Minireview

Plasmodium vivax transcriptomics: What is new?

Julia Weber Ferraboli, Gisele Tatiane Soares da Veiga and Letusa Albrecht

Laboratory of Apicomplexan Parasites Research, Carlos Chagas Institute, Oswaldo Cruz Foundation (FIOCRUZ), Curitiba 81310-020, Brazil
Corresponding author: Letusa Albrecht. Email: letusa.albrecht@fiocruz.br

Impact Statement

Given the rapid rise and improvement of gene sequencing technologies and omics science advancements, this review compiles information revealed in transcriptomics papers published in the last 5 years, updating the findings generated. Transcriptomics provides new insights into the parasite biology that causes vivax malaria, as there are many questions to be answered. The lack of *in vitro* culture limits the knowledge about the species, so it is necessary to use other strategies. The new findings indicate that different genes are expressed heterogeneously according to the parasite life cycle. Furthermore, it is postulated that *P. vivax* modulates the expression of these genes for its own benefit, facilitating parasite–host interactions and as an evasion mechanism, ensuring species survival. These new findings allow a better understanding of *P. vivax* pathogenesis and biology, ensuring the design of new disease control strategies.

Abstract

Malaria is the leading human parasitosis and is transmitted through the bite of anopheline mosquitoes infected with parasites of the genus *Plasmodium* spp. Among the seven species that cause malaria in humans, *Plasmodium vivax* is the most prevalent species in Latin America. In recent years, there have been an increasing number of reports of clinical complications caused by *P. vivax* infections, which were previously neglected and underestimated. *P. vivax* biology remains with large gaps. The emergence of next-generation sequencing technology has ensured a breakthrough in species knowledge. Coupled with this, the deposition of the *P. vivax* Sal-1 reference genome allowed an increase in transcriptomics projects by accessing messenger RNA. Thus, the regulation of differential gene expression according to the parasite life stage was verified, and several expressed genes were linked to different biological functions. Today, with the progress associated with RNA sequencing technologies, it is possible to detect nuances and obtain robust results. Discoveries provided by transcriptomic studies allow us to understand topics such as RNA expression and regulation and proteins and metabolic pathways involved during different stages of the parasite life cycle. The information obtained enables a better comprehension of immune system evasion mechanisms; invasion and adhesion strategies used by the parasite; as well as new vaccine targets, potential molecular markers, and others therapeutic targets. In this review, we provide new insights into *P. vivax* biology by summarizing recent findings in transcriptomic studies.

Keywords: *Plasmodium* vivax, malaria, transcriptomic, gene expression

***Experimental Biology and Medicine* 2023; 248: 1645–1656. DOI: 10.1177/15353702231198070**

Introduction

The high throughput of short reads generated by next-generation sequencing (NGS) has provided advances in several areas of knowledge, including biological applications.[1] Availability, ease of handling and more affordable costs are the benefits of using the tool. The genome sequencing projects of *Plasmodium* spp. have contributed to understanding parasite–host interactions, revealing large genetic diversity between species.[2] *P. falciparum*, which causes the most severe cases of malaria and is responsible for most deaths, has been the focus of control and the basis of most malaria research.[3] However, a single malaria eradication plan has not been effective for all species. Studies have shown that the mortality and lethality caused by *P. vivax* have been neglected and underestimated, leaving in the shadow of the enormous problem caused by *P. falciparum* in sub-Saharan Africa.[4,5] With this, research on vivax malaria faces several adversities, resulting in a general lack of knowledge about the specie biology.[6]

Following the deposition of the *P. vivax* Sal-1 reference genome, whole transcriptome sequencing (WTS) projects began to emerge.[7–10] Differential expression of gene clusters, according to the parasite life cycle, was noted by assessing *P. vivax* messenger RNA (mRNA) with microarray assays.[10] These genes were involved in parasite–host interactions, virulence and encode parasite development proteins. Corroborating these findings, extreme changes in mRNA co-expression of developmental genes have been found, hinting that they are responsible for modulating parasite progression.[8] Recently, RNA-seq has also been applied and can detect expression nuances, being a more sensitive and

complete technology compared to microarrays. The use of this new technology contrasted with previous findings indicating that, regardless of life stage, a homogeneous gene expression profile was found when analyzing patient isolates.[11]

Gene expression analyses of *P. vivax* transcripts are complicated, as it is a complex, dynamic and non-standardized system. In natural infections, parasites are mostly from heterogeneous populations, and different developmental stages are present simultaneously, which results in a distinct transcriptional profile.[12] To identify gene expression differences between cells in the same sample, single-cell RNA sequencing (scRNA-seq) is a valuable tool,[13] despite being a costly methodology and requiring the use of intact cells, which becomes complicated with patient samples.[14] However, scRNA-seq provides little information about gene isoforms and alternative splicing. These features can be observed when full-length isoform sequencing (iso-Seq) technology is applied.[13] It is known that during parasite development, *P. vivax* transcribes multiple gene isoforms, which can be noted by the variation in untranslated region (UTR) length or splicing.[13,15]

P. vivax transcriptomic analysis is limited on account of low parasitaemia and multiclonality infections. RNA isolation in terms of quantity and quality is a challenge due to the absence of *in vitro* culture combined with low quantity pure parasites.[16] Several research groups have been improving *Plasmodium* spp. transcriptomics area aiming to understand RNA expression and regulation, proteins and metabolic pathways involved in the life cycle[8,15,17–20] The information obtained provides clues to the immune evasion, invasion, and adhesion strategies used by the parasite as well as indicates new molecular targets for vaccine purposes or blocking parasite resistance to antimalarial drugs.

The lack of knowledge of *P. vivax* biology is enormous, and many of these are challenging. Herewith, the use of molecular tools such as transcriptomics allows an estimation of vivax malaria prevalence and incidence, parasite–host interactions, and gene expression.[12] In this review, we update the achievements made in recent years regarding the biology of *P. vivax* based on the transcriptome. In summary, the data are divided according to the *P. vivax* life cycle. We also indicate the current gaps and challenges that still exist related to vivax malaria.

Vivax malaria

In 2021, 247 million people contracted malaria, and 619,000 cases progressed to death.[21] Among the seven species that can cause human malaria, *P. falciparum* is responsible for the most severe clinical outcomes and fatalities, occurring mainly on the African continent.[3] However, *P. vivax* is the most distributed etiologic agent in densely populated regions and is the most prevalent specie in Latin America and Southeast Asia.[21] The mortality and lethality of *P. vivax* infection have been neglected and underestimated.[4] Critical clinical complications, including placental and cerebral malaria, thrombocytopenia, severe anemia, and acute respiratory syndrome have begun to be reported in recent years,[22–26] changing the view of *P. vivax* as a "benign" parasite that causes milder disease.

The decline in *P. falciparum* cases has brought an increase in *P. vivax* cases, especially affecting young children.[21] Chemotherapeutic treatment for malaria is already defined and recommended by the World Health Organization (WHO). However, parasites have acquired resistance to antimalarial drugs, despite the use of combination therapy in areas of high transmission.[21,27,28] Therefore, the development of new vaccine targets and effective drugs to combat malaria is increasingly necessary.[27–29] In addition, vivax malaria has re-emerged in regions considered malaria free.[21,30,31]

Among the *Plasmodium* species, *P. vivax* is most closely related to *P. cynomolgi*, the species responsible for infection in Asian monkeys.[12] *P. vivax* shows unique characteristics that distinguish it from *P. falciparum*, which can be explained by the distinct evolutionary pathway. These characteristics include the formation of hypnozoites that remain in the liver in a latent state, causing recurrent infections; the exclusivity for reticulocytes as host cells in the blood; and the appearance of sexual forms early in the infection, even before clinical symptoms, providing a rapid spread of the parasite in nature.[32–34]

P. vivax is known to deform infected erythrocytes (iRBCs),[35] and more recently, the cytoadhesion of iRBCs to the endothelium has been reported, primarily observed in lung and brain endothelial cells and placental tissue.[36] *Ex vivo* adhesion assays showed a positive correlation between the percentage of schizonts and cytoadhesion compared to other asexual stages.[37] Adhesion protects the parasite from destruction, as nonadherent infected mature erythrocytes are cleared by the spleen.[38,39] Host receptors are expressed in different organs and amounts by various endothelial cells, ensuring successful sequestration by the parasite.[40] It is known that the interaction of *P. vivax* with the host occurs through the glycophorin C receptor present in erythrocytes. However, the parasitic ligand in this interaction is unknown.[6]

The *Plasmodium* Interspersed Repeat (*pir*) multigene gene family is one of the largest identified gene families and is shared between simian malaria species, such as *P. vivax*, and rodents.[12] Some of the *pir* genes encode proteins expressed on the surface of infected erythrocytes. It is postulated that some of them may be involved in the host immune response, antigenic variation, and immune evasion, as well as having adhesion and signaling functions.[41] Among the *pir* subfamilies are the *vir* genes, which encode 20% of the total *P. vivax* genome[42] and codifying the VIR proteins. It is speculated that VIR proteins are mainly involved in cytoadherence in *P. vivax*.[38]

RNA-seq tools for *P. vivax* transcriptomics

The scRNA-seq data provide little information about the isoforms and alternative splicing of the parasite, since only a small portion of each transcript is sequenced and usually derived from 3′-end mRNA molecules, making it difficult to read genes from organisms with incomplete UTR annotations.[13,43] The iso-Seq can characterize gene isoforms and

their UTR using long read technology.[13] Hazzard et al.[13] showed that methodological combinations allowed us to robustly characterize the regulation of *P. vivax* gene expression, predicting complete and stage-specific transcripts. UTR variations were identified throughout the intraerythrocytic and sporozoite life cycle, with different stages using distinct sites to initiate transcription of the same gene. It is postulated that mRNA stability and translation efficiency may occur due to variations in the length of the 3′-UTR and the presence of introns in these regions.[13]

Bourgard et al.[43] outlined an RNA-seq methodology with *P. vivax* low parasitaemia isolates, combining parasite maturation and enrichment with efficient RNA extraction. The transcriptome results, although unbiased, showed a heterogeneous expression profile among the isolates, with four transcribed genes remaining functionally uncharacterized. The analysis revealed conserved proteins from the parasite's metabolism, membrane proteins and exported proteins. When compared with the *P. falciparum* transcriptome, several homologous genes were expressed, observing a common expression profile in trophozoites.[43]

In addition to scRNA-seq, an alternative method called gene expression deconvolution has emerged as a statistical approach to determine the cellular composition using bulk RNA-seq. This approach has the advantage of leveraging information from hundreds of genes and gene expression profiles obtained from reference cells in scRNA-seq data.[14] Tebben et al.[14] evaluated the performance of CIBERSORTx in gene expression deconvolution with *in vivo* and *in vitro* samples. The software provided information based on a reference scRNA-seq data set of *Plasmodium* spp. to estimate the proportion of each blood stage form present in the sample by bulk RNA-seq, generating a robust and in-depth characterization of the transcriptome.[14] The scRNA-seq data from the same *Plasmodium* spp. can reliably deconvolute bulk RNA-seq data and is species independent. This new methodology allows the analysis of numerous blood samples without the need for parasite culture, is sensitive to changes in the regulation of specific genes and is inexpensive.[14] However, it is important to be aware of biased estimates and to interpret the data carefully because they are dependent on the reference populations used—differences in proportions between samples can bias the analysis and mask differences in gene regulation.[14]

To remedy functional information scarcity, gene co-expression networks of different *Plasmodium* species were created by optimizing transcriptomic data and incorporated into a database.[17] Malaria tools (www.malaria.tools) enable the identification and comparison of clusters and check specific gene expression profiles according to life stage and co-expression networks between species. The platform facilitates the selection of relevant genes in functional characterization and is involved in biological processes, in addition to indicating potential drugs to be developed.[17]

At the genus level, the Malaria Cell Atlas (www.sanger.ac.uk/science/tools/mca/mca/) covers individual transcriptomic profiling of all *Plasmodium* spp. morphological stages from scRNA-seq analyses.[44] The platform is open and emerges as a reference for understanding developmental transcriptional patterns, making it a valuable resource for the study of gene transcription regulation. The atlas aims to inform gene function and usage throughout the life cycle, understand the mechanisms of gene regulation implicit in changes during development, and provide a reference database that can be used to understand parasite biology and be extended to various species of *Plasmodium*.[44] In addition, the combination of scRNA-seq technology with the Malaria Cell Atlas allows the gene expression characterization of species that were previously inaccessible.[44]

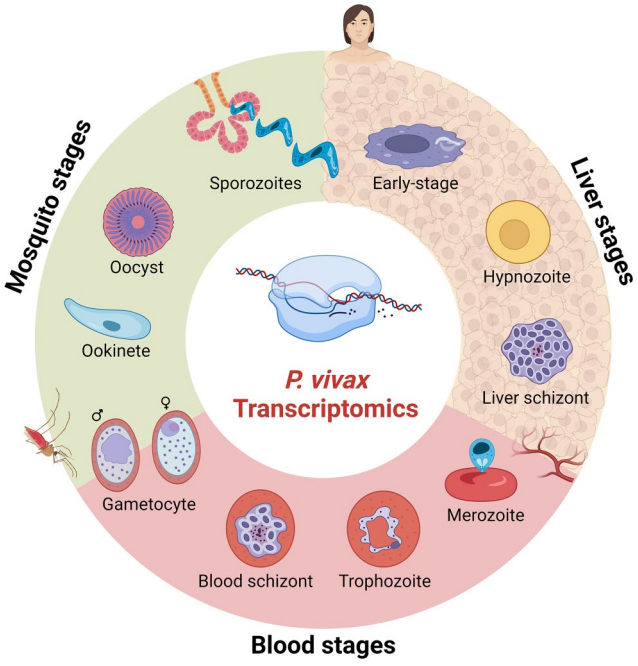

Figure 1. Transcriptomics through the *P. vivax* life cycle.
Different stages of the *P. vivax* life cycle highlighted in recent transcriptomics studies. The life stages which will be discussed in this review were didactically divided into liver stage (sporozoite, early stage, hypnozoite, and liver schizont) and blood stage (merozoite, trophozoite, blood schizont, and gametocyte), that happen inside of the vertebrate host; and the developmental stages into the mosquito (gametocyte, ookinete, oocyst, and sporozoite).

P. vivax biology still has several gaps regarding the development of the parasites. However, with advances in transcriptomics technologies, many questions have been elucidated. The findings of each parasite stage are highlighted according to new transcriptomic studies (Figure 1), which will be detailed below. Nevertheless, other issues remain elusive, and transcriptomics tools and their innovations can contribute to a better understanding into the *P. vivax* life cycle.

Unraveling transcriptomics of *P. vivax* liver stage

Liver stage infection is one of the key gaps concerning the *P. vivax* life cycle. Sporozoites are the transmissive forms of *Plasmodium* spp., initiating their development in *Anopheles* spp. salivary gland and finalizing with hepatocyte invasion in the vertebrate host.[45] *P. vivax* can develop hypnozoite forms that remain in the liver in a latent state, causing recurrent infections.[33,34] To date, the aspects concerning the development of sporozoites into liver schizonts or hypnozoite persistence and activation remain poorly understood.

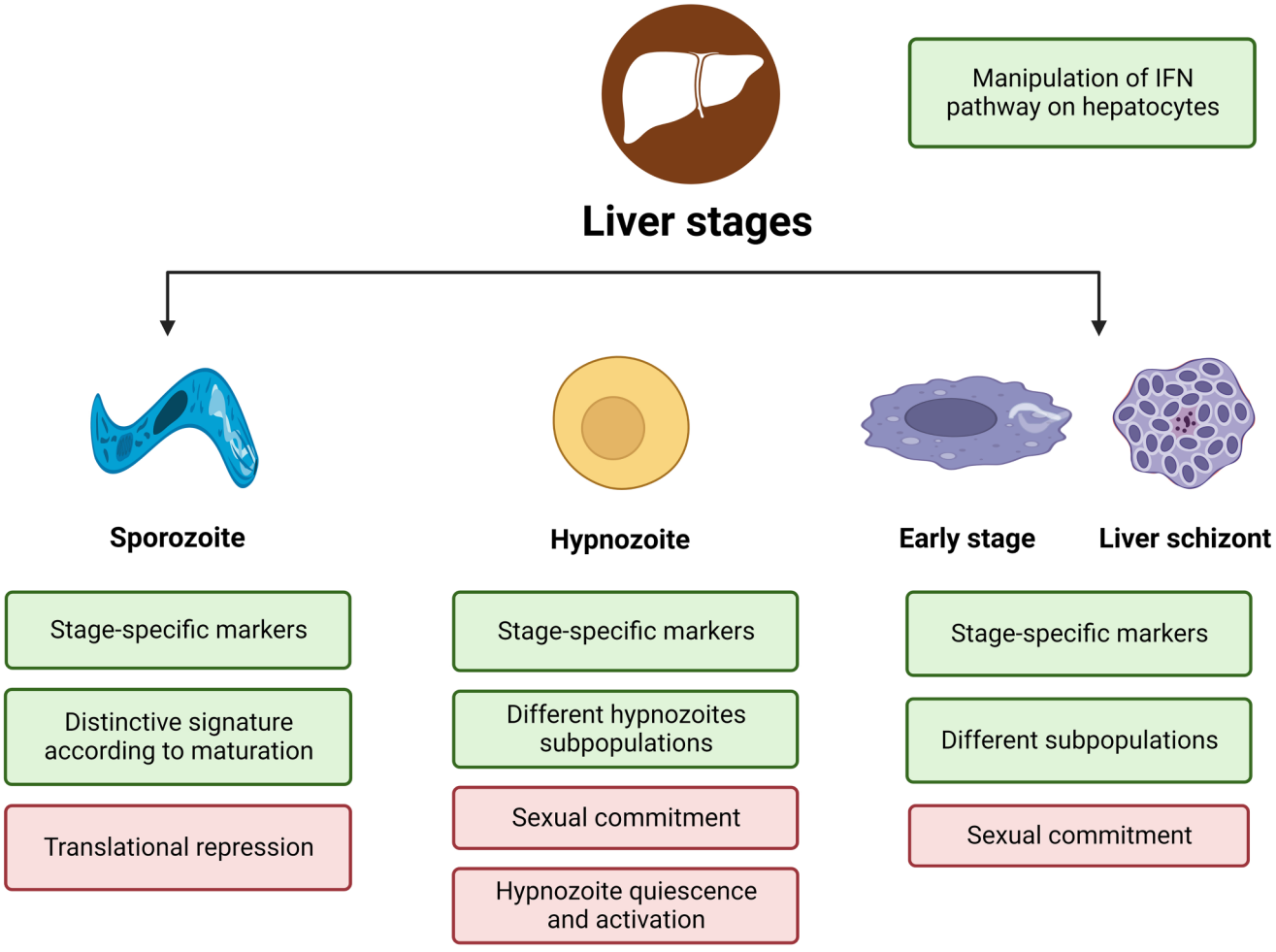

Figure 2. Transcriptomics of *P. vivax* liver stages.
Findings (green) and gaps (red) of the liver stage forms, divided into sporozoite, hypnozoite, early stage, and liver schizont in the vertebrate host, according to transcriptomic analyses.

The mosquito microenvironment is critical for sporozoite activation and for enhancing sporozoite infectivity to hepatocytes.[18] In addition, the mammalian microenvironment, in particular temperature and serum factors, is important for sporozoite transition into early liver stages.[18] Based on the transcriptomic data, we compiled some findings and gaps in the liver stage of the parasite life cycle (Figure 2), detailed as follows.

P. vivax sporozoite transcripts isolated from the mosquito salivary gland showed high transcriptomic heterogeneity with distinct markers that could group sporozoite subpopulations according to their maturation stage compared with annotated *P. falciparum* data.[46] The sporozoite-specific protein S10 (PVP01_0304200) is a marker for midgut/recently invaded sporozoites.[46] The genes that encode circumsporozoite protein: *csp* (PVP01_0835600), early transcribed membrane protein: *uis*4 (PVP01_0602100), thrombospondin-related anonymous protein (TRAP-like: PVP01_1132600) and sporozoite surface protein essential for liver stage development: *speld* (PVP01_0938800) are markers of salivary gland sporozoites.[46] The last group is represented by genes involved in proton transport (PVP01_0317600 and PVP01_1117400), redox response (PVP01_0835700 and PVP01_1249700), and heat shock proteins (PVP01_1440500 and PVP01_1011500), characterizing the activated sporozoites.[46] Altogether, *P. vivax* transcripts distinguish *P. falciparum* in genes associated with translational regulation and repression.[46]

A distinct signature between sporozoites and blood stage was observed, evidencing various stage-specific markers.[46,47] Comparing the scRNA-seq data obtained from sporozoite salivary glands with publicly available scRNA-seq data from the *P. vivax* blood stage, 208 differentially transcribed genes were found, among which 59 displayed sporozoite-specific transcription.[46] The sporozoite-specific markers were genes related to sporozoite development, maturation, and host infection (PVP01_1413500, PVP01_0835700, PVP01_0938800, PVP01_1212300, PVP01_1258000, PVP01_0611700, PVP01_0835600).[46] Only 8% of the genes were present in both sporozoite and erythrocyte forms (PVP01_1460700, PVP01_0710400, PVP01_0317600), but with higher transcriptional abundance in sporozoite.[46] Another study identified 1672 up- and 1958 downregulated genes in sporozoites in relation to blood stages.[47] Among them are genes related to RNA recognition motifs (RRM-1 and RRM-6), helicase domains (Helicase-C and DEAD box helicases), *puf*2 and *sap1* (PVP01_0947600), *alba*2, *alba*4, HoMu

(homolog of Musashi), PTB (polypyrimidine tract binding protein), nucleic acid binding domains such as bromodomains (PF00439), zinc fingers (PF13923) and EF hand domains (PF13499), a putative ApiAP2 transcription factor (PVP01_1211900), a homologue of the *Drosophila* zinc-binding protein "Yippee" (PVP01_0724100), thrombospondin-1 like repeats (TSR: PF00090) and von Willebrand factor type A domains (PF00092).[47]

In brief, *csp, etramps, celtos, gest, spect, siap*-1 genes as well as *alba*1, *alba*4 and *puf*2 genes, associated with translational repression, represented more than one-third of all RNA-seq transcripts in the salivary gland sporozoites.[47] However, many of them were not found by proteomic analysis, indicating that many transcribed genes in early infection were not translated to protein immediately, which supports the role of translational repression.[47] How this process might exactly regulate *P. vivax* liver stages development is still unclear; however, it is probably related to the regulation of the dormant stage.

Differential RNA-seq analysis revealed differences between *P. vivax* sporozoites, mixed liver stages and hypnozoites.[47] The sporozoite stage was enriched for Pfam domains related to the control and regulation of transcriptional, translational and chromatin levels, while the liver stages were enriched for domains related to replication, protein turn-over, protein export, membrane protein, metabolic activity, and merozoite formation.[47] Hypnozoites showed enrichment for gene domains associated with metabolic processes and mRNA/tRNA regulation and turnover.[47] The upregulated genes in hypnozoites were *uis*4, *speld*, and *puf*1 (PVP01_1015000). While *ap*2*s* (PVP01_0916300) was upregulated in hypnozoite compared with liver stages, it was also among the sporozoite transcripts and enriched relative to the blood stages.[47] These results suggested that hypnozoite is a transition point between sporozoites and schizonts, since it has several transcripts that are characteristic of sporozoites but also contains transcripts of key regulatory pathways and shares common genes with active schizonts.[47]

Analysis of two established platforms (micropatterned cocultures [MPCC] and Seq-Well S)[3] revealed host- and stage-dependent gene signatures in parasites and hepatocytes.[48] Mancio-Silva et al.[48] clustered different populations of *P. vivax* liver stages. The early stage is characterized by the expression of conserved genes with unknown function and residual genes specific to sporozoites related to gliding activity and cytoskeleton organization processes (PVP01_1262900, PVP01_1111400, PVP01_1301600, PVP01_0110400, PVP01_0941700). The core mid-stage has expression of well-known genes, such as liver-specific protein 1 (LISP1) and 2 (LISP2), and housekeeping function genes, such as translation (*eif*3*c*) and metabolism (*eno* and *ldh*).[48] Hypnozoite populations can be subclustered into different subpopulations according to their activation state, but in general, they are characterized by the expression of *puf*1 and genes encoding proteins with peptidase activity, such as *vivapain-1* and -2.[48] Finally, the late stage expresses genes encoding proteins that are essential for red blood cell invasion, such as merozoite surface protein (MSP), serine repeat antigen (SERA), rhoptry-associated membrane antigen (RAMA) and rhoptry neck (RON).[48]

A distinct cell group that co-expresses merozoite- and gametocyte-specific genes such as the tryptophan-rich protein family (TRAG), the gametocyte antigen G27/25, the gamete release protein (GAMER) and the homolog of gametocyte exported protein 5 (PHISTc)[48] was identified, in addition to these classical populations. Notably, the apicomplexan AP2 family of transcription factors was found to be highly expressed throughout the liver stages developmental phase.[48] Furthermore, genes specific to sexual stages (*pvs*16, *pvs*28, *pvs*25, *rubv*1, and *g*377) were found throughout the development of liver stages in various life forms.[48]

DAFT-seq (directional, amplification-free transcriptome sequencing) analysis characterized the heterogeneity between *P. vivax* schizont transcripts collected and purified from four Cambodian patient isolates.[49] The most highly expressed genes were related to host–parasite interactions, such as MSPs and early transcribed membrane proteins (ETRAMPs).[49] The most variably expressed genes belong to multigene families (*msp*, *phist*, *vir*, and *trag*), which are associated with immune evasion, antigenic variation, and virulence.[49] In addition, the genes involved in host cell recognition have differential expression profiles, such as reticulocyte binding proteins (RBPs), TRAGs, and Duffy Binding Protein (DBP),[49] which is a huge challenge for vaccine development.

While schizonts have an increase in gene expression associated with metabolic processes, proteasome subunits and protein processing, hypnozoites exhibit an increase in genes encoding histones, translation, transcription, and RNA regulatory mechanisms, including RNA-binding proteins.[50] The authors suggest that these proteins involved in cellular fating and translational repression could be related to hypnozoite quiescence.[50] Likewise, Mancio-Silva et al.[48] suggested that the dormant stage may depend on proteolytic activity and intracellular metabolites for long-lasting viability. The transcription signature of hypnozoites indicated that this is a stage with active metabolism and protein turnover.[47]

Interestingly, some liver stage subpopulations reveal early sexual commitment expressing gametocyte-specific genes, such as the transcription factor AP2-G, which is important for gametocyte differentiation during the blood stage.[48] Subpopulations of hypnozoites also express APG2-G, which could represent a reservoir of future gametocytes.[48] Consistent with this observation, Ruberto et al.[50] found the expression of sexual markers such as *pvs*25 (PVP01_0616100) and *g*377 (PVP01_1467200) in a subset of hypnozoites and *pvs*16 (PVP01_0305600) in schizonts, corroborating this hypothesis. Furthermore, Ruberto et al.[50] showed that hypnozoite transcripts reveal a variation between individual hypnozoites that can be clustered into three subpopulations, representing persisting, early activating, and late activating.

During early infection in human hepatocytes, there is strong activation of innate immune defense genes, including inflammation and stress response markers such as *LRG1, TNFSF10, NFKB1, SAA-1, SAA-2*, and *SAA-4*.[48] Throughout the infection, dysregulation of interferon alpha/beta (IFNα/β) and inflammatory signaling pathways in *P. vivax*-infected hepatocytes was noted,[48] indicating that successful *P. vivax* infection probably involves mechanisms to subvert host IFNα/β responses.[15,48] Overall, there was an

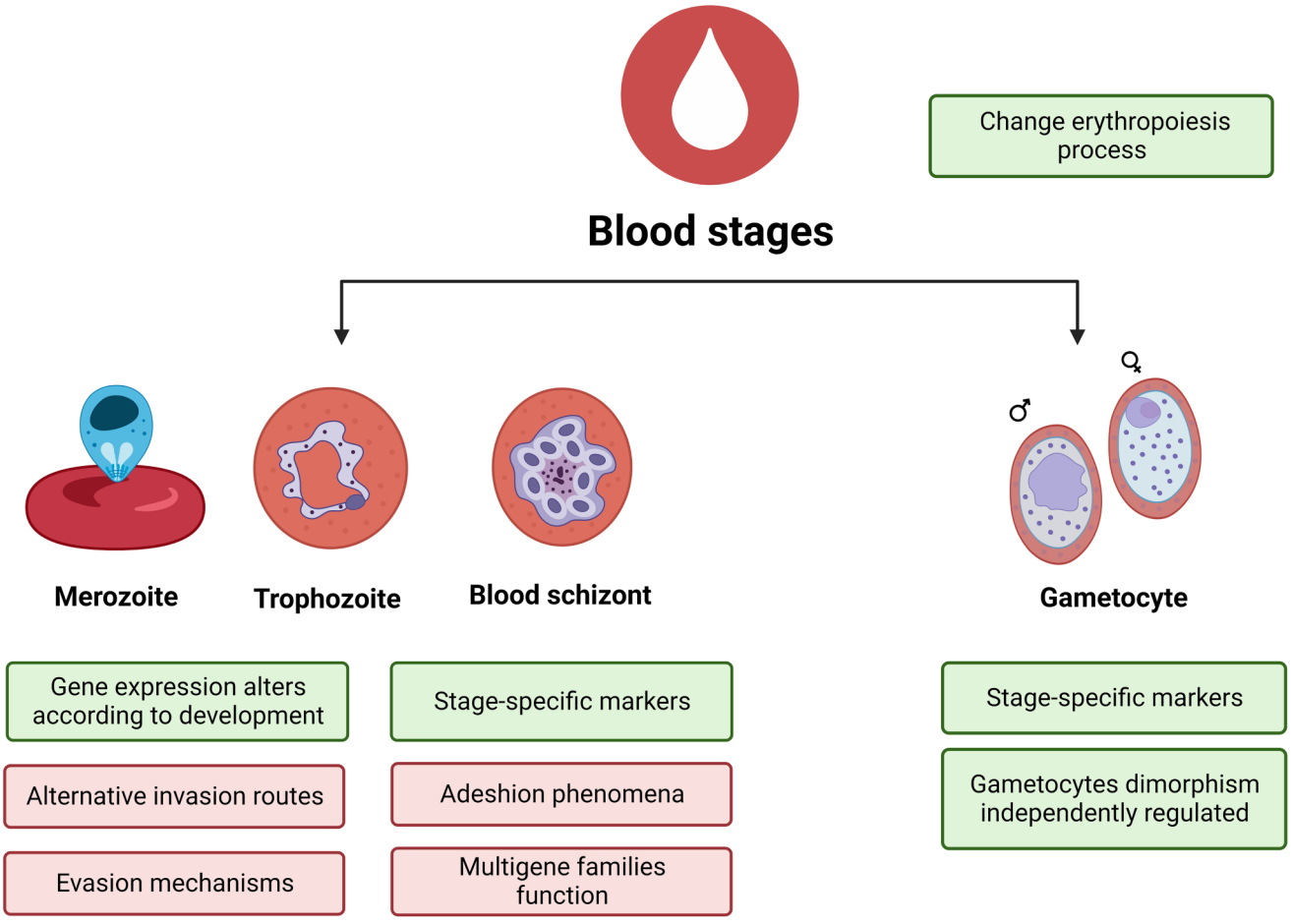

Figure 3. Transcriptomics of *P. vivax* blood stages.
Findings (green) and gaps (red) of the blood stage forms, divided into merozoite, trophozoite, blood schizont, and gametocyte in the vertebrate host, according to transcriptomic analyses.

upregulation of genes associated with energy metabolism and a downregulation of genes associated with IFN signaling and pathways related to the immune response.[48,50]

Elucidating the *P. vivax* blood stage transcripts

The infective liver stage form undergoes several modifications during the hepatic cycle, becoming a merozoite that invades the bloodstream and infects healthy erythrocytes, initiating asexual reproduction in looping.[45] The parasite progresses from the ring stage to the trophozoite stage and ultimately forms a schizont from which merozoites are released into the circulation upon infected erythrocyte disruption.[15] During each cycle, a small population of parasites produce sexual progeny that leave the peripheral circulation and enter the extravascular space of the bone marrow, where they mature into gametocytes inside of vertebrate host. Once mature, the male and female gametocytes return to the peripheral circulation.[45]

Recent advancements in transcriptomics have allowed for greater insight into the natural heterogeneity of blood stage infections and transcriptional regulation in *Plasmodium* species.[15,37,51] Despite the blood stage being the main stage recognized by the human host, the heterogeneity and antigenic variation of the *P. vivax* blood forms is one of the major difficulties faced by the immune system.[11,19] Supported by that, we show what is known and what is still open about this internship by evaluating the transcriptomic data (Figure 3).

One of the major challenges in the study of *P. vivax* blood stage is to clarify how the reticulocyte invasion process takes place. To date, little is known about this invasion process. Individuals who have a deletion in the band 3 gene and therefore a lack of band 3 protein on red blood cells, characterized by the Southeast Asian ovalocytosis (SAO) phenotype, have a lower incidence of vivax malaria. Because of this, band 3 on the surface of erythrocytes has been reported as an invading receptor.[51] De Meulenaere et al.[51] identified new candidates for band 3 ligands using differential expression analysis of *P. vivax* isolates. mRNA-seq from *P. vivax* schizonts revealed high transcriptional variability of multigene families (*phist*, *pir*, *pvtrag*) related to invasion, with considerable representation of *pvtrag* genes, especially *pvtrag*38.[51] Genes coding for PvTrag22, PvTRAG36 and PvGAMA also bound to band 3 *in vitro*, but only the last one was included in the ligand candidate list from differential expression analysis. The candidate list also highlights other invasion-related genes, such as *pvrbp*2a, *rpb*2c, *pvdbp*, *pvdbp*2, and *pvcyrpa*.[51]

P. vivax may also invade reticulocytes by interacting with Duffy antigen. However, Duffy-negative individuals, such as Africans, can be infected by *P. vivax*. Recent studies have identified potential alternative invasion routes, seeing that Duffy-negative individuals can also be infected by the parasite.[52] To identify potential ligands, Gunalan et al.[53] used RNA-seq data to compare the *P. vivax* transcriptome profile in *Saimiri* and *Aotus* monkey infections, as *Saimiri* lacks the DBP receptor and should use alternative receptors.[53] Genes belonging to the tryptophan-rich antigen (*Pv-fam-a*) and *msp* gene families, mainly *msp*-3 but also *msp*-1, *msp*-7, and *msp*-9, were more highly expressed in *Saimiri* infections than in *Aotus*.[53] These findings were also corroborated by Sá et al.[15] Interestingly, the *vir* genes on the erythrocyte surface were expressed at higher levels in *Aotus* than in *Saimiri* monkeys.[53] This finding suggests a splenectomy effect on the expression of these genes. In fact, Fernandez-Becerra et al.[38] showed that some *vir* genes are spleen-dependent and encode antigens associated with cytoadhesion.

During blood stage infection, multiple developmental stages are present, each with a unique profile of transcripts.[15] scRNA-seq analysis has revealed distinct gene expression patterns among these populations, with some expressing genes associated with transcription and translation, such as 60S ribosomal protein L34 and apical membrane antigen-1 (*ama*-1); expressing genes that encode invasion proteins, such as *msp3* (PVP01_1031100); and others expressing genes specific to male gametes, such as *mget* (PVP01_1236900) and *msp*-7 like (PVP01_1219900) or female gametocytes, such as *pvs*25.[15] Moreover, most of these genes have variable expression during the intraerythrocytic cycle and are restricted to a specific developmental stage.

Kim et al.[19] found different expression signatures of gametocytes with RNA-seq, clustering them into two subpopulations. One of them, with more expression of microtubule-associated genes, represented genes expressed in male gametocytes, such as male gamete fusion factor HAP2 (PVP01_0814300), *pvs*16 and gamete *egress and sporozoite traversal: gest (*PVP01_1258000), while genes associated with intracellular trafficking and histone remodeling were overexpressed in the second cluster, corresponding to female gametocyte genes, such as *pvs*25, CPW-WPC family protein (PVP01_0820000) and gametocyte-associated protein (PVP01_1403000).[19] These findings indicate that the proportion of male to female gametocytes varies among infections and imply that male and female gametocytogenesis are independently regulated in human host.[19] Corroborating these data, gametocyte-associated protein or the sexual antigen *pvs*16 was mostly found in asexual parasites by Sá et al.[15] Regarding gametocyte differentiation transcription, some genes were noted only in the intermediate population between the asexual parasites and the completely differentiated females, such as *gest*.[15] Meanwhile, male development gene 1 (PVP01_1435300) was detectable in male and female differentiating gametocytes.[15]

Transcriptomics studies can predict the expression profile of potential vaccine candidates and elucidate their important characteristics. Due to its cell surface position and salience in parasite–host interactions, MSP-7 is considered a promising vaccine candidate.[54] Cheng et al.[54] analyzed *msp*-7 gene expression in the blood of ten patients infected with *P. vivax* using RNA-seq data. MSP-7 family members have a different expression profile during blood infections: some members are expressed during the intraerythrocytic stage (*pvmsp-7A*, *pvmsp-7F*), while others are only expressed on the schizont (*pvmsp-7C*, *pvmsp-7H*, *pvmsp-7I*), reflecting developmental regulation within the gene family.[54] The authors' conclusions indicate that PvMSP-7A is the most immunogenic antigen, in addition to being expressed during blood infection, making it the best choice among the vaccine candidates evaluated.[54] These characteristics need to be considered during vaccine development, as they could impact the host's immune response.

In an RNA-seq study with 26 Cambodian individuals infected with *P. vivax*, a predominance of trophozoite transcripts was found, suggesting that this stage is more transcriptionally active in the blood than the others.[19] After chloroquine treatment, a large decrease in total parasite RNA was observed; however, the overall gene expression pattern was not significantly altered. Interestingly, many of the genes considerably downregulated after treatment encoded exported protein (*phist*), *pir* genes and numerous genes involved in erythrocyte invasion (*ama-1*, *dbp*, *msp*-5, *rbp2a*, *rbp2e*, or *rbp*3).[19]

P. vivax and its adhesion to host cells

P. vivax typically exhibits low parasitaemia in the peripheral blood, suggesting that the spleen and bone marrow may be utilized for parasite sequestration.[20] The hematopoietic niche1 is host to several infectious agents due to its nutrient-rich environment and anti-inflammatory and immunoprotected state. In addition, it is a place of blood cell productive capacity, which might harbor pathogens.[55,56] Parasite development is closely linked to erythropoiesis, the process of red blood cell production and maturation that occurs in the bone marrow parenchyma.[57] Sequencing and transcriptomic analysis of RNA extracted from patient bone marrow cells aspirated during acute infections demonstrated the presence of parasites and insufficient formation of red blood cells, altering the erythropoiesis process.[20] The RNA-seq of some patients indicated downregulated erythroid maturation genes (*nfe, tal1, arid3a*, and *gata)* and enzymes involved in heme biosynthesis (*ALAS1* and *ALAS2)*, together with upregulated immune response genes (TNF and TNF receptor superfamily 1A, 10A, and 10B) and complement activation proteins (Annexin A2 and A5), triggering erythropoiesis inhibition and phagocytosis of these cells.[20]

The development of chronic infections in *P. vivax* is associated with virulence factors linked to *vir* genes because of their immunogenic and immunovariant properties.[39] The *vir* genes are exclusive to *P. vivax* and share homologous sequences with other species of *Plasmodium* spp..[58] Studies have shown that several *vir* genes are related to the pathogenesis of vivax malaria, since gene transcription is increased in patients with severe malaria compared to non-severe cases.[59] However, the role of VIR proteins remains unclear, despite increasing research in recent years. In a global transcriptional analysis of parasites that grew in the presence or absence of the spleen in a nonhuman primate model, 67 spleen-dependent genes

were identified by Fernandez-Becerra[38] based on microarray analyses. *P. falciparum* transgenic lines were used to express two *P. vivax*-selected proteins belonging to the VIR and Pv-FAM-D multigene variant families. VIR14 protein was shown to be cytoadherent to human splenic fibroblasts.[38] Recombinant protein expression has shown that spleen-dependent antigens are immunogenic in natural infections and associated with clinical protection.[38] The author suggests that the parasite remains cytoadhered in the spleen to avoid splenic clearance, using adhesion as an immune evasion mechanism.[38]

In addition to cytoadhesion, rosette formation is another adhesion phenomenon common in *P. vivax* infection and occurs more frequently than in *P. falciparum* malaria. However, there is currently no observed correlation between rosetting and disease severity.[6] The phenomenon involves the interaction between iRBCs adhered to two or more healthy erythrocytes (RBCs). Albrecht et al.[60] demonstrated that *P. vivax* rosettes are dependent on plasma factors and that the plasma of *P. vivax*-infected patients enhanced rosetting rates. Rosette frequency correlated with total IgM but not with IgG levels.[60] It has been postulated that IgM acts as a bridge between infected and uninfected cells, stabilizing *P. falciparum* rosetting[61] and limiting opsonization and phagocytosis by masking IgG epitopes during their binding to iRBCs.[62] Despite this, the role of this immunoglobulin in adherence phenotypes has not been elucidated in vivax malaria. Albrecht et al.[60] identified differentially expressed genes related to the human phagocytosis pathway in RNA-seq analyses of *P. vivax* patients with distinct rosetting capacity. The identified genes included the immunoglobulin kappa constant (IGKC), immunoglobulin heavy constant gamma 1 (IGHG1) and actin-related protein 2/3 complex subunit 2 (ARPC20).[60]

Aiming to elucidate parasite–host interactions, Vallejo et al.[63] compared scRNA-Seq data from whole blood of controlled human malaria infection (CHMI) from healthy volunteers and exposed to vivax malaria to understand the responses induced at immune levels. Research has shown activation of innate immunity, antigen presentation and activation of the complement system during initial exposure to the parasite. However, dendritic cells mediate strong immunosuppression by inducing indoleamine 2,3-dioxygenase 1 (IDO1) and lymphocyte activation gene 3 (LAG3), and there is a depletion of neutrophil receptors.[63] Thus, the inflammatory response was attenuated in those exposed to malaria, with a decrease in the presentation of class II antigens in dendritic cells. The authors highlighted that the control of immunosuppression can be used for new formulations of vaccines against malaria, overcoming the immune evasion of the parasite.[63]

P. vivax mosquito stage in a transcriptomic view

During feeding, female and male gametocytes are ingested by mosquitoes and quickly develop into gametes. Inside the midgut, the male gametocyte divides into flagellated microgametes, while the female gametocyte develops into a single macrogamete. Zygote formation occurs with the fertilization of a macrogamete by a microgamete. The zygote undergoes meiosis and develops into an ookinete, which penetrates the mosquito gut wall. The ookinete forms an oocyst where the parasite reproduces asexually, forming several thousand sporozoites. With oocyst rupture, sporozoites migrate to the salivary glands, where they can be transmitted back to the vertebrate host.[45] *Anopheles–Plasmodium* interactions have also been explored by transcriptomics studies, which have also solved questions and raised new issues to be investigated (Figure 4).

The gametocytes in blood circulation can be taken up during a new *Anopheles* repast. Unsurprisingly, *P. vivax* infected and uninfected mosquitoes present a differential gene expression profile, which is altered during the infection. Boonkaew et al.[64] analyzed *P. vivax* infected *Anopheles dirus* at the ookinete and oocyst stages and identified different biological pathways regulated following the infection using RNA-seq. In *Anopheles dirus*, the most notably changed genes were associated with protein binding, integral components of the membrane and proteolysis. In *P. vivax*, transcripts were clustered as either those found only in ookinetes (*etramps*, *pv-fam*-a and putative DNA-directed RNA polymerase II), only in oocysts (histone 2B, putative 2-Cys peroxiredoxin, putative hydrolase, putative histone H3 and H4) or in both stages with the most significant upregulation (small subunit rRNAs, putative disulfide isomerase, putative ribonucleoside-diphosphate reductase large chain, and putative histone H2A) and the most significant downregulation (small subunit rRNA, putative aspartic protease PM4, putative DEAD/DEAH box helicase and putative FtsJ-like methyltransferase).[64]

Various biological processes were identified from *Anopheles* genes, indicating an activation of mosquito defense.[64] The data corroborate Roth et al.,[18] who used RNA-seq to demonstrate that genes related to the immune response were highly induced, as well as antimicrobial proteins (AMPs) such as gambicin, defensin, cecropin, attacin, and leucine-rich immune protein (LRIM1). Moreover, analysis of *Anopheles aquasalis* midgut transcripts showed that the mosquito midgut epithelium activates an autophagic response during invasion, with the majority of differentially expressed genes (*beclin*, *dram*, and *apg8)* being associated with this process, as measured by qRT-PCR from RNA-seq data.[65] In addition, the mosquito autophagic response contributes to reducing *P. vivax* infection.[65]

Interestingly, *P. vivax* activated several pathways to overcome the mosquito response, which were revealed by vesicle-mediated transport gene expression, such as multidrug resistance-associated protein 2 (MRP2) and phosphatidylinositol-4-phosphate 5-kinase (PIPK5).[64] Furthermore, RNA-seq analyses showed that the parasite may suppress the midgut microbial population early, altering iron metabolism and nutritional physiology, aiming to weaken host immunity and facilitate invasion and development in the mosquito.[66] In addition, it has been suggested that *P. vivax* overcomes mosquito tissue-specific responses by manipulating their own molecular architecture and expression of common salivary transcripts (ST1) to facilitate their survival.[67]

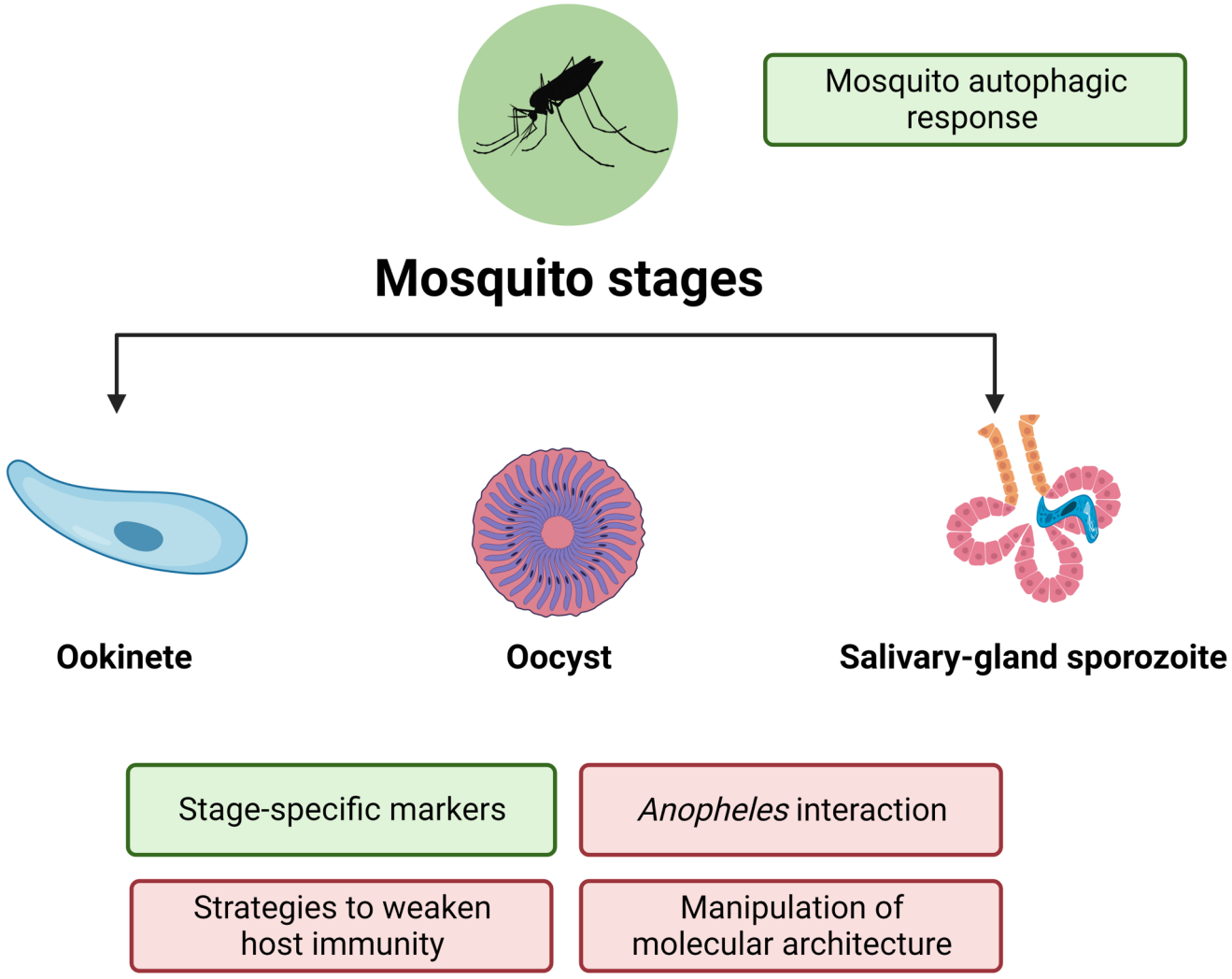

Figure 4. Transcriptomics of *P. vivax* mosquito stages.
Findings (green) and gaps (red) of the mosquito stages forms, divided into ookinetes, oocysts, and salivary glands in the mosquito, according to transcriptomic analyses.

A better understanding of all these mechanisms could aid in the design and development of new molecular strategies against vivax malaria.

Challenges in vivax malaria transcriptomics

P. vivax transcriptomics studies face several challenges from its biology to its parasite–host interaction. Some of these challenges include RNA quantities and quality, heterogeneous life cycle stages plus limited access to samples, high genetic diversity, and technical limitations. Because of low parasite density in the bloodstream, *P. vivax* transcriptomic analyses may be hampered by the difficulty in obtaining enough RNA.[16,43] In addition, false positives or false negatives in data analysis can be created in the case of host cell contamination.[20] To remedy this, new RNA extraction methodologies are being improved to achieve quality material with higher yields.[43]

In addition, *P. vivax* has a complex life cycle.[45] The correct identification of genes expressed in the various life forms is difficult and depends on isolation and individual analysis.[15] Large genetic diversity is also observed in *P. vivax*.[48] Identifying and comparing differentially expressed genes between distinct populations is an arduous task.[12] This can make it difficult to identify and validate candidate genes that are involved in parasite biology or in host–parasite interactions. Finally, transcriptomic studies need specialized techniques and equipment, such as RNA sequencing and bioinformatics analysis.[1] Coupled with this, if the RNA quality and quantity obtained from *P. vivax* samples were unsatisfactory, data analysis became complicated.[16] The lack of protocols to standardize methodologies used in transcriptomics studies makes the comparison of results between studies challenging, as well as the identification of gene expression patterns.[13]

Some specifics blank within each stage can be featured. In the liver stage, this fact could be exemplified by the notorious existence of genes associated with translational repression in salivary gland sporozoites.[47] Liver stage subpopulations and hypnozoites present gametocyte-specific gene expression, revealing early sexual commitment;[48,50]

and hypnozoite quiescence is linked to cellular fating proteins and translational repression.[50] During the blood stage, the evasion mechanisms of the immune system and receptors involved in adhesive phenomena are unclear;[38,60] gene expression analyses have revealed many representatives of multigenic families with unknown function;[39,41,42] and despite the existence of some receptor–ligand interactions known and responsible for erythrocyte invasion, there must be other routes used by the parasite.[51,53] In the mosquito stage, there are gaps related to the parasite-vector interaction;[18,64] activation of a defense response with expression of different genes involved in biological processes;[64,65] survival eased by its own molecular architecture changes to circumvent the specific tissue responses of mosquito;[67] and strategies to weaken the vector host immune system and enable its development.[66] Despite these challenges, improvements in RNA sequencing technology and the generation of new tools to study *P. vivax* gene expression have led to significant advances in understanding parasite biology.

Based on the papers included in this review, some genes specifically expressed in different developmental stages of the *P. vivax* life cycle have identified. In the liver stage, *csp*, *puf*2, and *alba*4 stood out in sporozoite forms;[46,47] *puf*1 in hypnozoite forms;[47,48] *lisp*-1, *lisp*-2, and housekeeping genes (*eif3c*, *eno*, and *ldh*) in liver schizont forms.[48] In the blood stage, *ama*-1, *msp*-3, and *msp*-7 stand out as markers for asexual blood forms;[15,54] *pvs*25 for female gametocytes and *pvs*16 for male gametocytes.[15,19] In the mosquito stage, *etramps* and *pv-fam*-a were cited for ookinete forms, and histone 2B was cited for oocyst forms.[64]

Conclusions

Transcriptome studies have provided new insights into the knowledge of *P. vivax* biology, including host–parasite interactions; the journey of sporozoites to the liver and their transition to the early liver stages; the activation/dormancy profiles of hypnozoites; the genetic signature of different stages and host cells; distinct markers for the different phases; the understanding of parasite heterogeneity; the proposition of new molecules involved in the reticulocyte invasion process; the ability to adhere and form rosettes, as well as the contribution of these phenotypes to the severity and evasion of the immune system; the ability of gametocytes to differentiate, the development of sexual stages within mosquitoes and the mechanism developed by parasites to avoid mosquito responses.

In this review, we divided the achievements from transcriptomic analyses according to the three major life cycle stages of *P. vivax*: liver, blood, and mosquito stages. We aimed to highlight the main findings and update the remaining gaps. Despite these advances, there are still several challenges and issues that need to be solved, mainly related to the quantity and quality of RNAs obtained and the technical limitations, the heterogeneity of the life cycle, and the high genetic diversity of the parasites. In summary, all findings obtained by transcriptomic analysis can significantly contribute to the development of new drugs and vaccines and facilitate the understanding of vivax malaria pathogenesis.

AUTHORS' CONTRIBUTIONS

All authors wrote, read, and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

We thank Carlos Chagas Institute (Fiocruz Paraná) for all support and to FGF and NC for the invitation.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: JWF and GTSV were supported by CAPES with a Master fellowship.

ORCID IDS

Julia Weber Ferraboli https://orcid.org/0009-0001-8652-9997

Gisele Tatiane Soares da Veiga https://orcid.org/0000-0003-1434-0922

Letusa Albrecht https://orcid.org/0000-0001-6406-2057

REFERENCES

1. Metzker ML. Sequencing technologies the next generation. *Nat Rev Genet* 2010;**11**:31–46
2. Hemingway J, Shretta R, Wells TN, Bell D, Djimdé AA, Achee N, Qi G. Tools and strategies for malaria control and elimination: what do we need to achieve a grand convergence in malaria? *PLoS Biol* 2016;**14**:e1002380
3. Weiss DJ, Lucas TCD, Nguyen M, Nandi AK, Bisanzio D, Battle KE, Cameron E, Twohig KA, Pfeffer DA, Rozier JA, Gibson HS, Rao PC, Casey D, Bertozzi-Villa A, Collins EL, Dalrymple U, Gray N, Harris JR, Howes RE, Kang SY, Keddie SH, May D, Rumisha S, Thorn MP, Barber R, Fullman N, Huynh CK, Kulikoff X, Kutz MJ, Lopez AD, Mokdad AH, Naghavi M, Nguyen G, Shackelford KA, Vos T, Wang H, Smith DL, Lim SS, Murray CJL, Bhatt S, Hay SI, Gething PW. Mapping the global prevalence, incidence, and mortality of *Plasmodium* falciparum, 2000–17: a spatial and temporal modelling study. *Lancet* 2019;**394**:322–31
4. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, George DB, Horby P, Wertheim HF, Price RN, Müeller I, Baird JK, Hay SI. A long neglected world malaria map: *Plasmodium* vivax endemicity in 2010. *PLoS Negl Trop Dis* 2012;**6**:e1814
5. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, del Portillo HA. Key gaps in the knowledge of *Plasmodium* vivax, a neglected human malaria parasite. *Lancet Infect Dis* 2009;**9**:555–66
6. Lee W-C, Malleret B, Lau Y-L, Mauduit M, Fong M-Y, Cho JS, Suwanarusk R, Zhang R, Albrecht L, Costa FTM, Preiser P, McGready R, Renia L, Nosten F, Russell B. Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes. *Blood* 2014;**123**:e100–9
7. Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, Russell B, Ginsburg H, Nosten F, Day NPJ, White NJ, Carlton JM, Preiser PR. The transcriptome of *Plasmodium* vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proc Natl Acad Sci* 2008;**105**:16290–5
8. Westenberger SJ, McClean CM, Chattopadhyay R, Dharia NV, Carlton JM, Barnwell JW, Collins WE, Hoffman SL, Zhou Y, Vinetz JM, Winzeler EA. A systems-based analysis of *Plasmodium* vivax lifecycle transcription from human to mosquito. *PLoS Negl Trop Dis* 2010;**4**:e653

9. Boopathi PA, Subudhi AK, Garg S, Middha S, Acharya J, Pakalapati D, Saxena V, Aiyaz M, Chand B, Mugasimangalam RC, Kochar SK, Sirohi P, Kochar DK, Das A. Revealing natural antisense transcripts from *Plasmodium* vivax isolates: evidence of genome regulation in complicated malaria. *Infect Genet Evol* 2013;**20**:428–43
10. Boopathi PA, Subudhi AK, Garg S, Middha S, Acharya J, Pakalapati D, Saxena V, Aiyaz M, Chand B, Mugasimangalam RC, Kochar SK, Sirohi P, Kochar DK, Das A. Dataset of natural antisense transcripts in *P. vivax* clinical isolates derived using custom designed strand-specific microarray. *Genom Data* 2014;**2**:199–201
11. Kim A, Popovici J, Vantaux A, Samreth R, Bin S, Kim S, Roesch C, Liang L, Davies H, Felgner P, Herrera S, Arévalo-Herrera M, Ménard D, Serre D. Characterization of *P. vivax* blood stage transcriptomes from field isolates reveals similarities among infections and complex gene isoforms. *Sci Rep* 2017;**7**:7761
12. Bourgard C, Albrecht L, Kayano ACAV, Sunnerhagen P, Costa FTM. *Plasmodium* vivax biology: insights provided by genomics, transcriptomics and proteomics. *Front Cell Infect Microbiol* 2018;**8**:34
13. Hazzard B, Sá JM, Ellis AC, Pascini TV, Amin S, Wellems TE, Serre D. Long read single cell RNA sequencing reveals the isoform diversity of *Plasmodium* vivax transcripts. *PLoS Negl Trop Dis* 2022;**16**:e0010991
14. Tebben K, Dia A, Serre D. Determination of the stage composition of *plasmodium* infections from bulk gene expression data. *Msystems* 2022;**7**:e0025822
15. Sà JM, Cannon MV, Caleon RL, Wellems TE, Serre D. Single-cell transcription analysis of *Plasmodium* vivax blood-stage parasites identifies stage- and species-specific profiles of expression. *PLoS Biol* 2020;**18**:e3000711
16. Orjuela-Sánchez P, Sá JM, Brandi MC, Rodrigues PT, Bastos MS, Amaratunga C, Duong S, Fairhurst RM, Ferreira MU. Higher microsatellite diversity in *Plasmodium* vivax than in sympatric *Plasmodium* falciparum populations in Pursat, Western Cambodia. *Exp Parasitol* 2013;**134**:318–26
17. Tan QW, Mutwil M. Malaria.Tools—comparative genomic and transcriptomic database for *Plasmodium* species. *Nucleic Acids Res* 2020;**48**:D768–75
18. Roth A, Adapa SR, Zhang M, Liao X, Saxena V, Goffe R, Li S, Ubalee R, Saggu GS, Pala ZR, Garg S, Davidson S, Jiang RHY, Adams JH. Unraveling the *Plasmodium* vivax sporozoite transcriptional journey from mosquito vector to human host. *Sci Rep* 2018;**8**:12183
19. Kim A, Popovici J, Menard D, Serre D. *Plasmodium* vivax transcriptomes reveal stage-specific chloroquine response and differential regulation of male and female gametocytes. *Nat Commun* 2019;**10**:371
20. Brito MAM, Baro B, Raiol TC, Ayllon-Hermida A, Safe IP, Deroost K, Figueiredo EFG, Costa AG, Armengol M, del P, Sumoy L, Almeida ACG, Hounkpe BW, De Paula EV, Fernandez-Becerra C, Monteiro WM, del Portillo HA, Lacerda MVG. Morphological and transcriptional changes in human bone marrow during natural *Plasmodium* vivax malaria infections. *J Infect Dis* 2022;**225**:1274–83
21. World Health Organization. World malaria report 2022. https://www.who.int/teams/global-malaria-programme
22. Aashish A, Manigandan G. Complicated vivax malaria, an often underestimated condition—case report. *J Family Community Med* 2015;**22**:180–2
23. Naing C, Whittaker MA, Nyunt Wai V, Mak JW. Is *Plasmodium* vivax malaria a severe malaria?: a systematic review and meta-analysis. *PLoS Negl Trop Dis* 2014;**8**:e3071
24. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. *Malar J* 2014;**13**:481
25. Costa FT, Lopes SC, Albrecht L, Ataíde R, Siqueira AM, Souza RM, Russell B, Renia L, Marinho CR, Lacerda MV. On the pathogenesis of *Plasmodium* vivax malaria: perspectives from the Brazilian field. *Int J Parasitol* 2012;**42**:1099–105
26. Tanwar GS, Khatri PC, Sengar GS, Kochar A, Kochar SK, Middha S, Tanwar G, Khatri N, Pakalapati D, Garg S, Das A, Kochar DK. Clinical profiles of 13 children with *Plasmodium* vivax cerebral malaria. *Ann Trop Paediatr* 2011;**31**:351–6
27. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant *Plasmodium* vivax: a systematic review and meta-analysis. *Lancet Infect Dis* 2014;**14**:982–91
28. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, Lampah DA, Price RN. Multidrug-resistant *plasmodium* vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med* 2008;**5**:e128
29. Veiga GTS, da Moriggi MR, Vettorazzi JF, Müller-Santos M, Albrecht L. *Plasmodium* vivax vaccine: what is the best way to go? *Front Immunol*. 2023;**13**:910236
30. Nasir SMI, Amarasekara S, Wickremasinghe R, Fernando D, Udagama P. Prevention of re-establishment of malaria: historical perspective and future prospects. *Malar J* 2020;**19**:452
31. Carrasco-Escobar G, Qquellon J, Villa D, Cava R, Llanos-Cuentas A, Benmarhnia T. Time-varying effects of meteorological variables on malaria epidemiology in the context of interrupted control efforts in the Amazon Rainforest, 2000–2017. *Front Med* 2021;**8**:721515
32. Kitchen SF, Boyd MF. On the infectiousness of patients infected with *Plasmodium* Vivax and *plasmodium* falciparum. *Am J Trop Med Hyg* 1937; s1-17:253–62
33. Krotoski WA, Collins WE, Bray RS, Garnham PC, Cogswell FB, Gwadz RW, Killick-Kendrick R, Wolf R, Sinden R, Koontz LC, Stanfill PS. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium* vivax infection. *Am J Trop Med Hyg* 1982;**31**:1291–3
34. Baird JK. Neglect of *Plasmodium* vivax malaria. *Trends Parasitol* 2007;**23**:533–9
35. Handayani S, Chiu DT, Tjitra E, Kuo JS, Lampah D, Kenangalem E, Renia L, Snounou G, Price RN, Anstey NM, Russell B. High deformability of *Plasmodium* vivax –infected red blood cells under microfluidic conditions. *J Infect Dis* 2009;**199**:445–50
36. Carvalho BO, Lopes SCP, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, Mamoni R, Leite JA, Rodrigues MM, Soares IS, Oliveira TR, Wunderlich G, Lacerda MVG, del Portillo HA, Araújo MOG, Russell B, Suwanarusk R, Snounou G, Rénia L, Costa FTM. On the cytoadhesion of *Plasmodium* vivax –infected erythrocytes. *J Infect Dis* 2010;**202**:638–47
37. Lopes SCP, Albrecht L, Carvalho BO, Siqueira AM, Thomson-Luque R, Nogueira PA, Fernandez-Becerra C, del Portillo HA, Russell BM, Rénia L, Lacerda MVG, Costa FTM. Paucity of *Plasmodium* vivax mature schizonts in peripheral blood is associated with their increased cytoadhesive potential. *J Infect Dis* 2014;**209**:1403–7
38. Fernandez-Becerra C, Bernabeu M, Castellanos A, Correa BR, Obadia T, Ramirez M, Rui E, Hentzschel F, López-Montañés M, Ayllon-Hermida A, Martin-Jaular L, Elizalde-Torrent A, Siba P, Vêncio RZ, Arevalo-Herrera M, Herrera S, Alonso PL, Mueller I, del Portillo HA. *Plasmodium* vivax spleen-dependent genes encode antigens associated with cytoadhesion and clinical protection. *Proc Natl Acad Sci* 2020;**117**:13056–65
39. del Portillo HA, Lanzer M, Rodriguez-Malaga S, Zavala F, Fernandez-Becerra C. Variant genes and the spleen in *Plasmodium* vivax malaria. *Int J Parasitol* 2004;**34**:1547–54
40. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. *Nature* 2002;**415**:673–9
41. Cunningham D, Lawton J, Jarra W, Preiser P, Langhorne J. The pir multigene family of *Plasmodium:* antigenic variation and beyond. *Mol Biochem Parasitol* 2010;**170**:65–73
42. del Portillo HA, Fernandez-Becerra C, Bowman S, Oliver K, Preuss M, Sanchez CP, Schneider NK, Villalobos JM, Rajandream M-A, Harris D, da Silva LHP, Barrell B, Lanzer M. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium* vivax. *Nature* 2001;**410**:839–42
43. Bourgard C, Lopes SCP, Lacerda MVG, Albrecht L, Costa FTM. A suitable RNA preparation methodology for whole transcriptome shotgun sequencing harvested from *Plasmodium* vivax-infected patients. *Sci Rep* 2021;**11**:5089
44. Howick VM, Russell AJC, Andrews T, Heaton H, Reid AJ, Natarajan K, Butungi H, Metcalf T, Verzier LH, Rayner JC, Berriman M, Herren JK, Billker O, Hemberg M, Talman AM, Lawniczak MKN. The malaria cell

atlas: single parasite transcriptomes across the complete *Plasmodium* life cycle. *Science* 2019;**365**:eaaw2619
45. Nilsson SK, Childs LM, Buckee C, Marti M. Targeting human transmission biology for malaria elimination. *PLoS Pathog* 2015;**11**:e1004871
46. Ruberto AA, Bourke C, Vantaux A, Maher SP, Jex A, Witkowski B, Snounou G, Mueller I. Single-cell RNA sequencing of *Plasmodium* vivax sporozoites reveals stage- and species-specific transcriptomic signatures. *PLoS Negl Trop Dis* 2022;**16**:e0010633
47. Vivax Sporozoite Consortium. Transcriptome and histone epigenome of *Plasmodium* vivax salivary-gland sporozoites point to tight regulatory control and mechanisms for liver-stage differentiation in relapsing malaria. *Int J Parasitol* 2019;**49**:501–13
48. Mancio-Silva L, Gural N, Real E, Wadsworth MH, Butty VL, March S, Nerurkar N, Hughes TK, Roobsoong W, Fleming HE, Whittaker CA, Levine SS, Sattabongkot J, Shalek AK, Bhatia SN. A single-cell liver atlas of *Plasmodium* vivax infection. *Cell Host Microbe* 2022;**30**:1048–10605
49. Siegel SV, Chappell L, Hostetler JB, Amaratunga C, Suon S, Böhme U, Berriman M, Fairhurst RM, Rayner JC. Analysis of *Plasmodium* vivax schizont transcriptomes from field isolates reveals heterogeneity of expression of genes involved in host-parasite interactions. *Sci Rep* 2020;**10**:16667
50. Ruberto AA, Maher SP, Vantaux A, Joyner CJ, Bourke C, Balan B, Jex A, Mueller I, Witkowski B, Kyle DE. Single-cell RNA profiling of *Plasmodium* vivax-infected hepatocytes reveals parasite- and host- specific transcriptomic signatures and therapeutic targets. *Front Cell Infect Microbiol* 2022;**12**:986314
51. De Meulenaere K, Prajapati SK, Villasis E, Cuypers B, Kattenberg JH, Kasian B, Laman M, Robinson LJ, Gamboa D, Laukens K, Rosanas-Urgell A. Band 3–mediated *Plasmodium* vivax invasion is associated with transcriptional variation in PvTRAg genes. *Front Cell Infect Microbiol* 2022;**12**:1011692
52. Kepple D, Pestana K, Tomida J, Abebe A, Golassa L, Lo E. Alternative invasion mechanisms and host immune response to *Plasmodium* vivax malaria: trends and future directions. *Microorganisms* 2020;**9**:15
53. Gunalan K, Sá JM, Moraes Barros RR, Anzick SL, Caleon RL, Mershon JP, Kanakabandi K, Paneru M, Virtaneva K, Martens C, Barnwell JW, Ribeiro JM, Miller LH. Transcriptome profiling of *Plasmodium* vivax in Saimiri monkeys identifies potential ligands for invasion. *Proc Natl Acad Sci* 2019;**116**:7053–61
54. Cheng CW, Jongwutiwes S, Putaporntip C, Jackson AP. Clinical expression and antigenic profiles of a *Plasmodium* vivax vaccine candidate: merozoite surface protein 7 (PvMSP-7). *Malar J* 2019;**18**:197
55. Nothelfer K, Sansonetti PJ, Phalipon A. Pathogen manipulation of B cells: the best defence is a good offence. *Nat Rev Microbiol* 2015;**13**:173–84
56. Venugopal K, Hentzschel F, Valkiūnas G, Marti M. *Plasmodium* asexual growth and sexual development in the haematopoietic niche of the host. *Nat Rev Microbiol* 2020;**18**:177–89
57. Lee RS, Waters AP, Brewer JM. A cryptic cycle in haematopoietic niches promotes initiation of malaria transmission and evasion of chemotherapy. *Nat Commun* 2018;**9**:1689
58. Lopez FJ, Bernabeu M, Fernandez-Becerra C, del Portillo HA. A new computational approach redefines the subtelomeric vir superfamily of *Plasmodium* vivax. *BMC Genomics* 2013;**14**:8
59. Gupta P, Sharma R, Chandra J, Kumar V, Singh R, Pande V, Singh V. Clinical manifestations and molecular mechanisms in the changing paradigm of vivax malaria in India. *Infect Genet Evol* 2016;**39**:317–24
60. Albrecht L, Lopes SCP, da Silva ABIE Barbosa V, Almeida RP, Siqueira AM, Leite JA, Bittencourt NC, dos Santos HG, Bourgard C, Garcia LFC, Kayano ACAV, Soares IS, Russell B, Rénia L, Lacerda MVG, Costa FTM. Rosettes integrity protects *Plasmodium* vivax of being phagocytized. *Sci Rep* 2020;**10**:16706
61. Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. Novel fibrillar structure confers adhesive property to malaria–infected erythrocytes. *Nat Med* 1996;**2**:204–8
62. Barfod L, Dalgaard MB, Pleman ST, Ofori MF, Pleass RJ, Hviid L. Evasion of immunity to *Plasmodium* falciparum malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. *Proc Natl Acad Sci* 2011;**108**:12485–90
63. Vallejo AF, Read RC, Arevalo-Herrera M, Herrera S, Elliott T, Polak ME. Malaria systems immunology: *Plasmodium* vivax induces tolerance during primary infection through dysregulation of neutrophils and dendritic cells. *J Infect* 2018;**77**:440–7
64. Boonkaew T, Mongkol W, Prasert S, Paochan P, Yoneda S, Nguitragool W, Kumpitak C, Sattabongkot J, Kubera A. Transcriptome analysis of Anopheles dirus and *Plasmodium* vivax at ookinete and oocyst stages. *Acta Trop* 2020;**207**:105502
65. Santana RAG, Oliveira MC, Cabral I, Junior RCAS de Sousa DRT, Ferreira L, Lacerda MVG, Monteiro WM, Abrantes P, Guerra M, das GVB, Silveira H. Anopheles aquasalis transcriptome reveals autophagic responses to *Plasmodium* vivax midgut invasion. *Parasit Vectors* 2019;**12**:261
66. Sharma P, Rani J, Chauhan C, Kumari S, Tevatiya S, Das De T, Savargaonkar D, Pandey KC, Dixit R. Altered gut microbiota and immunity defines *Plasmodium* vivax survival in Anopheles stephensi. *Front Immunol* 2020;**11**:609
67. Kumari S, Chauhan C, Tevatiya S, Singla D, De TD, Sharma P, Thomas T, Rani J, Savargaonkar D, Pandey KC, Pande V, Dixit R. Genetic changes of *Plasmodium* vivax tempers host tissue-specific responses in Anopheles stephensi. *Curr Res Immunol* 2021;**2**:12–22