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



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ABSTRACT

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

Biological significance

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

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1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Reagents

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

2.2. Animal handling

Monkeys kept at Fundación Instituto de Immunología 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) \times 100. The *P. vivax* infected blood samples were collected for proteomic studies once parasitaemia percentage was found to be between 2 and 5. Monkeys were treated with paediatric doses of chloroquine (10 mg/kg on the first day and 7.5 mg/kg/day until the fifth day) and primaquine (0.25 mg/kg/day from the third to the fifth day) at the end of the study to guarantee parasite clearance from blood. Once experiments were over, CorpoAmazonia officers supervised the primates' return to their natural habitat in excellent health.

2.3. Isolating *P. vivax* blood stages

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

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

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

2.4. Protein extraction and precipitation

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

2.5. Protein digestion and purification

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

2.6. Mass spectrometry

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

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

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

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

The LTQ-Orbitrap Velos was operated in a data-dependent MS/MS mode using Xcalibur 2.1.0.1140 software (Thermo-Fisher, San Jose, CA) at 2.10 kV spray voltage, 325 °C and 60% S-lens RF level. Survey scans were acquired in the mass range 400 to 1600 m/z with 60,000 resolution at m/z 400 with lock mass option enabled for the 445.120025 ion [62]. The 20 most intense peaks having ≥ 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 BaCellLo [73] were considered when selecting proteins destined for the secretory pathway. The Interpro database [74] was scanned in the search for putative domains in the whole protein sequence. The presence of transmembrane and glycosylphosphatidylinositol (GPI) anchor sequences was determined by using Phobius [75] and FragAnchor [76] tools, respectively. Adhesine-like proteins were predicted using MAAP software, using >0.7 score, according to the recommendations [77].

3. Results

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

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

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

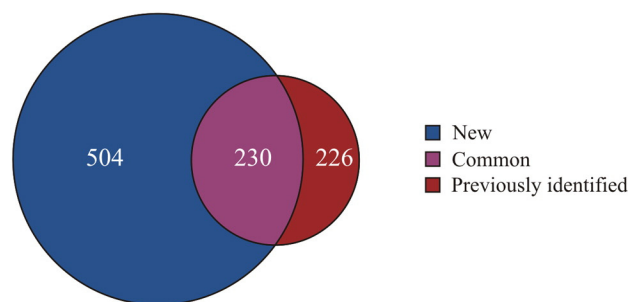


Fig. 1 – *P. vivax* proteins identified to date. Venn diagram showing the proteins identified in this study compared to early proteomic and immunoproteomic studies.

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

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

New members of the *Pv-fam* family (not found previously) predicted in the *P. vivax* genome *in silico* analysis [91] were detected (Supplementary Data 3). Rhoptry (RAP-1 (PVX_085930), -2 (PVX_097590), RON2 (PVX_117880), Clag (PVX_121885)) and surface (MSP-8 (PVX_097625), -9 (PVX_124060), Pv41 (Pfs230) (PVX_000995) and Pv12 (PVX_113775)) proteins which have already been identified and considered as good candidates for inclusion in a *P. falciparum* vaccine were also identified [92–94]. A recently reported pre-erythrocytic (liver stage antigen (PVX_091675)) protein was found; although this molecule is immunogenic, its role during blood cycle has not been studied [44].

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

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

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

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

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

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

3.4. Pharmacological target prediction

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

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

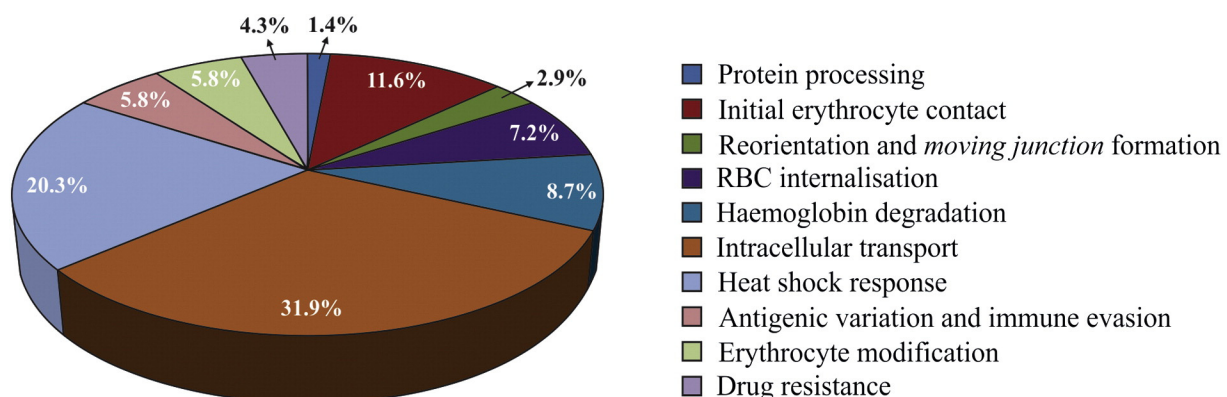


Fig. 2 – Pie chart showing the *P. vivax* proteins distribution related to functional classes.

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

Biological process	Protein name and PlasmoDB ID	References
Protein processing	Subtilisin-like protease (PVX_097935)	[78,79]
Initial erythrocyte contact	MSP-1 (PVX_099980), -7 (PVX_082675), -7H (PVX_082680), -7I (PVX_082685) ^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 (rhopty neck protein), EXP (exported protein), HSP (heat shock protein), vir (variable surface protein), *etramp* (early transcribed membrane protein), and *mrp* (multidrug resistance protein).

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

3.5. In silico predicted vaccine candidates

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

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

previously been studied in pre-clinical assays [101], others had already been described as surface (Pu12 and Pu41) [26,38] and rhopty (PuRON2) [34] proteins, 8 were hypothetical proteins and other rhopty 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.

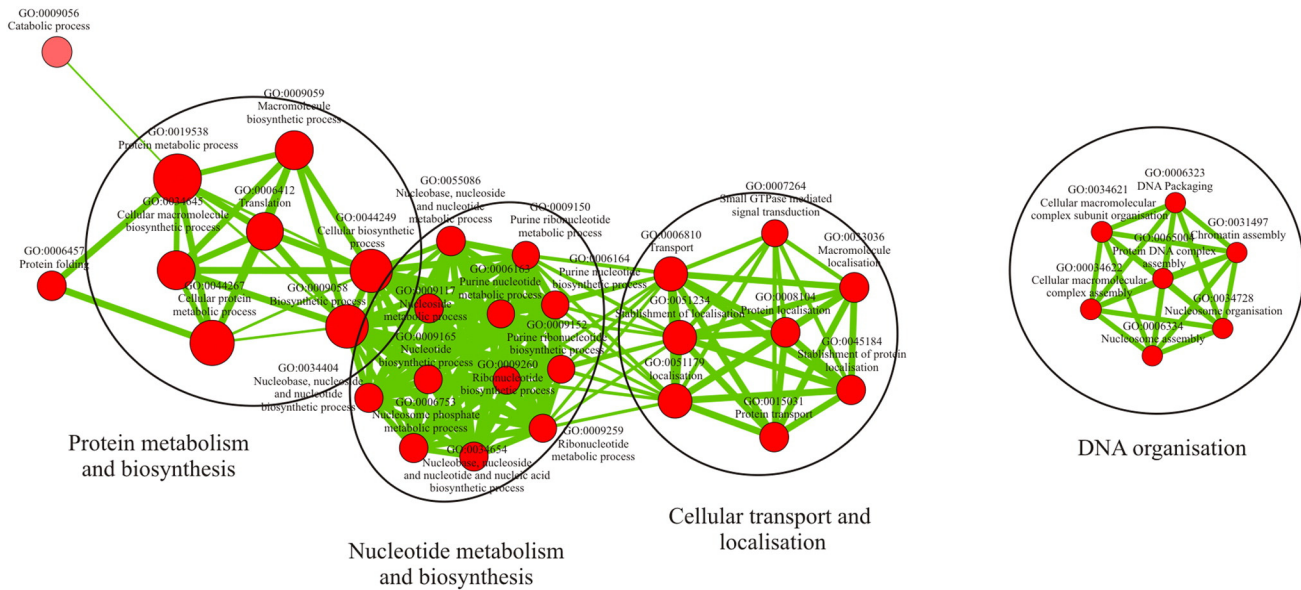


Fig. 3 – Enrichment map for the *P. vivax* proteins identified here. Map displaying *P. vivax* proteins grouped according to their function. The size of the red node represents the number of proteins by term.

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	Glutamyl-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 of their encoding mRNA abundance.

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

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

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

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

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

Rhoptry and surface proteins are important candidates given that they are required for host cell attachment and parasite invasion [109,110]; therefore, RON-2 (PVX_117880), -3 (PVX_101485), -5 (PVX_089530), the rhoptry protein above mentioned (PVX_084720), one member of the cytoadherence protein family (PVX_121885), and Pv12 (PVX_113775) and Pv41 (PVX_000995) could be good candidates.

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

Table 4 – Proteins recognised by stage and their amount.

Stage	Total proteins	<i>P. vivax</i>	Primate	Contaminants ^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.

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, C-terminal (IPR024781)	–	–	–
PVX_082680 ^a	Merozoite surface protein 7 (MSP7), putative	35	x	x	–	–	–	–
PVX_084720 ^a	Hypothetical protein, conserved	40	x	x	–	–	x	–
PVX_086915	Early transcribed membrane protein (ETRAMP)	35	x	x	<i>etramp</i> family (IPR006389)	1	–	–
PVX_089530	Rhoptry neck protein 5, putative (RON5)	35	x	x	–	2	–	–
PVX_090230	Early transcribed membrane protein (ETRAMP)	35	x	x	<i>etramp</i> family (IPR006389)	2	–	–
PVX_090945	Hypothetical protein, conserved	35	x	x	–	1	–	–
PVX_091700 ^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	<i>etramp</i> family (IPR006389)	1	x	–
PVX_096245	Rhoptry-associated leucine zipper-like protein 1	35	x	x	–	–	x	–
PVX_096950	Tryptophan-rich antigen (Pv-fam-a)	43	x	x	Tryptophan/threonine-rich domain (IPR022089)	–	–	–
PVX_096990	<i>Pv-fam-d</i> protein	40	x	x	–	1	–	–
PVX_097935	Subtilisin-like protease precursor, putative	40	x	x	S8/S53 domain (IPR000209)	–	–	–
PVX_099710	Hypothetical protein, conserved	40	x	x	Calycin-like domain (IPR011038)	–	–	–
PVX_099980 ^a	Major blood-stage surface antigen Pv200	35	x	x	EGF domain (IPR010901, IPR024730, IPR024731)	1	x	HP
PVX_101485	Rhoptry neck protein 3, putative (RON3)	35	x	x	–	3	–	–
PVX_112665	Tryptophan-rich antigen (<i>Pv-fam-a</i>)	43	x	x	Tryptophan/threonine-rich domain (IPR022089)	–	–	–
PVX_112685 ^{a,b}	Tryptophan-rich antigen (<i>Pv-fam-a</i>)	40	x	x	–	–	x	–
PVX_113225	Plasmodium exported protein, unknown function	40	x	x	Protein of unknown function DUF3671 (IPR022139)	3	–	–
PVX_113775 ^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}	<i>Pv-fam-d</i> protein	40	x	x	–	2	–	–
PVX_122910	Hypothetical protein, conserved	43	x	x	–	1	–	–
PVX_124090	Hypothetical protein, conserved	35	x	x	–	1	–	–

MET: maximum expression time; SP: signal peptide; GPI: glycosylphosphatidylinositol; HP: highly probable.
^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.

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Competing interests

The authors have declared that no competing interests exist.

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