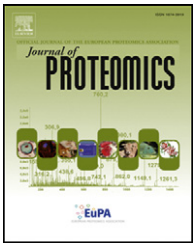
JOURNAL OF PROTEOMICS 113 (2015) 268–280

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot



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

D.A. Moreno-Pérez[a,b,c], R. Dégano[d], N. Ibarrola[d], A. Muro[c], M.A. Patarroyo[a,b,*]

[a]Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50 No. 26-20, Bogotá, Colombia
[b]Universidad del Rosario, Calle 63D No. 24-31, Bogotá, Colombia
[c]IBSAL-CIETUS (Instituto de Investigación Biomédica de Salamanca-Centro de Investigación en Enfermedades Tropicales de la Universidad de Salamanca), Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain
[d]Unidad de Proteómica, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca, Spain

ARTICLE INFO

Article history:
Received 12 June 2014
Accepted 2 October 2014
Available Online 12 October 2014

Keywords:
P. vivax
Proteome
Mass spectrometry
Proteins
Biology

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.

* *Corresponding author.*
E-mail addresses: darandmorper@gmail.com (D.A. Moreno-Pérez), romade@usal.es (R. Dégano), nibarrola@usal.es (N. Ibarrola), ama@usal.es (A. Muro), mapatarr.fidic@gmail.com (M.A. Patarroyo).

http://dx.doi.org/10.1016/j.jprot.2014.10.003

1. Introduction

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

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

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

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

On the other hand, earlier proteomic studies have helped to characterise the protein composition of *P. vivax*. Acharya et al. identified 154 proteins in clinically isolated *P. vivax* parasites from information derived from mass spectrometry (MS); some were hypothetical proteins, metabolic enzymes, chaperones and molecules involved in virulence [47,52]. Roobsoong et al. identified 316 proteins in schizont-enriched parasite samples obtained from symptomatic malaria patients. After separating the complex sample on a 2D gel and digesting it, analysis revealed proteins having different functions, such as binding, synthesis, cell transport and metabolism [35]. Two immunoassay-based studies for identifying *P. vivax* antigenic proteins have also been developed. Chen et al. used the wheat germ cell-free system (WGCF) for the mass expression of 86 molecules; 18 of them were recognised by sera from *P. vivax* infected patients (11 of them having no functional evidence) [53]. Lu et al. expressed 152 proteins using the same WGCF expression system, 44 of which were immunoreactive [43]. The proteomic and immunoproteomic studies described above led to identifying 457 *P. vivax* proteins, this being a third of the *P. falciparum* molecules detected during different parasite stages (1289 proteins, of which 714 have been identified in asexual blood stages, 931 in gametocytes and 645 in gametes) [54].

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

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

2. Materials and methods

2.1. Reagents

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

2.2. Animal handling

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

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

2.3. Isolating *P. vivax* blood stages

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

Leukocytes and platelets were removed by filtering through a CF11 column, as previously described by Sriprawat et al. [58] and parasite percentage was confirmed again using acridine staining (Table 1). Samples enriched in each stage (ring, trophozoite and schizont) were pooled accordingly and selected for proteomics analysis. Ring and/or trophozoite stages could not be enriched to >90% purity since no density gradient protocol was available for such purpose; however, schizonts were enriched using a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden), as previously described [59]. Parasites were isolated from cells by incubating them for 5 min in 0.02 mM saponin buffer containing 7 mM K_2HPO_4, 1 mM NaH_2PO_4, 11 mM $NaHCO_3$, 58 mM KCl, 56 mM NaCl, 1 mM $MgCl_2$ and 14 mM glucose, pH 7.5 and then were washed intensively with PBS pH 7.0.

2.4. Protein extraction and precipitation

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

2.5. Protein digestion and purification

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

2.6. Mass spectrometry

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

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

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

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

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

2.7. Peptide identification by database search

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

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

2.8. Protein annotation

The Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.7) 2003–2014 from the National Institute of Allergy and Infectious Diseases (NIAID) [69] was used for functional annotation. The parameters selected here were as follows: GOTERM_BP_ALL or GOTERM_MF_ALL from the Gene Ontology section. The analysis involved a count of 2 and EASE score threshold was set at 0.05. Results were saved in Microsoft Excel and txt format. Enriched Map with DAVID output was generated using Cytoscape 3.1 software [70]. Analysis parameters involved a 0.05 *p* value, FDR = 0.1 and overlap coefficient = 0.6. Clusters were circled manually and labelled to highlight the prevalent biological functions amongst a set of related gene-sets. Parasite proteins having orthologues in humans were searched using the Kyoto Encyclopedia of Genes and Genomes ortholog clusters (KEGG OC) database for drug target analysis [71].

2.9. *In silico* protein characterisation

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

3. Results

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

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

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

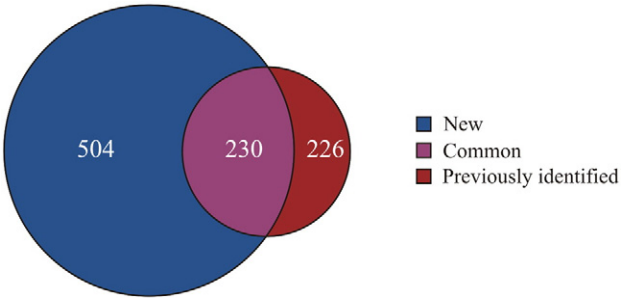


Fig. 1 – *P. vivax* proteins identified to date. Venn diagram showing the proteins identified in this study compared to early proteomic and immunoproteomic studies.

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

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

New members of the *Pv-fam* family (not found previously) predicted in the *P. vivax* genome *in silico* analysis [91] were detected (Supplementary Data 3). Rhoptry (RAP-1 (PVX_085930), -2 (PVX_097590), RON2 (PVX_117880), Clag (PVX_121885)) and surface (MSP-8 (PVX_097625), -9 (PVX_124060), *Pv*41 (Pfs230) (PVX_000995) and *Pv*12 (PVX_113775)) proteins which have already been identified and considered as good candidates for inclusion in a *P. falciparum* vaccine were also identified [92–94]. A recently reported pre-erythrocytic (liver stage antigen (PVX_091675)) protein was found; although this molecule is immunogenic, its role during blood cycle has not been studied [44].

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

GO terms were initially used for categorising whole proteins identified in the *P. vivax* VCG-1 strain through gene-annotation enrichment analysis using DAVID software. A total of 314 proteins were related to biological processes; the enrichment map revealed that most of them were functionally-involved in four processes (statistical significance: $p < 0.05$): protein metabolism and biosynthesis, nucleotide metabolism and biosynthesis, cellular transport and localisation and DNA organisation (Fig. 3) (Supplementary Data 4). On the other hand, 310 molecules were predicted as being related to a molecular function; the most significant related functions derived from DAVID analysis were: structural molecule activity (67 proteins, $p = 1.26E^{-10}$), structural constituent of ribosome (58 proteins, $p = 8.28E^{-09}$), unfolded binding protein (22 proteins, $p = 2.17E^{-06}$), hydrolytic (12 proteins, $p = 3.14E^{-05}$) and translation (24 proteins, $p = 4.41E^{-04}$) activity, and nucleotide binding (149 proteins, $p = 7.92E^{-04} - 2.03E^{-03}$) (Supplementary Data 4). Some proteins could not be classified by DAVID, which may have been because most were not seen to be similar to molecules for which biological knowledge has been reported in databases.

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

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

Interestingly, 6 proteins were found for which there was no evidence of transcripts in Bozdech's study; one hypothetical conserved protein (accession number PVX_086055) was identified in a later study by Westenberger et al. [68]. The remaining 5 proteins consisted of three hypothetical proteins (PVX_091652, PVX_091992 and PVX_118162), one HAM1 domain-containing protein (PVX_096292) and one putative arginyl-tRNA synthetase (PVX_123597) (Supplementary Data 5).

3.4. Pharmacological target prediction

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

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

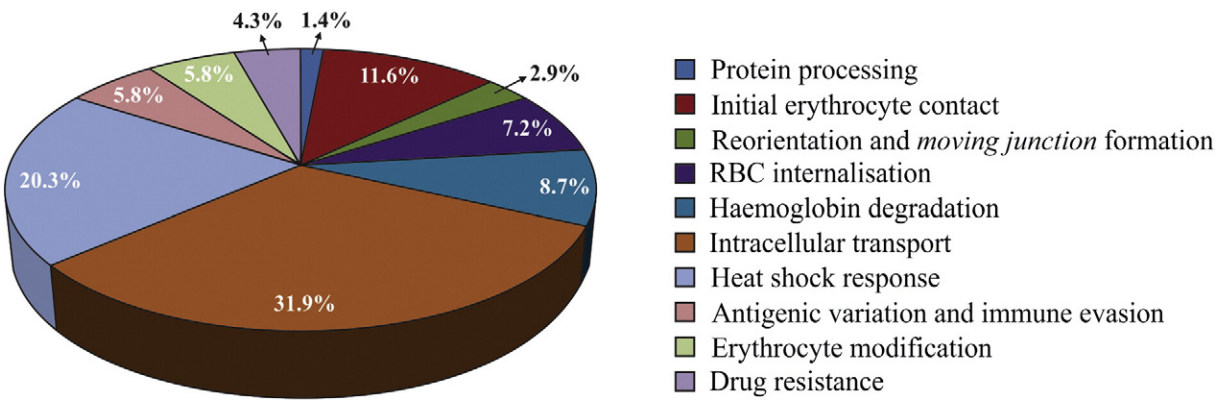


Fig. 2 – Pie chart showing the *P. vivax* proteins distribution related to functional classes.

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

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

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

A total of 177 proteins participating in 87 metabolic pathways were found by using a recently updated PlasmoDB application designed for such purpose in a second analysis [67]; 36 proteins did not have human orthologues and 16 of them were participating in only one pathway (Table 3). The M1-family aminopeptidase (PVX_122425) was common with the drug targets identified in the *P. vivax* studies reported by Acharya et al. [47]. S-adenosyl-L-homocysteine hydrolase (PVX_080200), malate:quinone oxidoreductase (PVX_113980) and leucine aminopeptidase (PVX_118180) have previously been considered as attractive drug targets for *P. falciparum* [96–98]. Other molecules have been predicted representing major metabolic pathways required for *P. falciparum* parasite replication and growth: adenosine deaminase (PVX_111245) and phosphoethanolamine N-methyltransferase (PVX_083045) involved in purine salvage [99] and glycerophospholipid metabolism [100].

3.5. *In silico predicted vaccine candidates*

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

The analysis led to identifying 31 molecules having the characteristics described above (Table 5). The MSP-1 had previously been studied in pre-clinical assays [101], others had already been described as surface (*Pv*12 and *Pv*41) [26,38] and rhoptry (*Pv*RON2) [34] proteins, 8 were hypothetical proteins and other rhoptry proteins not described as yet. Six hypothetical proteins have not been studied in any *Plasmodium* species; PVX_001780 had a domain involved in proteolysis, PVX_092070 appeared to be restricted to the *Plasmodium* genus and PVX_099710 had a domain characteristic of extracellular proteins which are cell binding ligands (Table 5). Proteins linked to parasite invasion and growth (subtilisin-like protease, EXP, and SERA proteins) and components of multigene families (MSP-7, *Pv-fam* and *etramp*) were also predicted as vaccine candidates.

4. Discussion

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

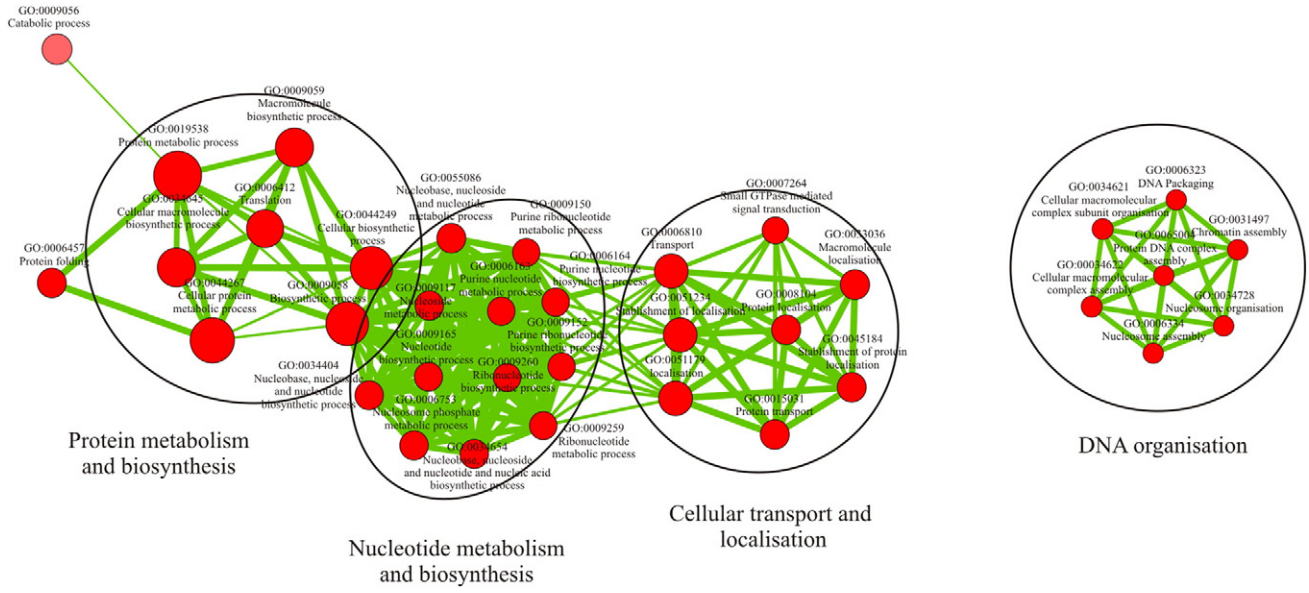


Fig. 3 – Enrichment map for the *P. vivax* proteins identified here. Map displaying *P. vivax* proteins grouped according to their function. The size of the red node represents the number of proteins by term.

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

Proteins having N-terminal acetylation were also found. This represents a major post-translational modification which is prevalent in enzymes catalysing intermediate metabolism in human cells [102]. Further analysis of these proteins is thus needed to study their role in regulating metabolic processes concerning *P. vivax*.

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

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

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

[a] Proteins which have been suggested as being good drug targets in *P. falciparum*.

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

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

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

The search for therapeutic targets against malaria has become an important line of research, given that resistant *P. vivax* strains continue emerging and threatening the health of millions of people in endemic areas [104]. Sixteen candidates were predicted in this study, some of them being orthologous to *P. falciparum* proteins which have been considered potential pharmacological targets (Table 3). Although several molecules have been suggested as possible *P. vivax* drug targets by Acharya et al. [52] not all were identified here because such proteins did not meet the inclusion criteria established for this study [95]. The absence of these predicted proteins in mammals makes them ideal targets for designing novel antimalarial drugs. However, further assays orientated towards evaluating structural homology with other human proteins and the toxicity of the drugs used against these targets in *in vitro* controlled trials are needed to ascertain pharmacological potential.

Table 4 – Proteins recognised by stage and their amount.

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

Numbers in brackets indicate the percentage of total proteins detected by stage.
[a] Main contaminants were human keratins.

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

The *in silico* prediction led to identifying *Pv-fam-a* proteins in which some members have been shown to bind erythrocytes [105], *Pv-fam-d* for which there is no functional evidence data to date and *etramps* orthologues to *P. falciparum* proteins whose red blood cell binding role has been shown (Table 5) [106]. Other important proteins found were two MSP-7, two SERA and five malarial adhesins, which have been considered good vaccine candidates as they mediate cell binding [79,107]. One *Pv-fam* (PVX_112685) and one *etramp* (PVX_096070) proteins were predicted by MAAP, as well as one hypothetical protein (PVX_084720), the MSP-1 (PVX_099980) which has been extensively studied in *Plasmodium* species, and one conserved rhoptry protein (PVX_096245) which is important but not essential for *P. falciparum* invasion, as shown in a gene knockout study [108].

Rhoptry and surface proteins are important candidates given that they are required for host cell attachment and parasite invasion [109,110]; therefore, RON-2 (PVX_117880), -3 (PVX_101485), -5 (PVX_089530), the rhoptry protein above mentioned (PVX_084720), one member of the cytoadherence protein family (PVX_121885), and *Pv*12 (PVX_113775) and *Pv*41 (PVX_000995) could be good candidates.

Interestingly, according to the PlasmoDB information, 2 *Pv-fam* family proteins (PVX_112685 and PVX_121910) and one hypothetical protein (PVX_096055) had no orthologues in *P. falciparum* but were present in *Plasmodium cynomolgi*, a monkey parasite which is a closely *P. vivax*-related species and also infects reticulocytes (Table 5) [111]. This supports the notion that these proteins are possibly related to *P. vivax* cellular preference for invasion. Further characterisation of all the aforementioned molecules should be considered for testing their role in reticulocyte adhesion or invasion.

5. Conclusions

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

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

PlasmoDB ID	Description	MET	SP	BacelLo	Interpro Scan	Phobius	MAAP	GPI-anchor
PVX_000995[a]	Transmission-blocking target antigen Pfs230, putative (P41)	35	x	x	s48/45 domain (IPR010884)	–	–	–
PVX_001780	Hypothetical protein, conserved	35	x	x	Aspartic peptidase domain (IPR021109)	1	–	–
PVX_003805[a]	Serine-repeat antigen (SERA), putative	35	x	x	Papain domain (IPR000668)	–	–	–
PVX_003810	Serine-repeat antigen 5 (SERA), putative	35	x	x		–	–	–
PVX_082675	Merozoite surface protein 7 (MSP7)	40	x	x	Merozoite surface protein,	–	–	–
PVX_082680[a]	Merozoite surface protein 7 (MSP7), putative	35	x	x	C-terminal (IPR024781)	–	–	–
PVX_084720[a]	Hypothetical protein, conserved	40	x	x	–	–	x	–
PVX_086915	Early transcribed membrane protein (ETRAMP)	35	x	x	*etramp* family (IPR006389)	1	–	–
PVX_089530	Rhoptry neck protein 5, putative (RON5)	35	x	x	–	2	–	–
PVX_090230	Early transcribed membrane protein (ETRAMP)	35	x	x	*etramp* family (IPR006389)	2	–	–
PVX_090945	Hypothetical protein, conserved	35	x	x	–	1	–	–
PVX_091700[a]	Circumsporozoite-protein related antigen, putative (EXP1)	40	x	x	Circumsporozoite-related antigen family (IPR009512)	1	–	–
PVX_092070[a]	Hypothetical protein, conserved	40	x	x	Protein of unknown function DUF3271 (IPR021689)	–	–	–
PVX_096055[b]	Hypothetical protein	43	x	x	–	2	–	–
PVX_096070	Early transcribed membrane protein (ETRAMP)	43	x	x	*etramp* family (IPR006389)	1	x	–
PVX_096245	Rhoptry-associated leucine zipper-like protein 1	35	x	x	–	–	x	–
PVX_096950	Tryptophan-rich antigen (Pv-fam-a)	43	x	x	Tryptophan/threonine-rich domain (IPR022089)	–	–	–
PVX_096990	*Pv-fam-d* protein	40	x	x	–	1	–	–
PVX_097935	Subtilisin-like protease precursor, putative	40	x	x	S8/S53 domain (IPR000209)	–	–	–
PVX_099710	Hypothetical protein, conserved	40	x	x	Calycin-like domain (IPR011038)	–	–	–
PVX_099980[a]	Major blood-stage surface antigen *Pv*200	35	x	x	EGF domain (IPR010901, IPR024730, IPR024731)	1	x	HP
PVX_101485	Rhoptry neck protein 3, putative (RON3)	35	x	x	–	3	–	–
PVX_112665	Tryptophan-rich antigen (*Pv-fam-a*)	43	x	x	Tryptophan/threonine-rich	–	–	–
PVX_112685[a,b]	Tryptophan-rich antigen (*Pv-fam-a*)	40	x	x	domain (IPR022089)	–	x	–
PVX_113225	Plasmodium exported protein, unknown function	40	x	x	Protein of unknown function DUF3671 (IPR022139)	3	–	–
PVX_113775[a]	6-cysteine protein (P12)	35	x	x	s48/45 Domain (IPR010884)	1	–	HP
PVX_117880	Rhoptry neck protein 2 (RON2)	35	x	x	–	3	–	–
PVX_121885	Cytoadherence linked asexual protein, CLAG, putative	35	x	x	Cytoadherence-linked asexual protein family (IPR005553)	2	–	–
PVX_121910[a,b]	*Pv-fam-d* protein	40	x	x	–	2	–	–
PVX_122910	Hypothetical protein, conserved	43	x	x	–	1	–	–
PVX_124090	Hypothetical protein, conserved	35	x	x	–	1	–	–

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

involved in parasite growth, antigenic variability, invasion and others having a GO term linked to metabolic pathways. The study has presented an important source of information for molecule selection, providing the potential for establishing suitable control strategies aimed at preventing or treating *P. vivax* malaria infection. Further studies are needed to confirm the potential use of the *in silico* predicted drug targets and vaccine candidates here described.

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

Competing interests

The authors have declared that no competing interests exist.

Acknowledgements

We would like to thank Alvaro Mongui for the experimental design, Diego Alonso Lopez and Carlos Fernando Suárez for bioinformatics support, Jason Garry for translating and reviewing this manuscript and especially Professor Manuel Elkin Patarroyo for his invaluable comments and suggestions. We would also like to thank the ProteoRed, PRB2-ISCIII proteomics facility at the Instituto de Biología Molecular y Celular del Cáncer USAL-CSIC, financed by grant PT13/0001. This research was supported by the "Instituto Colombiano para el Desarrollo de la Ciencia 'Francisco José de Caldas'" (COLCIENCIAS) through contracts RC#309-2013 and 709-2013.

REFERENCES

[1] WHO. World malaria report 2013. WHO global malaria programme; 2013.
[2] Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, et al. The international limits and population at risk of *Plasmodium vivax* transmission in 2009. PLoS Negl Trop Dis 2010;4:e774.
[3] Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, Alpers MP, et al. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS Med 2008;5:e127.
[4] Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, et al. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med 2008;5:e128.
[5] Moreno-Perez DA, Ruiz JA, Patarroyo MA. Reticulocytes: *Plasmodium vivax* target cells. Biol Cell 2013;105:251–60.
[6] Nobrega de Sousa T, Carvalho LH, Alves de Brito CF. Worldwide genetic variability of the Duffy binding protein: insights into *Plasmodium vivax* vaccine development. PLoS One 2011;6:e22944.
[7] Moon SU, Na BK, Kang JM, Kim JY, Cho SH, Park YK, et al. Genetic polymorphism and effect of natural selection at domain I of apical membrane antigen-1 (AMA-1) in *Plasmodium vivax* isolates from Myanmar. Acta Trop 2010; 114:71–5.
[8] Urquiza M, Patarroyo MA, Mari V, Ocampo M, Suarez J, Lopez R, et al. Identification and polymorphism of *Plasmodium vivax* RBP-1 peptides which bind specifically to reticulocytes. Peptides 2002;23:2265–77.
[9] Prajapati SK, Kumari P, Singh OP. Molecular analysis of reticulocyte binding protein-2 gene in *Plasmodium vivax* isolates from India. BMC Microbiol 2012;12:243.
[10] Gutierrez A, Vicini J, Patarroyo ME, Murillo LA, Patarroyo MA. *Plasmodium vivax*: polymorphism in the merozoite surface protein 1 gene from wild Colombian isolates. Exp Parasitol 2000;95:215–9.
[11] Imwong M, Boel ME, Pagornrat W, Pimanpanarak M, McGready R, Day NP, et al. The first *Plasmodium vivax* relapses of life are usually genetically homologous. J Infect Dis 2012;205:680–3.
[12] Hulden L. Activation of the hypnozoite: a part of *Plasmodium vivax* life cycle and survival. Malar J 2011;10:90.
[13] Wertheimer SP, Barnwell JW. *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. Exp Parasitol 1989;69:340–50.
[14] del Portillo HA, Longacre S, Khouri E, David PH. Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different Plasmodium species. Proc Natl Acad Sci U S A 1991;88:4030–4.
[15] Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell 1992;69:1213–26.
[16] Cheng Q, Saul A. Sequence analysis of the apical membrane antigen I (AMA-1) of *Plasmodium vivax*. Mol Biochem Parasitol 1994;65:183–7.
[17] Black CG, Barnwell JW, Huber CS, Galinski MR, Coppel RL. The *Plasmodium vivax* homologues of merozoite surface proteins 4 and 5 from *Plasmodium falciparum* are expressed at different locations in the merozoite. Mol Biochem Parasitol 2002;120:215–24.
[18] Vargas-Serrato E, Barnwell JW, Ingravallo P, Perler FB, Galinski MR. Merozoite surface protein-9 of *Plasmodium vivax* and related simian malaria parasites is orthologous to p101/ABRA of *P. falciparum*. Mol Biochem Parasitol 2002;120:41–52.
[19] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. *Plasmodium vivax* merozoite surface protein 8 cloning, expression, and characterisation. Biochem Biophys Res Commun 2004;324:1393–9.
[20] Patarroyo MA, Perez-Leal O, Lopez Y, Cortes J, Rojas-Caraballo J, Gomez A, et al. Identification and characterisation of the *Plasmodium vivax* rhoptry-associated protein 2. Biochem Biophys Res Commun 2005;337:853–9.
[21] Jalah R, Sarin R, Sud N, Alam MT, Parikh N, Das TK, et al. Identification, expression, localization and serological characterization of a tryptophan-rich antigen from the human malaria parasite *Plasmodium vivax*. Mol Biochem Parasitol 2005;142:158–69.
[22] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. Identifying and characterising the *Plasmodium falciparum* merozoite surface protein 10 *Plasmodium vivax* homologue. Biochem Biophys Res Commun 2005;331:1178–84.
[23] Perez-Leal O, Mongui A, Cortes J, Yepes G, Leiton J, Patarroyo MA. The *Plasmodium vivax* rhoptry-associated protein 1. Biochem Biophys Res Commun 2006;341:1053–8.
[24] Mongui A, Perez-Leal O, Soto SC, Cortes J, Patarroyo MA. Cloning, expression, and characterisation of a *Plasmodium vivax* MSP7 family merozoite surface protein. Biochem Biophys Res Commun 2006;351:639–44.
[25] Mongui A, Perez-Leal O, Rojas-Caraballo J, Angel DI, Cortes J, Patarroyo MA. Identifying and characterising the *Plasmodium falciparum* RhopH3 *Plasmodium vivax* homologue. Biochem Biophys Res Commun 2007;358:861–6.
[26] Angel DI, Mongui A, Ardila J, Vanegas M, Patarroyo MA. The *Plasmodium vivax Pv*41 surface protein: identification and characterization. Biochem Biophys Res Commun 2008;377: 1113–7.
[27] Mongui A, Angel DI, Guzman C, Vanegas M, Patarroyo MA. Characterisation of the *Plasmodium vivax Pv*38 antigen. Biochem Biophys Res Commun 2008;376:326–30.
[28] Siddiqui AA, Bora H, Singh N, Dash AP, Sharma YD. Expression, purification, and characterization of the immunological response to a 40-kilodalton *Plasmodium vivax* tryptophan-rich antigen. Infect Immun 2008;76:2576–86.
[29] Alam MT, Bora H, Singh N, Sharma YD. High immunogenecity and erythrocyte-binding activity in the tryptophan-rich domain

(TRD) of the 74-kDa *Plasmodium vivax* alanine-tryptophan-rich antigen (PvATRAg74). Vaccine 2008;26:3787–94.
[30] Mongui A, Angel DI, Gallego G, Reyes C, Martinez P, Guhl F, et al. Characterization and antigenicity of the promising vaccine candidate *Plasmodium vivax* 34 kDa rhoptry antigen (Pv34). Vaccine 2009;28:415–21.
[31] Mongui A, Angel DI, Moreno-Perez DA, Villarreal-Gonzalez S, Almonacid H, Vanegas M, et al. Identification and characterization of the *Plasmodium vivax* thrombospondin-related apical merozoite protein. Malar J 2010;9:283.
[32] Moreno-Perez DA, Mongui A, Soler LN, Sanchez-Ladino M, Patarroyo MA. Identifying and characterizing a member of the RhopH1/Clag family in *Plasmodium vivax*. Gene 2011;481:17–23.
[33] Moreno-Perez DA, Montenegro M, Patarroyo ME, Patarroyo MA. Identification, characterization and antigenicity of the *Plasmodium vivax* rhoptry neck protein 1 (PvRON1). Malar J 2011;10:314.
[34] Arevalo-Pinzon G, Curtidor H, Patino LC, Patarroyo MA. PvRON2, a new *Plasmodium vivax* rhoptry neck antigen. Malar J 2011;10:60.
[35] Roobsoong W, Roytrakul S, Sattabongkot J, Li J, Udomsangpetch R, Cui L. Determination of the *Plasmodium vivax* schizont stage proteome. J Proteomics 2011;74:1701–10.
[36] Bitencourt AR, Vicentin EC, Jimenez MC, Ricci R, Leite JA, Costa FT, et al. Antigenicity and immunogenicity of *Plasmodium vivax* merozoite surface protein-3. PLoS One 2013;8:e56061.
[37] Jiang J, Barnwell JW, Meyer EV, Galinski MR. *Plasmodium vivax* merozoite surface protein-3 (PvMSP3): expression of an 11 member multigene family in blood-stage parasites. PLoS One 2013;8:e63888.
[38] Moreno-Perez DA, Areiza-Rojas R, Florez-Buitrago X, Silva Y, Patarroyo ME, Patarroyo MA. The GPI-anchored 6-Cys protein Pv12 is present in detergent-resistant microdomains of *Plasmodium vivax* blood stage schizonts. Protist 2013;164: 37–48.
[39] Moreno-Perez DA, Saldarriaga A, Patarroyo MA. Characterizing PvARP, a novel *Plasmodium vivax* antigen. Malar J 2013;12:165.
[40] Wang B, Lu F, Cheng Y, Li J, Ito D, Sattabongkot J, et al. Identification and characterization of the *Plasmodium falciparum* RhopH2 ortholog in *Plasmodium vivax*. Parasitol Res 2013;112:585–93.
[41] Arevalo-Pinzon G, Curtidor H, Abril J, Patarroyo MA. Annotation and characterization of the *Plasmodium vivax* rhoptry neck protein 4 (PvRON4). Malar J 2013;12:356.
[42] Cheng Y, Wang Y, Ito D, Kong DH, Ha KS, Chen JH, et al. The *Plasmodium vivax* merozoite surface protein 1 paralog is a novel erythrocyte-binding ligand of *P. vivax*. Infect Immun 2013;81:1585–95.
[43] Lu F, Li J, Wang B, Cheng Y, Kong DH, Cui L, et al. Profiling the humoral immune responses to *Plasmodium vivax* infection and identification of candidate immunogenic rhoptry-associated membrane antigen (RAMA). J Proteomics 2014;102C:66–82.
[44] Goo YK, Seo EJ, Choi YK, Shin HI, Sattabongkot J, Ji SY, et al. First characterization of *Plasmodium vivax* liver stage antigen (PvLSA) using synthetic peptides. Parasit Vectors 2014;7:64.
[45] Patarroyo MA, Calderon D, Moreno-Perez DA. Vaccines against *Plasmodium vivax*: a research challenge. Expert Rev Vaccines 2012;11:1249–60.
[46] Raza A, Beg MA. Population genetics and drug resistance markers: an essential for malaria surveillance in Pakistan. J Coll Physicians Surg Pak 2013;23:878–84.
[47] Acharya P, Pallavi R, Chandran S, Dandavate V, Sayeed SK, Rochani A, et al. Clinical proteomics of the neglected human malarial parasite *Plasmodium vivax*. PLoS One 2011; 6:e26623.
[48] Patarroyo ME, Bermudez A, Patarroyo MA. Structural and immunological principles leading to chemically synthesized, multiantigenic, multistage, minimal subunit-based vaccine development. Chem Rev 2011;111:3459–507.
[49] Patarroyo ME, Patarroyo MA. Emerging rules for subunit-based, multiantigenic, multistage chemically synthesized vaccines. Acc Chem Res 2008;41:377–86.
[50] Restrepo-Montoya D, Becerra D, Carvajal-Patino JG, Mongui A, Nino LF, Patarroyo ME, et al. Identification of *Plasmodium vivax* proteins with potential role in invasion using sequence redundancy reduction and profile hidden Markov models. PLoS One 2011;6:e25189.
[51] Frech C, Chen N. Genome comparison of human and non-human malaria parasites reveals species subset-specific genes potentially linked to human disease. PLoS Comput Biol 2011;7:e1002320.
[52] Acharya P, Pallavi R, Chandran S, Chakravarti H, Middha S, Acharya J, et al. A glimpse into the clinical proteome of human malaria parasites Plasmodium falciparum and Plasmodium vivax. Proteomics Clin Appl 2009;3:1314–25.
[53] Chen JH, Jung JW, Wang Y, Ha KS, Lu F, Lim CS, et al. Immunoproteomics profiling of blood stage *Plasmodium vivax* infection by high-throughput screening assays. J Proteome Res 2011;9:6479–89.
[54] Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, Sauerwein RW, et al. Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry. Nature 2002;419:537–42.
[55] Ray S, Kamath KS, Srivastava R, Raghu D, Gollapalli K, Jain R, et al. Serum proteome analysis of *vivax* malaria: an insight into the disease pathogenesis and host immune response. J Proteomics 2012;75:3063–80.
[56] Ray S, Renu D, Srivastava R, Gollapalli K, Taur S, Jhaveri T, et al. Proteomic investigation of *falciparum* and *vivax* malaria for identification of surrogate protein markers. PLoS One 2012;7:e41751.
[57] Pico de Coana Y, Rodriguez J, Guerrero E, Barrero C, Rodriguez R, Mendoza M, et al. A highly infective *Plasmodium vivax* strain adapted to *Aotus* monkeys: quantitative haematological and molecular determinations useful for *P. vivax* malaria vaccine development. Vaccine 2003;21:3930–7.
[58] Sriprawat K, Kaewpongsri S, Suwanarusk R, Leimanis ML, Lek-Uthai U, Phyo AP, et al. Effective and cheap removal of leukocytes and platelets from *Plasmodium vivax* infected blood. Malar J 2009;8:115.
[59] Andrysiak PM, Collins WE, Campbell GH. Concentration of *Plasmodium ovale*- and *Plasmodium vivax*-infected erythrocytes from nonhuman primate blood using Percoll gradients. Am J Trop Med Hyg 1986;35:251–4.
[60] Prieto JH, Koncarevic S, Park SK, Yates III J, Becker K. Large-scale differential proteome analysis in *Plasmodium falciparum* under drug treatment. PLoS One 2008;3:e4098.
[61] Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc 2007;2:1896–906.
[62] Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, et al. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 2005;4:2010–21.
[63] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20:3551–67.
[64] Brosch M, Yu L, Hubbard T, Choudhary J. Accurate and sensitive peptide identification with Mascot Percolator. J Proteome Res 2009;8:3176–81.
[65] Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat Methods 2007;4:923–5.
[66] Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, Russell B, et al. The transcriptome of *Plasmodium vivax* reveals divergence and diversity of transcriptional regulation in

malaria parasites. Proc Natl Acad Sci U S A 2008;105:16290–5.
[67] Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: a functional genomic database for malaria parasites. Nucleic Acids Res 2009;37:D539–43.
[68] Westenberger SJ, McClean CM, Chattopadhyay R, Dharia NV, Carlton JM, Barnwell JW, et al. A systems-based analysis of *Plasmodium vivax* lifecycle transcription from human to mosquito. PLoS Negl Trop Dis 2010;4:e653.
[69] Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37:1–13.
[70] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13:2498–504.
[71] Nakaya A, Katayama T, Itoh M, Hiranuka K, Kawashima S, Moriya Y, et al. KEGG OC: a large-scale automatic construction of taxonomy-based ortholog clusters. Nucleic Acids Res 2013;41:D353–7.
[72] Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 2011;8:785–6.
[73] Pierleoni A, Martelli PL, Fariselli P, Casadio R. BaCelLo: a balanced subcellular localization predictor. Bioinformatics 2006;22:e408–16.
[74] Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, et al. InterPro: the integrative protein signature database. Nucleic Acids Res 2009;37:D211–5.
[75] Kall L, Krogh A, Sonnhammer EL. A combined transmembrane topology and signal peptide prediction method. J Mol Biol 2004;338:1027–36.
[76] Poisson G, Chauve C, Chen X, Bergeron A. FragAnchor: a large-scale predictor of glycosylphosphatidylinositol anchors in eukaryote protein sequences by qualitative scoring. Genomics Proteomics Bioinformatics 2007;5:121–30.
[77] Ansari FA, Kumar N, Bala Subramanyam M, Gnanamani M, Ramachandran S. MAAP: malarial adhesins and adhesin-like proteins predictor. Proteins 2008;70:659–66.
[78] Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. Nature 2002;419:520–6.
[79] Cowman AF, Berry D, Baum J. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. J Cell Biol 2012;198:961–71.
[80] Goldberg DE. Hemoglobin degradation. Curr Top Microbiol Immunol 2005;295:275–91.
[81] Zerial M, McBride H. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2001;2:107–17.
[82] Ayong L, Raghavan A, Schneider TG, Taraschi TF, Fidock DA, Chakrabarti D. The longin domain regulates the steady-state dynamics of Sec22 in *Plasmodium falciparum*. Eukaryot Cell 2009;8:1330–40.
[83] Lee MC, Moura PA, Miller EA, Fidock DA. *Plasmodium falciparum* Sec24 marks transitional ER that exports a model cargo via a diacidic motif. Mol Microbiol 2008;68:1535–46.
[84] Adisa A, Albano FR, Reeder J, Foley M, Tilley L. Evidence for a role for a *Plasmodium falciparum* homologue of Sec31p in the export of proteins to the surface of malaria parasite-infected erythrocytes. J Cell Sci 2001;114:3377–86.
[85] Pesce ER, Acharya P, Tatu U, Nicoll WS, Shonhai A, Hoppe HC, et al. The *Plasmodium falciparum* heat shock protein 40, *Pfj4*, associates with heat shock protein 70 and shows similar heat induction and localisation patterns. Int J Biochem Cell Biol 2008;40:2914–26.
[86] Muralidharan V, Oksman A, Pal P, Lindquist S, Goldberg DE. *Plasmodium falciparum* heat shock protein 110 stabilizes the asparagine repeat-rich parasite proteome during malarial fevers. Nat Commun 2012;3:1310.
[87] Acharya P, Kumar R, Tatu U. Chaperoning a cellular upheaval in malaria: heat shock proteins in Plasmodium falciparum. Mol Biochem Parasitol 2007;153:85–94.
[88] del Portillo HA, Fernandez-Becerra C, Bowman S, Oliver K, Preuss M, Sanchez CP, et al. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. Nature 2001;410:839–42.
[89] Spielmann T, Gardiner DL, Beck HP, Trenholme KR, Kemp DJ. Organization of ETRAMPs and EXP-1 at the parasite-host cell interface of malaria parasites. Mol Microbiol 2006;59:779–94.
[90] Koenderink JB, Kavishe RA, Rijpma SR, Russel FG. The ABCs of multidrug resistance in malaria. Trends Parasitol 2010;26:440–6.
[91] Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature 2008;455:757–63.
[92] Rodriguez LE, Curtidor H, Urquiza M, Cifuentes G, Reyes C, Patarroyo ME. Intimate molecular interactions of *P. falciparum* merozoite proteins involved in invasion of red blood cells and their implications for vaccine design. Chem Rev 2008;108:3656–705.
[93] Curtidor H, Patino LC, Arevalo-Pinzon G, Vanegas M, Patarroyo ME, Patarroyo MA. *Plasmodium falciparum* rhoptry neck protein 5 peptides bind to human red blood cells and inhibit parasite invasion. Peptides 2014;53:210–7.
[94] Garcia J, Curtidor H, Pinzon CG, Vanegas M, Moreno A, Patarroyo ME. Identification of conserved erythrocyte binding regions in members of the *Plasmodium falciparum* Cys6 lipid raft-associated protein family. Vaccine 2009;27:3953–62.
[95] Zheng C, Han L, Yap CW, Xie B, Chen Y. Progress and problems in the exploration of therapeutic targets. Drug Discov Today 2006;11:412–20.
[96] Nakanishi M. S-adenosyl-L-homocysteine hydrolase as an attractive target for antimicrobial drugs. Yakugaku Zasshi 2007;127:977–82.
[97] Uyemura SA, Luo S, Vieira M, Moreno SN, Docampo R. Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in *Plasmodium yoelii yoelii* mitochondria *in situ*. J Biol Chem 2004;279:385–93.
[98] Skinner-Adams TS, Stack CM, Trenholme KR, Brown CL, Grembecka J, Lowther J, et al. *Plasmodium falciparum* neutral aminopeptidases: new targets for anti-malarials. Trends Biochem Sci 2010;35:53–61.
[99] Madrid DC, Ting LM, Waller KL, Schramm VL, Kim K. *Plasmodium falciparum* purine nucleoside phosphorylase is critical for viability of malaria parasites. J Biol Chem 2008;283:35899–907.
[100] Pessi G, Kociubinski G, Mamoun CB. A pathway for phosphatidylcholine biosynthesis in *Plasmodium falciparum* involving phosphoethanolamine methylation. Proc Natl Acad Sci U S A 2004;101:6206–11.
[101] Sierra AY, Barrero CA, Rodriguez R, Silva Y, Moncada C, Vanegas M, et al. Splenectomised and spleen intact *Aotus* monkeys' immune response to *Plasmodium vivax* MSP-1 protein fragments and their high activity binding peptides. Vaccine 2003;21:4133–44.
[102] Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, et al. Regulation of cellular metabolism by protein lysine acetylation. Science 2010;327:1000–4.
[103] Kolker E, Higdon R, Hogan JM. Protein identification and expression analysis using mass spectrometry. Trends Microbiol 2006;14:229–35.
[104] Baird JK. Chloroquine resistance in *Plasmodium vivax*. Antimicrob Agents Chemother 2004;48:4075–83.
[105] Tyagi RK, Sharma YD. Erythrocyte binding activity displayed by a selective group of *Plasmodium vivax* tryptophan rich

antigens is inhibited by patients' antibodies. PLoS One 2012; 7:e50754.
[106] Garcia J, Curtidor H, Obando-Martinez AZ, Vizcaino C, Pinto M, Martinez NL, et al. Synthetic peptides from conserved regions of the *Plasmodium falciparum* early transcribed membrane and ring exported proteins bind specifically to red blood cell proteins. Vaccine 2009;27:6877–86.
[107] Triglia T, Tham WH, Hodder A, Cowman AF. Reticulocyte binding protein homologues are key adhesins during erythrocyte invasion by *Plasmodium falciparum*. Cell Microbiol 2009;11:1671–87.
[108] Haase S, Cabrera A, Langer C, Treeck M, Struck N, Herrmann S, et al. Characterization of a conserved rhoptry-associated leucine zipper-like protein in the malaria parasite *Plasmodium falciparum*. Infect Immun 2008;76:879–87.
[109] Counihan NA, Kalanon M, Coppel RL, de Koning-Ward TF. *Plasmodium* rhoptry proteins: why order is important. Trends Parasitol 2013;29:228–36.
[110] Tonkin ML, Arredondo SA, Loveless BC, Serpa JJ, Makepeace KA, Sundar N, et al. Structural and biochemical characterization of *Plasmodium falciparum* 12 (*Pf*12) reveals a unique interdomain organization and the potential for an antiparallel arrangement with *Pf*41. J Biol Chem 2013;288:12805–17.
[111] McCutchan TF, Dame JB, Miller LH, Barnwell J. Evolutionary relatedness of *Plasmodium* species as determined by the structure of DNA. Science 1984;225:808–11.