv*ARP synthetic peptide (Figure 2) were used for checking protein expression and localization in the schizont-enriched sample. *Pv*ARP was recombinantly expressed excluding the signal peptide and then purified (Figure 3A). Once human anti-$PvARP_{38582}$ 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 *Pv*ARP 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

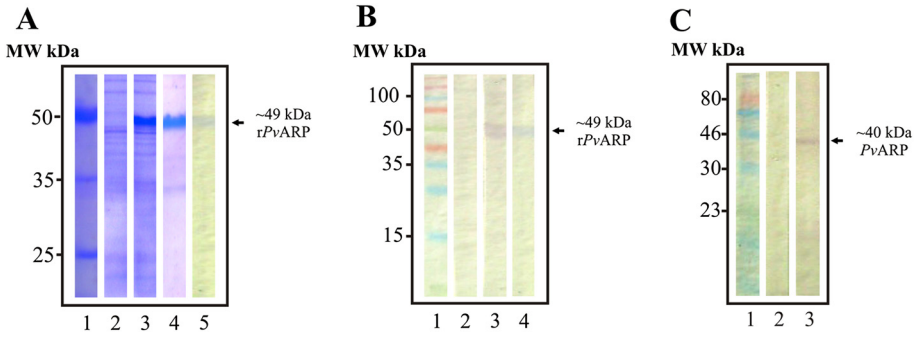

Figure 3 Detecting recombinant and parasite protein by human antibodies. (**A**) Recombinant protein expression and purification. Lanes 2–3 show non-induced and induced cell lysate, respectively (Coomassie staining). Lanes 4–5 show purified *rPv*ARP stained with Coomassie or analyzed by Western blot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

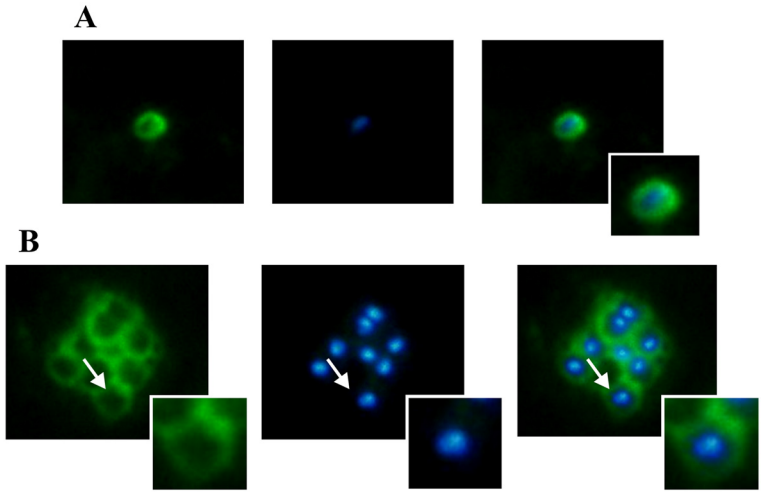

Figure 4 *Pv*ARP sub-cellular localization in mature schizonts. (**A**) Shows the detection of the protein on free merozoite surface. (**B**) *Pv*ARP labelling on mature schizonts. The nuclei are labelled with DAPI (blue). An amplified image of a merozoite (indicated by an arrow) is shown in small boxes.

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 *Pv*ARP 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

*Pv*ARP 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, *Pv*ARP 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.

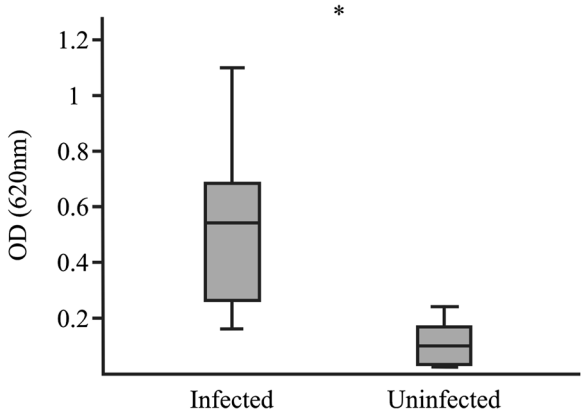

Figure 5 r*Pv*ARP antigenicity. The box diagram shows OD distribution (Y axis) for detecting r*Pv*ARP by sera from non-infected and infected individuals (X axis). *: Infected individuals (n = 38; $\overline{X}\pm S = 0.5 \pm 0.2$; 95%CI = 0.16-1.1) and control (n = 15; $\overline{X}\pm S = 0.1 \pm 0.07$; 95%IC = 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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Parasites & Vectors

RESEARCH Open Access

Characterising *Pv*RBSA: an exclusive protein from *Plasmodium* species infecting reticulocytes

Darwin A. Moreno-Pérez[1,2], Luis A. Baquero[1], Diana M. Chitiva-Ardila[1] and Manuel A. Patarroyo[1,3*]

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. r*Pv*RBSA 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 ($CD71^{hi}$).

Conclusions: This study describes for the first time, a molecule involved in host cell binding that is exclusive in reticulocyte-infecting *Plasmodium* species. This suggest that *Pv*RBSA 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 BaCelLo [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 PMSF, 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_{600}. 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 *Pv*RBSA 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^{+2}-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 *Pv*RBSA sequence was used for designing two 20 aa-long peptides (CG-KRNSSVSSLDSDMGS 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 $NaHCO_3$, 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-*Pv*RBSA rabbit antibodies (1:30) or anti-*Pv*RON2 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 florescence 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-*Pv*RBSA 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 r*Pv*RBSA 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-r*Pv*RBSA 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 typified for determining the Duffy phenotype (Fya^+/Fyb^-; Fya^-/Fyb^+; Fya^+/Fyb^+) by standard blood banking methods using anti-Fya and Fyb sera. Five μL of cells were then incubated with 25 μg r*Pv*RBSA 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 ($CD71^{lo}$) and high ($CD71^{hi}$) 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. GradhPad 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 (*Pv*RBSA) according to the results showed in this study.

Regarding the *pvrbsa* gene, it 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. *Pv*RBSA 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 *Pv*RBSA 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].

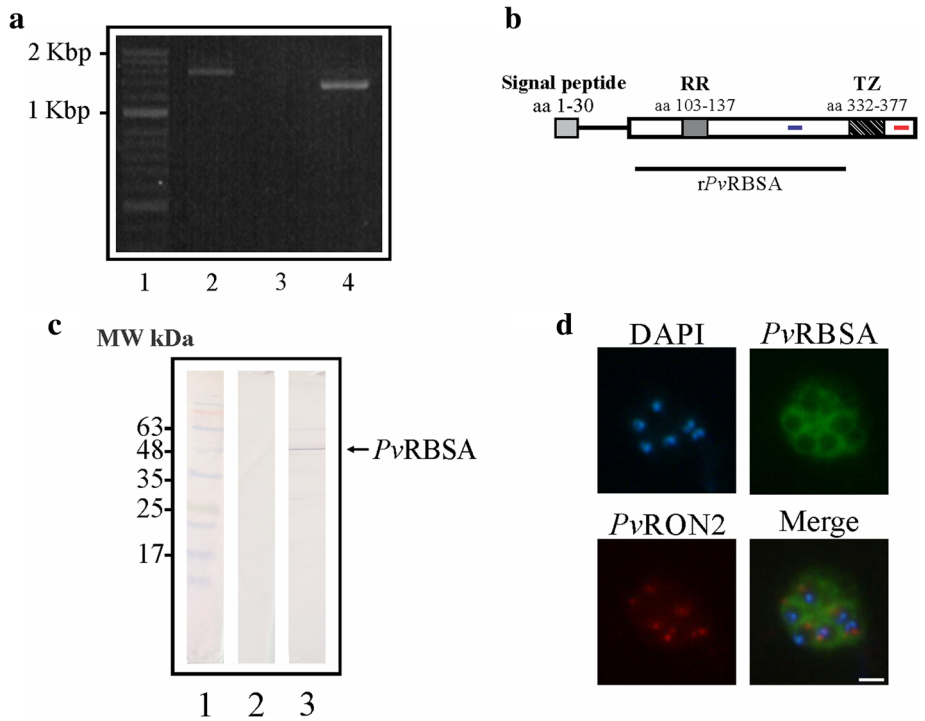

Fig. 1 *Pv*RBSA characterisation in the *P. vivax* VCG-I strain. **a** *pvrbsa* gene presence and transcription in schizonts. Lane 1 indicates the molecular weight pattern. Lanes 2 to 4 show gene amplification using gDNA, cDNA-RT- and cDNA-RT+, respectively. **b** *In silico* characterisation of *Pv*RBSA. The diagram shows signal peptide, repeat region (RR) and transmembrane zone (TZ) location. The peptides selected for rabbit immunisation are highlighted in *blue* (peptide 39478) and *red* (peptide 39480) lines. The recombinant protein region expressed is shown by a *black line*. **c** Recognition of *Pv*RBSA in parasite lysate. Lane 1 indicates the molecular weight (MW) marker. Lanes 2 and 3 show recognition of the protein using pre-immune and hyper-immune sera, respectively. **d** Sub-cellular location of *Pv*RBSA in schizonts. The images show recognition of *Pv*RBSA (*green*), *Pv*RON2 (*red*) and the nuclei (*blue*). *Scale-bar.* 1 μm

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

Changes in *Pv*RBSA nucleotide sequence in Sal-I and VCG-I *P. vivax* strains[a]	Changes in *Pv*RBSA aa sequences in Sal-I and VCG-I *P. vivax* strains[a]	Mutation
c.29A > G	p.Tyr10Cys	Non-synonymous
c.391_411delCTAACAGGAAGTAATGAATCC	p.Leu131_Ser137del[b]	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

[a]Nucleotide and amino acid positions are numbered according to the Sal-I reference strain sequence alignment with the VCG-I strain
[b]Relative location for a region having 4 identical tandem repeats from amino acid position 103 to 130 in the VCG-I strain

*Pv*RBSA 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-*Pv*RBSA 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 (*Pv*RON2). 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 r*Pv*RBSA 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.

*Pv*RBSA interaction with human reticulocytes

Flow cytometry was used for quantifying r*Pv*RBSA ability to bind cord blood reticulocytes using a gating strategy to exclude cell debris and select the CD71 + CD45- cell population (Fig. 3). The recombinant protein had a curve shift when PE signals from r*Pv*RBSA binding assay and control (using CD71 + CD45- cells) were compared in a histogram. r*Pv*RBSA 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 r*Pv*RBSA 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, r*Pv*RBSA had higher interaction with $CD71^{hi}$ than $CD71^{lo}$ cells (Fig. 4c) (*t*-test: $t_{(4)} = 16.44$, $P = 0.0001$), suggesting that this molecule binds better to the more immature reticulocyte stages.

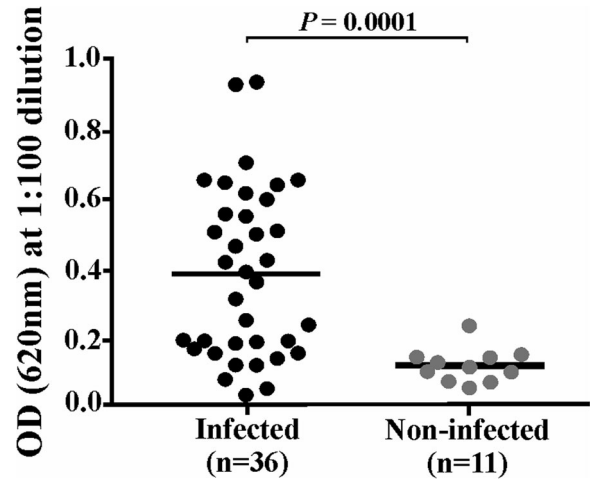

Fig. 2 Antigenicity assay. The dot plot shows OD distribution (*Y-axis*) for detecting the *Pv*RBSA in infected and non-infected patients' sera (X-axis). A statistically significant difference between groups was observed (Mann-Whitney *U*-test: $U = 52$, $Z = -3.66$, $P = 0.0001$)

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 *Pv*RBSA, 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 *Pv*RBSA 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 *Pv*RBSA 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 *Pv*RBSA 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 *Pv*RBSA 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.

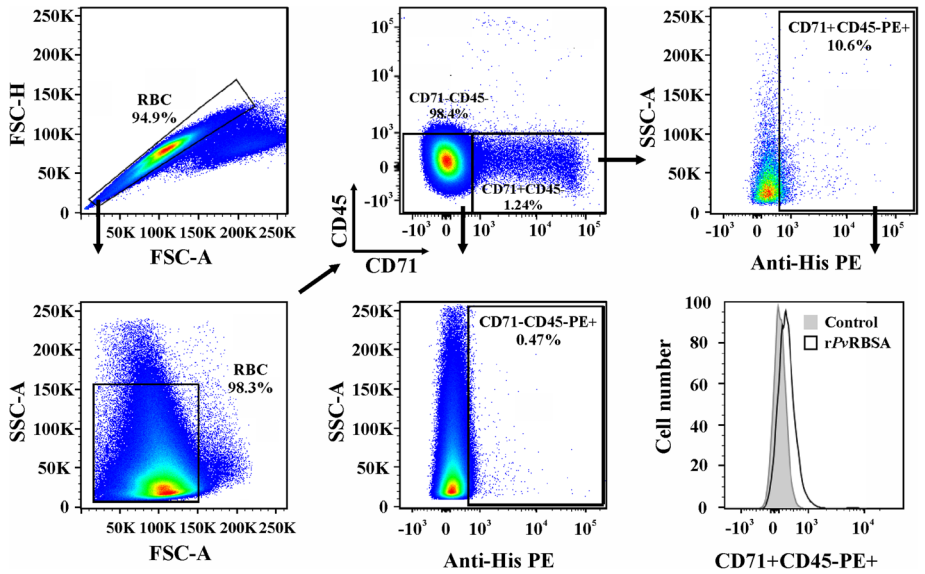

Fig. 3 Gating strategy for selecting reticulocytes and mature erythrocyte cell population. Doublets were excluded comparing FSC-H to FSC-A cytogram. Cells were then selected by their granularity by plotting SSC-A against FSC-A. The CD45 and CD71 signals were plotted for selecting reticulocyte (CD71 + 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 r*Pv*RBSA 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 *Pv*RBSA 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 *Pv*MSP-10 [37], *Pv*12 [38] and *Pv*ARP [30]. Once *Pv*RBSA 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-

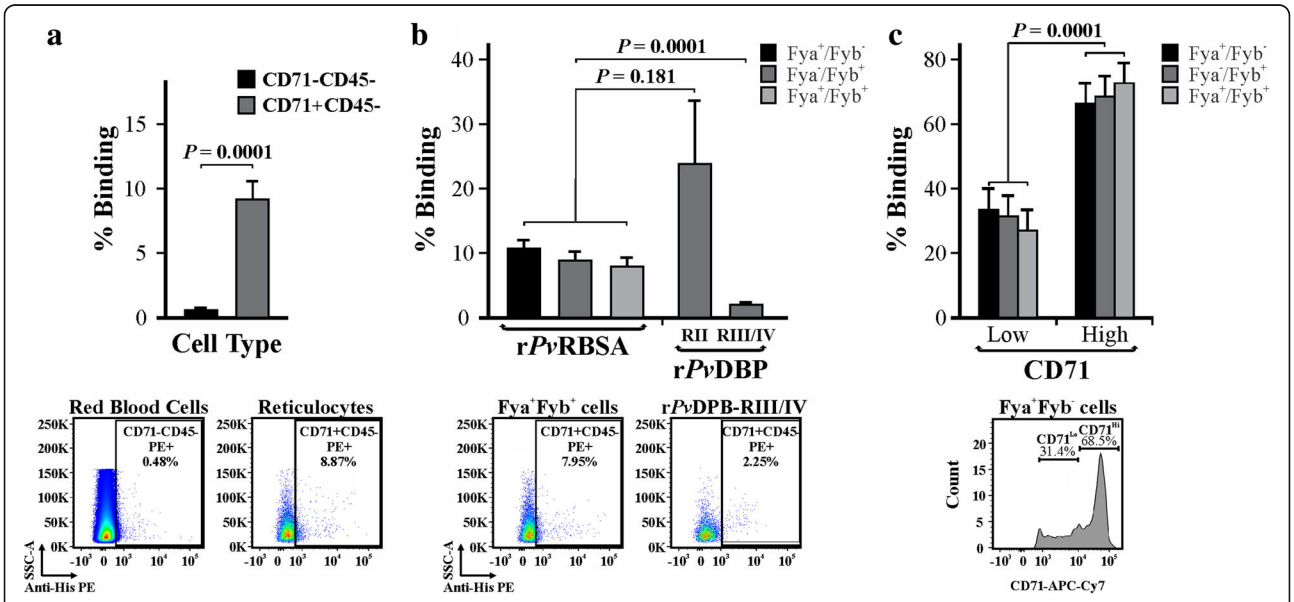

Fig. 4 r*Pv*RBSA binding activity to target cells. **a** r*Pv*RBSA binding percentage to erythrocytes (CD71-CD45-) and reticulocytes (CD71 + CD45-). **b** Percentage r*Pv*RBSA binding to reticulocytes with a different Duffy phenotype and **c** regarding CD71-APCH7 signal. A representative dot plot or 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 ± SD of three independent experiments

Table 2 r*Pv*RBSA 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
r*Pv*RBSA	Fya^+/Fyb^-	0.47 ± 0.01	10.7 ± 1.29
	Fya^-/Fyb^+	0.48 ± 0.22	8.87 ± 0.65
	Fya^+/Fyb^+	0.79 ± 0.23	7.95 ± 1.94

CD71 monoclonal antibody (a specific marker for the cells [39]). r*Pv*RBSA was able to interact with the youngest reticulocyte population ($CD71^{hi}$) 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 r*Pv*RBSA 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 *Pv*MSP-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, *Pv*RBSA 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 preselecting this cell population, such as *Pv*MSP-1, EBP, DBP and now, r*Pv*RBSA.

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 *Pv*RBSA is present in the *P. vivax* VCG-I strain genome, produces a transcript and encodes a protein having a surface location pattern. *Pv*RBSA 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 *Pv*RBSA in the *Aotus* monkey experimental model.

Additional file

Additional file 1: Recombinant *Pv*RBSA 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 r*Pv*RBSA by Western blot using anti-polyhistidine antibodies. (TIF 1539 kb)

Abbreviations

ANOVA: Analysis of variance.; $CD71^{hi}$: CD71 high; $CD71^{lo}$: 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; *Pv*RBSA: *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.

Publisher's Note

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RESEARCH Open Access

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 (*Pv*GAMA) 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, *Pv*GAMA 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 *Pv*GAMA 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 *Pv*GAMA 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 (*Pf*GAMA) 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* (*Tg*GAMA) 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 ($dN > dS$ 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 (π) 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 (ω) at each codon using a statistical test to determine whether ω 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′)[a]	MT (°C)	Product size (bp)	aa position
pvgama	Fwd: ATGAAGTGCAACGCCTCC Rev: AAAAATGAATAGGAGCAACG	58	2313	1 to 771
pvgama -Nt	Fwd: ATACGGAATGGAAACAACC Rev: AGTCGGTTCGTTATTCTCG		1284	22 to 449
pvgama -Ct	Fwd: CTGCTCAAGAACACGAAC Rev: GCTTCCACTCTGCAATTC		948	434 to 749
pvgama -CR1	Fwd: GACGATCATCTGTGTTCAAAAA Rev: GACCTCATTTTTGGACTTCTC	60	666	87 to 308
pvgama -VR1	Fwd: GGCGCCTTCCTGCAGTC Rev: CATTAACATGGTGTTGTCGCT		438	330 to 475
pvgama -CR2	Fwd: CAGGCGGCCATCTTACTAA Rev: GCTCCCGTTGACGCCCTT		321	482 to 588
pvgama -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

[a]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 μl PCR reactions containing 1× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA, USA), 0.3 μM 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, Tm °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

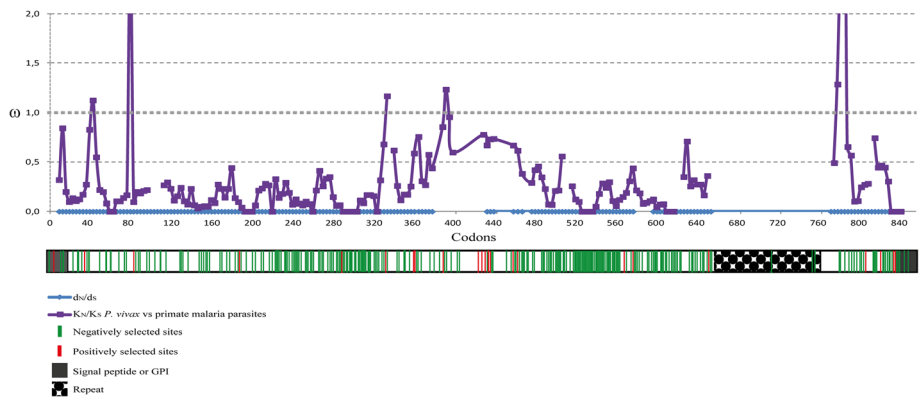

Fig. 1 Evolutionary rate (ω) sliding window. Intra-species ω values (d_N/d_S) are represented in *blue* whilst inter-species ω values (K_N/K_S between *P. vivax* and malarial parasites infecting primates) are shown in *purple*. A ω value equal 1 means neutral evolution, ω < 1 negative selection whilst ω > 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_{600}. 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 *Pv*GAMA-Nt and *Pv*GAMA-Ct fragments was homogenised in denaturing extraction buffer (DEB) (6 M urea, 10 mM Tris, 100 mM NaH_2PO_4 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

*Pv*GAMA-CR1, *Pv*GAMA-VR1, *Pv*GAMA-CR2 and *Pv*GAMA-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^{+2}-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)

*Pv*GAMA 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 r*Pv*GAMA-Nt, or r*Pv*GAMA-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 (± 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× 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^-Fyb^+ phenotype). Briefly, 5 μl samples were incubated with 25 μg of each recombinant protein (*Pv*GAMA-CR1, *Pv*GAMA-VR1, *Pv*GAMA-CR2 and *Pv*GAMA-VR2) for 16 h at 4 °C at 4× *rpm*. Twenty-five μg 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 *Pv*GAMA 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 ($CD71^{lo}$) and high ($CD71^{hi}$) 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. GradhPad Software (San Diego, CA) was used for all statistical analysis.

Results

*Pv*GAMA 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 ω (dN/dS 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, r*Pv*GAMA-Nt and r*Pv*GAMA-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. r*Pv*GAMA-Nt reacted

positively with 64.8% of the sera in screening (0.26 cut-off point) whilst 67.5% of them recognised r*Pv*GAMA-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 r*Pv*GAMA was recognised by 54.5% of the sera used in the array [41]. The statistical test for the assay with r*Pv*GAMA-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 r*Pv*GAMA-Ct was detected by human sera (Fig. 2a). There was also a statistically significant difference when analysing the means of recognition for r*Pv*GAMA-Nt and r*Pv*GAMA-Ct (ANOVA: $F_{(1,72)} = 16.01$, $P = 0.0002$). Taking into account that the response was higher against *Pv*GAMA-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 *Pv*GAMA-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 *Pv*GAMA carboxyl region.

*Pv*GAMA bound to human reticulocytes

Red blood cell samples having the $Fya^{-}Fyb^{+}$ 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 *Pv*GAMA 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 $CD71^{hi}$ 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])

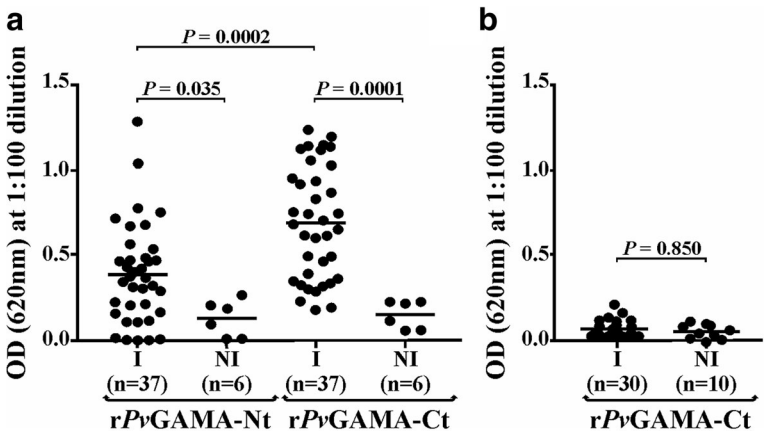

Fig. 2 *Pv*GAMA antigenicity during natural malaria infection. The *dot plot* shows OD distribution (Y-axis) for detecting r*Pv*GAMA-Nt or r*Pv*GAMA-Ct by *P. vivax* (**a**) or r*Pv*GAMA-CT by *P. falciparum* (**b**) infected (I) and non-infected (NI) patients' sera (X-axis). r*Pv*GAMA-Nt: infected individuals $n = 37$, $m \pm SD = 0.38 \pm 0.29$; control individuals $n = 6$, $m \pm SD = 0.12 \pm 0.1$. r*Pv*GAMA-Ct: infected individuals $n = 37$, $m \pm SD = 0.67 \pm 0.32$; control individuals $n = 6$, $m \pm SD = 0.14 \pm 0.08$. r*Pv*GAMA-Ct recognised by *P. falciparum* infected patients' sera: infected individuals $n = 30$, $m \pm SD = 0.06 \pm 0.04$; control individuals $n = 10$, $m \pm SD = 0.06 \pm 0.03$

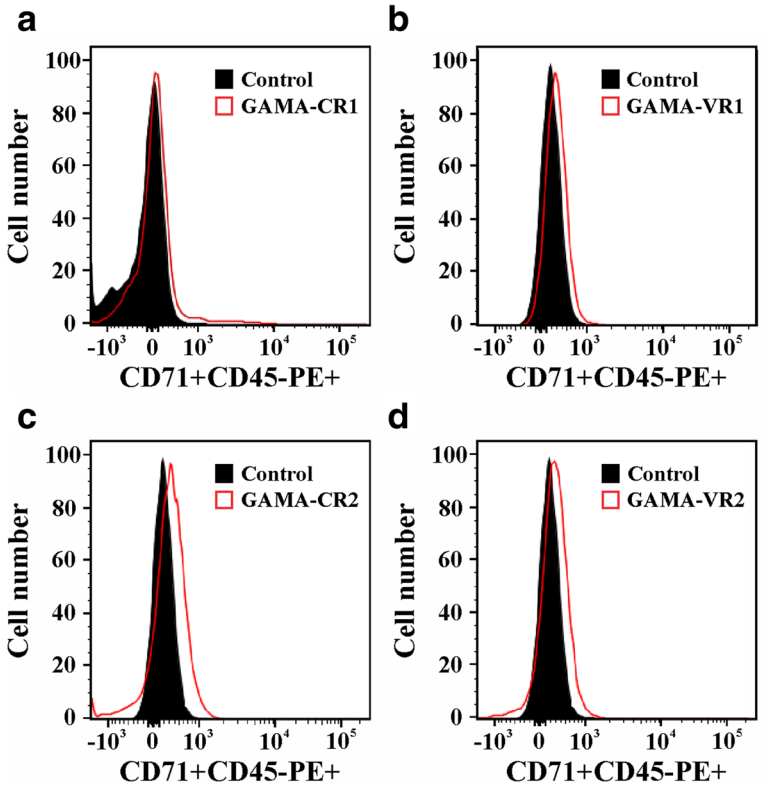

Fig. 3 Flow cytometry analysis. Histograms of conserved (**a** and **c**) and variable (**b** and **d**) GAMA fragments compared to control (cells not incubated with the protein). Each figure is representative from three independent experiments

(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 *Pv*GAMA 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.

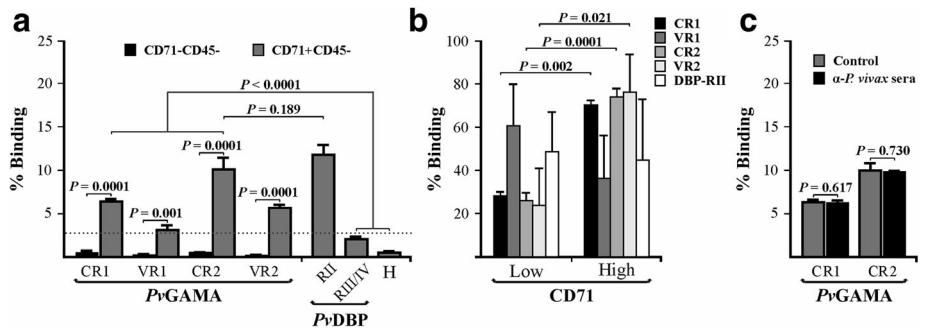

Fig. 4 *Pv*GAMA human reticulocyte binding activity. Flow cytometry analysis showing the recombinant binding percentage to CD71-CD45- and CD71 + CD45- cells (**a**) and regarding CD71-APCH7 signal (only for CD71 + CD45- cells) (**b**). Positive (DBP-RII) and negative (DBP-RIII/IV and H (6 histidine peptide)) binding controls are also shown. **c** CR1 and CR2 reticulocyte binding inhibition assay using human sera (α-*P. vivax* sera). Binding percentage in both analyses were expressed as mean ± 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 r*Pv*GAMA-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 (ω) (Fig. 1).

Given the polymorphism and selection analysis, it was decided to determine *Pv*GAMA 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 *Pv*GAMA 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 *Pv*GAMA 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 *Pv*GAMA 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 $CD71^{hi}$ reticulocyte binding percentages than to $CD71^{lo}$ (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 *Pv*GAMA receptor is less abundant in $CD71^{lo}$ cells unlike $CD71^{hi}$, as a consequence of cell maturation. The fact that more than 69% of the CD71 + CD45- cells were $CD71^{lo}$ ($m \pm$

SD = 69.3 ± 3.3) can be the explanation of why *Pv*GAMA 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 *Pv*GAMA.

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 *Pv*GAMA (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 *Pv*GAMA 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 *Pv*GAMA reticulocyte binding properties for the first time. The *Pv*GAMA antigenic response was principally directed against its carboxyl fragment which comprises by a repetitive region. On the other hand, it was shown that *Pv*GAMA 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. ω^+ model: ω rate values. Pr [ω = ω +]: 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 r*Pv*GAMA-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) *Pv*GAMA 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; $CD71^{hi}$: CD71 high; $CD71^{lo}$: 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; *Pv*GAMA: *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-I 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.

Publisher's Note

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CONCLUSIONES, RECOMENDACIONES Y PERSPECTIVAS GENERALES

Conclusiones

En esta investigación, se analizó el proteoma de la cepa VCG-1 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 permitió 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 estudió el proteoma de eritrocitos del primate *A. nancymaae*. Los análisis comparativos permitieron 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 soportan 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 información importante para entender 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 este es el primer estudio de proteómica realizado en Colombia donde se evaluó, 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 *Pv*ARP, *Pv*RBSA y *Pv*GAMA, 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, *Pv*ARP*, Pv*RBSA y *Pv*GAMA son capaces de desencadenar una respuesta inmune durante la infección natural. De éstas, *Pv*RBSA y *Pv*GAMA presentaron actividad de unión a reticulocitos, al igual que se demostró en previos estudios para otras moléculas como *Pv*DBP, *Pv*MSP-1, *Pv*RBPs y *Pv*RON5. Los hallazgos destacan que *Pv*RBSA y *Pv*GAMA 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 contribuyó 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 esta investigación 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 fueron 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 *Pv*RBSA 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 *Pv*RBSA y *Pv*GAMA 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. nancymaae* usando tecnología de alto rendimiento.

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 por la FIDIC para *P. falciparum*.

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ANEXOS

Anexo 1

"Vacunas contra *P. vivax*: Desafío en la Investigación"

Nota: Se muestra sólo la primera hoja, ya que la versión completa del artículo de revisión no se encuentra disponible debido a las políticas derecho a copia de la revista. Sin embargo, en caso de requerir una copia, esta puede ser solicitada a los siguientes correos: darandmorper@gmail.com, mapatarr.fidic@gmail.com.

EXPERT REVIEWS

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*.

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Anexo 2

"Proteoma de los Eritrocitos de *A. nancymaae*"

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The *Aotus nancymaae* erythrocyte proteome and its importance for biomedical research

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ABSTRACT

The *Aotus nancymaae* 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. nancymaae* 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. nancymaae* 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. nancymaae* 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. nancymaae* 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.

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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Anexo 3

"Caracterización de *Pv*GAMA en la cepa VCG-1 de *P. vivax*"

Caracterización de *Pv*GAMA 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* (*Pf*GAMA: 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.

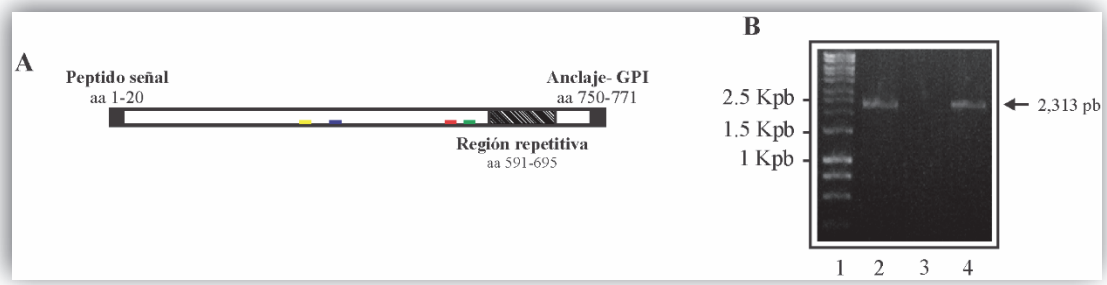

Figura 1: Identificación de GAMA en la cepa VCG-1 de *P. vivax.* (A) Caracterización *in silico* de *Pv*GAMA. El diagrama muestra la localización del péptido señal, la región repetitiva, la secuencia de anclaje GPI y los péptidos seleccionados para el ensayo de inmunización en conejos (líneas de color). (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 ADNc sintetizado con retrotranscriptasa (RT+) y ADNg, respectivamente. El control de una síntesis de ADNc 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 *Pv*GAMA 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 *Pv*GAMA (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 *Pv*GAMA, 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 hidrofilicidad. 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).

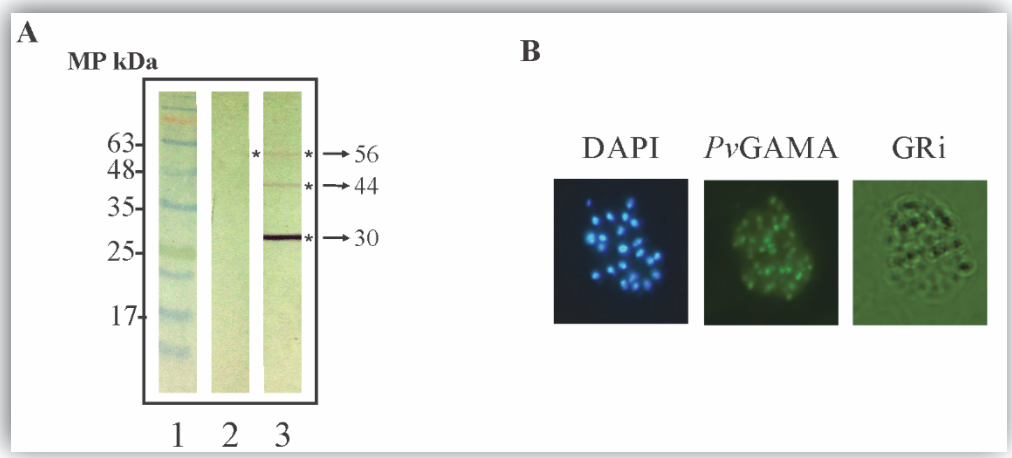

Figura 2. Detección de *Pv*GAMA nativa en esquizontes. (A) Reconocimiento de *Pv*GAMA usando anticuerpos generados en conejo. Carril 1 indica el marcador de peso molecular en kilodaltons (MP kDa). Carriles 2 y 3 muestran el reconocimiento de *Pv*GAMA usando suero pre-inmune y pos-III, respectivamente. (B) Localización sub-celular de *Pv*GAMA 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-*Pv*GAMA 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 *Pv*GAMA 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*"

Nota: Se muestra sólo la primera hoja, ya que la versión completa del artículo de revisión no se encuentra disponible debido a las políticas derecho a copia de la revista. Sin embargo, en caso de requerir una copia, esta puede ser solicitada a los siguientes correos: darandmorper@gmail.com, mapatarr.fidic@gmail.com.

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Review

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 establishing *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.