Journal of Proteomics 218 (2020) 103719

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Review

Inferring *Plasmodium vivax* protein biology by using omics data

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ARTICLE INFO

Keywords:
Plasmodium vivax
Proteomics
Immunomics
Interactomics
Control methods

ABSTRACT

Deciphering *Plasmodium vivax* biology has long been a challenge for groups working on this parasite, mainly due to the complications involved in propagating it *in vitro*. However, adapting *P. vivax* strains in non-human primates and the arrival of high-performance analysis methods has led to increased knowledge regarding parasite protein composition and the ability of some molecules to trigger an immune response or participate in protein-protein interactions. This review describes the state of the art concerning proteomics-, immunomics- and interatomics-related *P. vivax* omic studies, discussing their potential use in developing disease control methods.

1. Introduction

Plasmodium vivax is the second epidemiologically important species causing malaria in humans [1]. The parasite's complex biology has been the main cause of delay regarding knowledge of the molecules needed for developing new diagnostic methods and vaccines [2]. Consequently, knowledge regarding *P. vivax* has always lagged behind that concerning *P. falciparum* and, although interesting findings have been made, it was not until 2008 that it was reported that these species has a different evolutionary pattern, as found when using high-throughput omic methods [3–5].

Omics technologies have thus enabled improving the understanding of parasites' nature and content through the global study of their set of molecules. These tools have been useful for ascertaining biological systems' genome (genomic), transcripts (transcriptomic), proteins (proteomic) and metabolites (metabolomic) [6] and, more currently, measuring an antigenic response against parasite-derived proteins (immunomic) [7] or protein-protein interactions (interactomic) [8]. Incorporating this technology in *P. vivax* led to identifying the species' genome and transcriptome a decade ago [4,9]. However, only recently have other biological aspects of the species been studied in greater depth through proteomics, immunomics and interatomics [10–18]. These methods, together with the use of protein enrichment techniques and bioinformatics analysis have particularly offered an extraordinary alternative for resolving the problem of *P. vivax* molecules' functional evaluation in continuous *in vitro* culture, although this has not yet been standardised.

This review is aimed at reporting the latest advances regarding *P. vivax* omic studies and highlighting their contribution to identifying molecules which could be used for improving diagnostic or disease prevention methods.

2. *P. vivax* protein composition

Following the advent of high performance sequencing techniques there has been a boom in analysing *P. vivax* molecular composition regarding its genome and transcriptome [19]. However, and due to a lack of a *P. vivax* continuous *in vitro* culture, advances regarding knowledge of the parasite's protein composition have progressed slowly compared to those concerning *P. falciparum*, *i.e.* such knowledge can be summed up in just seven reports to date (Additional file 1). In spite of this, the few studies in this area have significantly contributed to deepening knowledge of *P. vivax* protein composition and function through *in silico* prediction and functional annotation tools, using different types of software, mainly the Database for Annotation, Visualization and Integrated Discovery (DAVID) [20].

Particularly interesting have been the discrepancies regarding the amount of proteins reported in proteomics studies (Fig. 1; Add. file 1) [10,21–28]. These can be explained by various parameters. A first consideration concerns the source of *P. vivax*-infected blood samples, human samples being extremely heterogeneous and having few parasites (*i.e.* a 2–20 mL mixture of blood from different patients having 0.1% parasitaemia) [21,22,24], unlike those from primates (*i.e.* 2–3 mL of blood having 0.5–3% parasitaemia from non-human primates

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https://doi.org/10.1016/j.jprot.2020.103719
Received 30 November 2019; Received in revised form 10 February 2020; Accepted 21 February 2020
Available online 21 February 2020

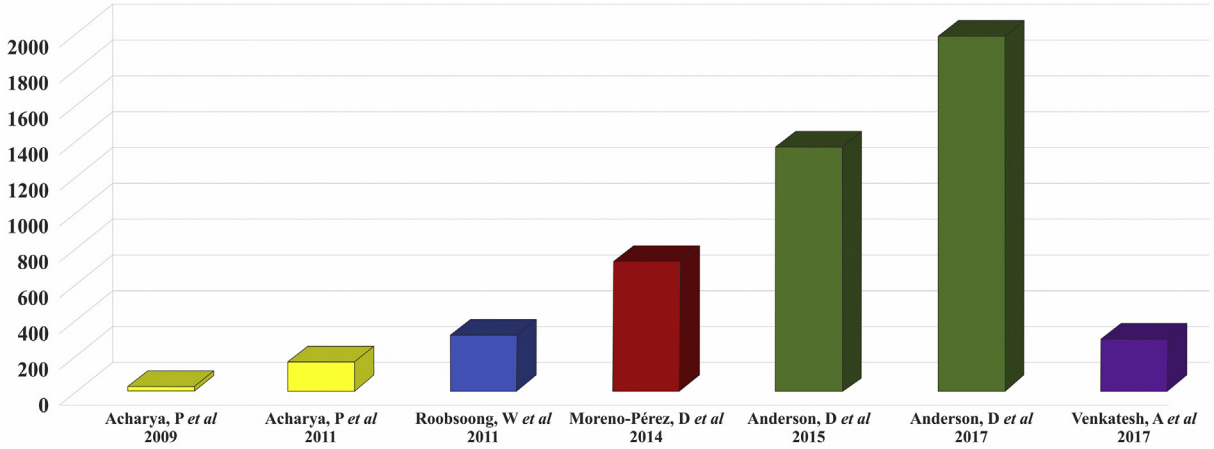

Fig. 1. Bar chart showing the amount of *P. vivax* proteins identified by proteomics over the last ten years. The X axis indicates the main authors and the year when each study was carried out and the Y axis the number of molecules identified.

infected with *P. vivax* strains (VCG-1 or Sal-1)) [10,11,27]. Blood samples from primates also promote a higher percentage of enriched schizonts being obtained compared to short-term cultured ones from humans. Secondly, the SDS-PAGE technique which is used in some studies for reducing sample complexity could not facilitate analysis given that it causes a loss of molecules to be detected by MS/MS due to difficulty in separating low abundance, acidic and basic proteins and hydrophobic molecules (mainly membrane proteins). Another important parameter concerns contamination by highly abundant host proteins (such as haemoglobin and cytoskeletal molecules) masking parasite peptide detection, proteome coverage thus becoming considerably decreased. The different ionization techniques used, along with the types of mass spectra identification software could also affect the results reported.

Although the amount of *P. vivax* proteins identified by proteomics has considerably increased (Fig. 1), it must be pointed out that such data does not represent the amount of transcripts reported to date [4]. This is because proteomic data is higher for the trophozoite/schizont stages and because the relative abundance of each molecule in a particular stage is not known with certainty [10]. A continuous *in vitro* culture is thus required for knowing in detail how *P. vivax* protein expression is regulated throughout the intra-reticulocyte life-cycle. Such culture must enable a sufficient amount of parasites to be obtained, density gradients to be standardised for enriching the different stages and highly robust quantitative proteomic techniques to be incorporated. For the moment, the data obtained by proteomics continues being a source of invaluable information for the *in silico* prediction of biomarkers and/or vaccine candidates.

3. Antigen response during *P. vivax* natural infection

The immunogenic approach considers that immunodominant proteins are the most appropriate ones for inclusion in a vaccine since they could be useful in a protection-inducing response against the parasites. Studying humoral responses, and even more so those associated with protection against clinical malaria, has become a *P. vivax*-related task which research groups worldwide have been working on for many years now (Additional file 2). Simultaneously studying a humoral response against various *P. vivax* antigens (immunoproteomics) became a reality with the integration of reverse vaccinology (enabling *in silico* genome, transcriptome and proteome data mining), eukaryotic or prokaryotic cell expression systems (used for efficiently expressing and obtaining proteins) and protein array technology (for printing hundreds of molecules on a chip and enabling simultaneous analysis).

The first high performance arrays for analysing *P. vivax* immunomics were constructed with proteins expressed in the wheat germ cell-free (WGCF) expression system. A first study by Chen, J et al., printed 90 proteins in an array which had previously been selected *in silico* as good vaccine candidates. Eighteen molecules were seroreactive to 20 sera from individuals exposed to *P. vivax* infection; 4 were recognised by more than 60% of the sera whilst the remainder were detected by 5% to 35% of them. Particularly interesting was the fact that 16 of these molecules were found to have transcripts abundantly expressed during the schizont stage [28]. Subsequently, Lu, F et al., constructed an array having 152 proteins (85% having high expression during schizont stages), 44 of which reacted to sera from 22 individuals, 14 of them were recognised by more than 60% of the sera whilst the remaining 30 were detected by 23% to 59% [16].

Han, J's group evaluated the antigenicity of molecules involved in *P. vivax* selective invasion of human reticulocytes, known as reticulocyte binding proteins (RBPs). RBPs were detected by more than 50% of the patients' sera (56.3% to 87.5%), although some fragments derived from them were poorly immunogenic (37.5 to 43.8% detection percentage) [29]. Chen, J et al., used the *Escherichia coli* cell-free expression system in 2015 for obtaining and immobilising 1936 molecules in an array. They found that 151 molecules (53% expressed during schizont stage) were recognised by sera from 15 individuals; half of the 18 seroreactive proteins were positive for detection by more than 60% of the sera and the other half by 27% to 53% of them [13].

The cell-free *in vitro* transcription/translation (IVTT) system is another technique which has been used for obtaining proteins to be immobilised in an array. The study by Baum et al., evaluated the reactivity of 153 sera to 515 *P. vivax* molecules; 105 proteins were detected by 60% to 94% of the sera whilst the remaining were detected by 28% to 59%. Interestingly, two molecules were associated with protection as antigen response was more reactive in asymptomatic individuals compared to individuals showing symptoms of the disease (Additional file 3) [30]. An expanded study by the same group analysed age-related antibody (Ab) response, finding that 142 of the 298 sera were variably recognised (13 by 60% to 78% and 129 by 20% to 58%). Seven molecules' Ab response was abundant in asymptomatic individuals and became reduced in symptomatic ones; they can thus be considered protective response stimulators (Additional file 3) [12]. Arévalo-Herrera, M et al., (2016) compared the Ab profiles triggered in healthy (naive) or semi-immune humans following experimental infection with *P. vivax* sporozoites (Spz) *via* female mosquito bite. They found that sera from both groups collected on day 45 recognised 236 proteins (*i.e.* where the highest fever peak occurred: 188 naive and 181 semi-immune). However, reactivity declined on day 145 to 138 molecules (103 naive and 88 semi-immune). According to the analysis, 8 antigens were associated with protection becoming more abundant in asymptomatic individuals (Additional file 3) [31].

Human embryonic kidney (HEK) 293E cells have also been used for expressing and constructing *P. vivax* molecule libraries. Hostetler, J et al., expressed 37 complete ectodomains from protein vaccine candidates. They found that 26 of them were recognised by the pool of sera from 14 Cambodian individuals by enzyme-linked immunosorbent assay (ELISA) [15]. One year later, Franca, C et al., reported recognising 28/34 immunoreactive molecules by ELISA having a high degree of variability regarding IgG levels for each of them, according to the data obtained by Luminex bead array; 3 of them triggered a response having high Ab titres associated with protection (Additional file 3) [32]. The same group integrated proteins expressed by different systems (HEK293E and *E. coli* cells or WGCF expression system) in Luminex analysis. They found that the 38 fragments expressed were recognised by sera from 255 children having great variability regarding Ab seroprevalence; 31 of them had species-specific association with reduced risk of *P. vivax* malaria, 5 of them having low Ab levels (Additional file 3) [14].

Integrating the results obtained from the immunomics studies described here, 339 antigenic molecules have been reported to date, 24 of them reacting against more than 90% of the sera evaluated; 4 have been identified in 5 to 7 studies (out of 10) and have been found to be associated with protection (Fig. 2 and Additional file 4). The foregoing makes it clear that such approach has provided some clues for understanding the pathogenesis of *P. vivax* natural infection. However, it is worth noting that this relies heavily on the initial bioinformatics selection and relevant targets of natural immunity could thus be excluded.

The variability in the serological recognition of some molecules and the prediction of proteins associated with protection reported in different studies is very heterogeneous (Additional files 3 and 4), suggesting that it is risky to consider these molecules good vaccine candidates. Even more so, if one considers that some of them associated with protection do participate in parasite metabolic processes (Additional file 3: highlighted in pink). Greater precision is certainly required regarding the *in silico* selection of proteins related to cell invasion and experimentally confirming their function during the life-cycle before prioritising them as candidates.

Immunoproteomic findings represent a source of invaluable information for identifying markers of infection and/or disease severity, as has been the described in various studies [13,16,29,32]. These data are undoubtedly useful for finding proteins that improve rapid diagnostic tests in samples having very low parasitaemia levels, thereby enabling timely diagnosis and early treatment.

4. Screening protein-protein interactions by interactomics

Identifying protein-protein interactions represents a potential point of intervention for designing control methods against different microorganisms. Particularly regarding *P. vivax,* little information has been reported concerning intra- (molecular complex formation) and intermolecular interactions (receptor-ligand interaction) occurring between merozoite (Mrz) proteins and their target cells (Fig. 3).

Intramolecular interactions have been carried out/studied through binary interaction assays between *P. vivax* molecules involved in invasion such as AMA1-RONs [33]. However, only recently have methodologies begun to be used for the parallel analysis of multiple molecules, *i.e.* Avidity-based extracellular interaction screen (AVEXIS) and nucleic acid programmable protein array (NAPPA) [18,34]. Hostetler's group (2015) reported interactions between *Pv*12-*Pv*41, *Pv*MSP3.10-*Pv*MSP7.1 and *Pv*12-PVX_110945 for the first time by constructing a library of 34 molecules in the AVEXIS system expressed with HEK293E cells. *Pv*12-*Pv*41 and *Pv*MSP3.10-*Pv*MSP7.1 were validated by saturation assays measured by surface plasmon resonance (SPR), showing binding specificity between such complexes and high affinity regarding *Pv*12-*Pv*41 interaction [15]. More recently, Arévalo-Pinzón et al., determined *Pv*12 interaction with various parasite surface- (*Pv*41, PvMSP1$_{42}$ and *Pv*MSP8), rhoptry- (*Pv*RAP1) and microneme-located molecules (*Pv*RBP1aIV) by NAPPA, using the IVTT system for *in situ* expression. *Pv*12 particularly had greater interaction with *Pv*41, PvMSP1$_{42}$ and *Pv*RBP1a [17], thereby supporting the idea that, unlike

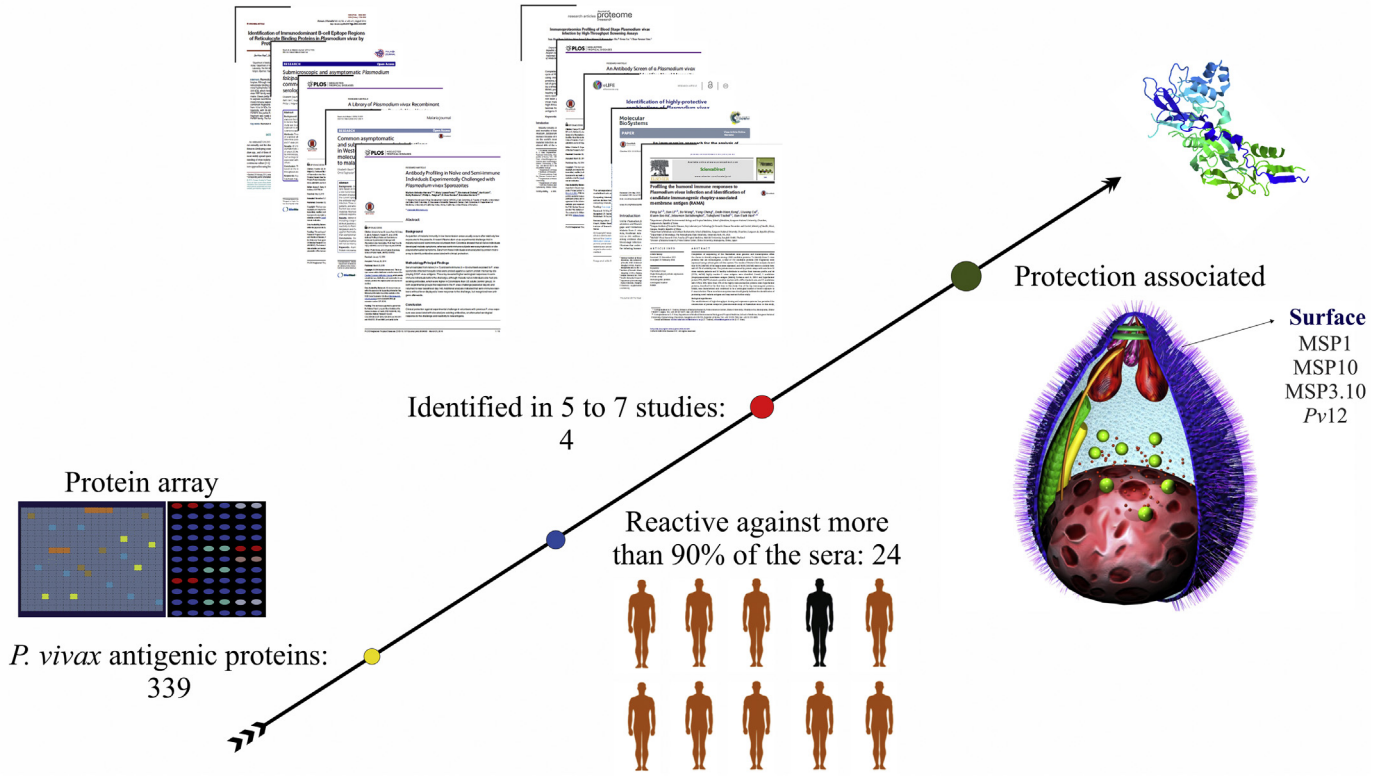

Fig. 2. Flow chart showing the identification of *P. vivax* molecules which could be considered as diagnostic markers of the disease.

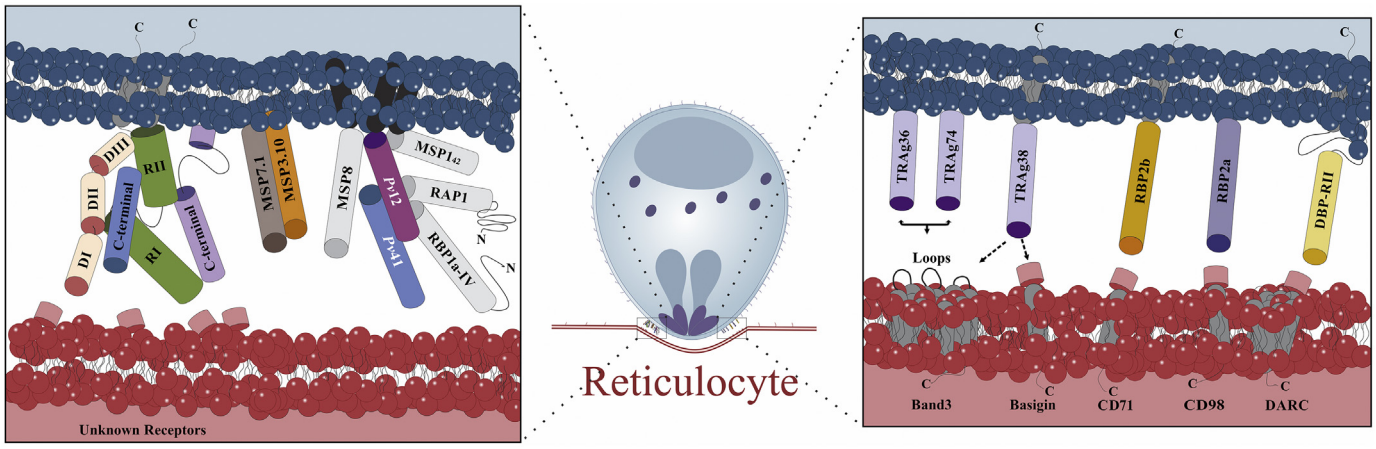

Fig. 3. *P. vivax* molecules described as participating in intra and inter-molecular interactions. The intramolecular interactions shown in grey have not been confirmed by high sensitivity techniques. GPI anchor (Black) and transmembrane (Dark grey) regions, as well as AMA1 (beige), RON2 (green), RON4 (purple), RON5 (dark blue) proteins are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P. falciparum [35], this molecule could play an important role during *P. vivax* Mrz interaction with human reticulocytes once becoming associated with detergent-resistant membrane (DRM) regions where most parasite protein complexes involved in invasion are built [36].

Inter-molecular interactions between *P. vivax* Mrz proteins and receptors on human reticulocytes have not been studied with high-throughput techniques. However, robust methodologies have been used to confirm and analyse ligand-receptor interaction in-depth, one-by-one [37,38]. For example, the DBP-DARC interaction was confirmed by rosetting formation and *in vitro* inhibition assays [39,40]. TRAg38 binding to basigin and band 3 receptors was evaluated using pulldown, multidimensional protein identification technology (MudPIT), label transfer, solid phase binding, SPR and yeast two-hybrid assays [41]. Cellular ELISA, solid-phase binding assay, SPR and growth inhibition assay were used to study TRAg36/TRAg74 binding to band3. Other technics such as competition ELISA, protein binding to enzymatically-treated reticulocytes, recombinant binding inhibition using Abs targeting cell receptors, fluorescence resonance energy transfer (FRET), receptor knockout on JK-1 cells *via* clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and *in vitro* inhibition assay in short-term culture have also been used for confirming RBP2b-CD71 [42] and RBP2a-CD98 [43] intermolecular interactions.

The foregoing techniques, opening up new horizons for studying important *P. vivax* receptor-ligand interactions, compensate for the difficulties regarding continuously propagating the parasite *in vitro*. Furthermore, taking the advances made by AVEXIS and NAPPA into account, such techniques could be extremely useful for screening multiple protein-protein interactions which are critical for parasite survival.

5. Concluding remarks

Important advances related to *P. vivax* biology have been made during the last decade *via* omic techniques. The results have particularly opened up new horizons in the field of studying diagnostic methods and anti-*P. vivax* vaccine candidates due to the infection biomarkers and molecules capable of exclusively binding to the reticulocytes reported so far. Immunomics data can be used for resolving relevant clinical problems such as the lack of a certain diagnosis due to current gold standard methods' inaccuracy (such as microscopy and rapid diagnosis tests) for detecting sub-microscopic parasite densities. For example, molecules detected by more than 90% of sera from individuals suffering natural infection could be considered potential disease markers, *i.e. Pv*MSP1, *Pv*MSP10, *Pv*MSP3.10 and *Pv*12.

The results described here reinforces the need to integrate omic (proteomics and interactomics) data for reducing the large bottle neck regarding the functional characterisation of the antigens involved in essential protein-protein interactions for *P. vivax* development. Basically, initial *in silico* analysis of the proteomic data and subsequent screening by AVEXIS or NAPPA of whole proteins or their fragments governing *P. vivax* parasite (Spz and Mrz) interaction with their target cells (hepatocytes and reticulocytes) undoubtedly represents the best strategy for seeking the most useful regions to be used in a fully protective multi-antigen, multi-stage vaccine.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103719.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We would like to thank FIDIC's Molecular Biology and Immunology group for their support, as well as Jason Garry for translating this manuscript. We also thank Gabriela Arevalo-Pinzón and Maritza Bermúdez for their assistance with Fig. 3.

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