RESEARCH ARTICLE

Novel insights from the Plasmodium falciparum sporozoite-specific proteome by probabilistic integration of 26 studies

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Abstract

Plasmodium species, the causative agent of malaria, have a complex life cycle involving two hosts. The sporozoite life stage is characterized by an extended phase in the mosquito salivary glands followed by free movement and rapid invasion of hepatocytes in the human host. This transmission stage has been the subject of many transcriptomics and proteomics studies and is also targeted by the most advanced malaria vaccine. We applied Bayesian data integration to determine which proteins are not only present in sporozoites but are also specific to that stage. Transcriptomic and proteomic Plasmodium data sets from 26 studies were weighted for how representative they are for sporozoites, based on a carefully assembled gold standard for Plasmodium falciparum (Pf) proteins known to be present or absent during the sporozoite life stage. Of 5418 Pf genes for which expression data were available at the RNA level or at the protein level, 975 were identified as enriched in sporozoites and 90 specific to them. We show that Pf sporozoites are enriched for proteins involved in type II fatty acid synthesis in the apicoplast and GPI anchor synthesis, but otherwise appear metabolically relatively inactive in the salivary glands of mosquitoes. Newly annotated hypothetical sporozoite-specific and sporozoite-enriched proteins highlight sporozoite-specific functions. They include PF3D7_0104100 that we identified to be homologous to the prominin family, which in human has been related to a quiescent state of cancer cells. We document high levels of genetic variability for sporozoite proteins, specifically for sporozoite-specific proteins that elicit antibodies in the human host. Nevertheless, we can identify nine relatively well-conserved sporozoite proteins that elicit antibodies and that together can serve as markers for previous exposure. Our understanding of sporozoite biology benefits from identifying key pathways that are enriched during this life stage. This work can guide studies of molecular mechanisms underlying sporozoite biology and potential well-conserved targets for marker and drug development.
Author summary

When a person is bitten by an infectious malaria mosquito, sporozoites are injected into the skin with mosquito saliva. These sporozoites then travel to the liver, invade hepatocytes and multiply before the onset of the symptom-causing blood stage of malaria. By integrating published data, we contrast sporozoite protein expression with other life stages to filter out the unique features of sporozoites that help us understand this stage. We used a “guideline” that we derived from the literature on individual proteins so that we knew which proteins should be present or absent at the sporozoite stage, allowing us to weigh 26 data sets for their relevance to sporozoites. Among the newly discovered sporozoite-specific genes are candidates for fatty acid synthesis while others might play a role keeping the sporozoites in an inactive state in the mosquito salivary glands. Furthermore, we show that most sporozoite-specific proteins are genetically more variable than non-sporozoite proteins. We identify a set of conserved sporozoite proteins against which antibodies can serve as markers of recent exposure to sporozoites or that can serve as vaccine candidates. Our predictions of sporozoite-specific proteins and the assignment of previously unknown functions give new insights into the biology of this life stage.

Introduction

Malaria is a mosquito transmittable disease resulting in over 220 million clinical cases and half a million deaths annually. Most deaths are caused by *Plasmodium falciparum* (*Pf*), one of the five species of *Plasmodium* that can infect humans. The infection begins with the deposition of liver-infective sporozoite forms in the skin by blood-feeding mosquitoes. These sporozoites travel to the liver where they invade, differentiate and multiply asymptomatically inside hepatocytes for approximately a week before releasing red blood cell (RBC)-infective merozoites into the circulation. The subsequent asexual multiplication, rupture and re-invasion of the parasites into circulating RBCs cause the symptoms associated with malaria.

Identifying specific sporozoite proteins will aid in the understanding the biology of this highly motile stage in which the parasite is directly exposed to the body’s immune system, similar to blood stage-infective merozoites. Sporozoites deposited in the skin can take up to 90 minutes to reach the liver [1], a much longer potential exposure to immune system than the 90 seconds of merozoites. Mosquito midgut-derived and circulating sporozoites are less infectious for hepatocytes than those that have resided in the salivary gland [2,3]. The ability to improve and remain infective in the salivary gland of mosquitoes for an extended period of time (approximately 1 week) is an intriguing phenomenon that is poorly understood [4]. It is suggestive of a molecular landscape where the parasite is kept in low activity but can quickly activated to evade immune systems, invade and develop in hepatocytes. Understanding the specific molecular make-up of sporozoites will shed light on this aspect of its biology and may reveal new targets for interventions.

A number of studies have identified genes that are expressed in sporozoites during both their development in mosquito midgut (oocyst) and in the salivary gland. They have identified expression at the RNA level[5] and at the protein level[6] with a specific emphasis on surface proteins[7]. The studies varied in their focus and resolution, with RNA level studies facing the challenge of their relevance for protein expression[8] while proteomics studies face challenges in detecting low abundance proteins. More importantly, while such studies address the question what is present in the sporozoite, they do not address what is specific to it. Therefore, to
prioritize sporozoite specific proteins, we implemented a naïve Bayesian data integration in which both transcriptomics and proteomic data were included. This method has been applied before to identify transcripts and proteins that are specific to *P. falciparum*’s gametocyte life stage [9] and to detect genes involved in the cilium in eukaryotes [10]. For informed data integration, gold-standard lists representing proteins that are either sporozoite specific or non-sporozoite specific were manually assembled from the existing literature on individual proteins. Using this gold standard, published *Plasmodium* datasets were weighted by the presence of sporozoite specific proteins and the absence of proteins known to be absent from sporozoites, allowing us to obtain an extended list of predicted sporozoite specific proteins. We classified 90 proteins as sporozoite-specific, of which 67 were not part of the gold standard.

“Conserved, hypothetical proteins with unknown functions” were examined using sensitive homology and orthology detection tools [11,12] to predict their function and shed light on the biology of sporozoites.

From the proteins that in the assembled proteomics data were identified to be present in sporozoites, we examined whether we can identify a limited set of proteins for which human antibodies are generated [13] and that can serve as or markers of exposure to sporozoites. As attractive targets would be conserved between different *P. falciparum* strains, we sequenced three *P. falciparum* strains, NF54, NF135 and NF166, and required selected proteins to have limited genetic diversity between those strains as well as in sequenced genomes in PlasmoDB [14].

Methods

Sporozoite protein and transcriptomic data sets

The data integration was performed using transcriptomic and proteomic data sets from 22 studies describing all life cycle stages of *P. falciparum* (S1 Table). Data sets were obtained from PlasmoDB version 43 [14], and literature (S1 Table). In addition to that, transcriptomics data on the liver and blood stage of *Plasmodium cynomolgi* and the sporozoites of *Plasmodium vivax* were included, as well as proteomics studies covering the sporozoite stage of *P. vivax* and the liver stage of *P. yoelii*, respectively, resulting in a total of 48 data samples from 26 different studies (S1 Table). Data from non-*falciparum* studies were converted into *P. falciparum* IDs using the orthologs lists available on PlasmoDB, favouring orthologs that are syntenic in the case of multiple options.

Gold standards

Bayesian data integration requires gold standards, in this case of proteins known to be either highly enriched in sporozoites or depleted from them. We required positive gold standard proteins to be present dominantly in sporozoites based on western blot or immunofluorescent assay data, resulting in a selection of 31 sporozoite-specific proteins (Table 1).

The negative gold standard was curated by searching literature for non-sporozoite proteins. We selected 19 proteins based on their literary evidence of absence from sporozoites. Additionally, 20 gametocyte specific proteins [41] were added to the negative gold standard, increasing the total number to 39 negative gold standard proteins (S2 Table). These proteins spanned the remaining *P. falciparum* life cycle stages in the human host, with the majority being found in (a)sexual blood stages.

Bayesian data integration

The used data sets were examined for their correlations with each other (S1 Fig). By and large most data sets show little correlation. We did leave the few correlated data sets in to keep the
Proteomic data were converted into unique peptide counts for each protein identified and transcriptomic data were converted into expression percentiles for a total of 5668 *P. falciparum* gene IDs. Proteomic and transcriptomic data was binned consistently for all data sets, with 0, 1, or >1 identified unique peptides, or into four bins, containing transcripts that are in the percentile > 80, the “80 > percentile > 60”, the “60 > percentile > 40” and the 40 > percentile, respectively. The data sets were then weighted according to their presence of gold standard proteins and their absence of negative gold standard proteins. Each bin in each data set was given a log2 score based on the likelihood of it containing sporozoite gold standard proteins and the likelihood of it containing non-sporozoite proteins, according to the right side of Eq (1), where B = present in bin, S = sporozoite specific and nonS = not sporozoite

Table 1. Positive gold standard members, based on the presence of proteins in western blot or immunofluorescent assay data.

| Gene ID   | Name                                                                 | Evidence                        |
|-----------|----------------------------------------------------------------------|---------------------------------|
| PF3D7_0104000 | Thrombospondin-related sporozoite protein                 | Labaied et al.[15] |
| PF3D7_0107600 | Eukaryotic translation initiation factor 2-α kinase 2, putative | Matuschewski et al.[16] |
| PF3D7_0207300 | Serine repeat antigen 8                                         | Arisue et al.[17] |
| PF3D7_0304000 | Inner membrane complex protein 1a, putative                 | Khater et al.[18] |
| PF3D7_0304600 | Circumsporozoite (CS) protein                                  | Singh et al.[19] |
| PF3D7_0408600 | Sporozoite invasion-associated protein 1                      | Arevalo-Pinzon et al.[20] |
| PF3D7_0408700 | Perforin-like- protein 1                                       | Yang et al.[21] |
| PF3D7_0417100 | mRNA-binding protein PUF2                                      | Gomes-Santos et al.[22] |
| PF3D7_0511400 | Unknown function protein                                        | Schlarmann et al.[23] |
| PF3D7_0517600 | F-actin-capping protein subunit β, putative                  | Ganter et al.[24] |
| PF3D7_0615100 | Enoyl-acyl carrier reductase                                    | Vaughan et al.[25] |
| PF3D7_0626300 | 3-oxoacyl-acyl-carrier protein synthase I/II                   | Vaughan et al.[25] |
| PF3D7_0702300 | Sporozoite threonine and asparagine-rich protein               | Fidock et al.[26] |
| PF3D7_0809100 | Erythrocyte membrane protein 1                                  | Zanghi et al.[27] |
| PF3D7_0812300 | Sporozoite surface protein 3, putative                        | Harupa et al.[28] |
| PF3D7_0816500 | Small heat shock protein HSP20, putative                       | Montagna et al.[29] |
| PF3D7_0822700 | Thrombospondin-related protein 1, putative                    | Klug et al.[30] |
| PF3D7_0830300 | Sporozoite invasion-related protein 2                          | Siau et al.[31] |
| PF3D7_1137800 | Sporozoite surface protein essential for liver stage development | Al-Nihmi et al.[32] |
| PF3D7_1138200 | Unknown function protein                                        | Schlarmann et al.[33] |
| PF3D7_1147000 | Sporozoite and liver stage asparagine-rich protein             | Silvie et al.[34] |
| PF3D7_1201300 | Liver stage associated protein 1                               | Siau et al.[31] |
| PF3D7_1207300 | LIMP protein, putative                                         | Santos et al.[35] |
| PF3D7_1208200 | Cysteine repeat modular protein 3                              | Douradinha et al.[36] |
| PF3D7_1216600 | Cell traversal protein for ookineteis and sporozoites          | Bergmann-Leitner et al.[37] |
| PF3D7_1221400 | Inner membrane complex protein 1b, putative                   | Tremp et al.[38] |
| PF3D7_1302200 | Protein UIS3                                                   | Matuschewski et al.[16] |
| PF3D7_1335900 | Thrombospondin-related anonymous protein                      | Ejigiri et al.[39] |
| PF3D7_1342500 | Sporozoite protein essential for cell traversal                | Yang et al.[21] |
| PF3D7_1442600 | TRAP-like protein                                               | Steinbuechel et al.[40] |
| PF3D7_1475400 | Cysteine repeat modular protein 4                              | Douradinha et al.[36] |

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specific. The Bayesian score for an individual protein is then the sum of the scores for the bins in which it occurs (one bin per data set).

\[
\log_2 \left( \frac{P(S|B)}{P(\text{non } S|B)} \right) = \sum^N_i \log_2 \left( \frac{P(B_i|S)}{P(B_i|\text{non } S)} \right)
\]  

(1)

As the expected number of sporozoite-specific proteins was unknown, no prior was included, rather we used cutoffs based on the position of known sporozoite specific proteins to define sporozoite specific proteins and sporozoite enriched proteins.

We calculated a false discovery rate, corrected for the prior probability that a protein is enriched in sporozoites using Eq 2, see ref [10] for details. Here specificity and sensitivity are based on positions of the gold standard proteins in a 5 fold cross validation.

\[
cFDR = \frac{1 - \text{specificity}}{1 - \text{specificity} + \text{sensitivity} \cdot Q_{\text{prior}}}
\]  

(2)

**Overrepresentation of function categories in sporozoite proteins**

GO terms were acquired from PlasmoDB and formatted into a .gmt file according to the format specified by the GSEA server at the Broad Institute[42]. GSEA Preranked on the ranked list of sporozoite-specific proteins was used with the conservative "preranked" option in the "classic mode", i.e. using Kolmogorov Smirnoff statistics to determine enriched GO terms. The large variable protein families RIFIN, STEVOR and PfEMP1 (var genes) were left out of the analysis.

A new GO term for gliding motility in *Plasmodium* was assembled by searching literature for proteins associated with gliding motility in sporozoites, ookinetes and merozoites. For this we extended the list of glideosome associated proteins assembled by Lindner *et al.*[43] with 10 new proteins possibly associated with the glideosome. These proteins were either annotated with the motility GO term GO:0071976 (SSP3, CelTOS, LIMP protein, plasmepsin VII and glideosome-associated connector), or otherwise known to be involved in gliding motility (CTRP [44], SIAP1[45], GAPDH[46], GAP40[47], IMC11[48]). Similarly we added a list of GPI-anchored proteins to the set of processes for which we examined enrichment using the list of Gilson *et al.*[49], based on the hypothesis that the type II fatty acid synthesis was required for the creation of GPI anchors [50]. To investigate Pfam domain enrichment in sporozoite proteins, Pfam annotations for all proteins were downloaded from PlasmoDB and were used as gene sets in the GSEA. To prevent the GSEA analysis results for enriched pathways to be affected by the large numbers of hypothetical proteins in *P. falciparum*, those were filtered out before the analysis. Furthermore, proteins that were part of the gold standard were left out of the analysis to prevent circular arguments and therewith overestimating the enrichment. For the enrichment of type II fatty acid synthesis we originally relied on the GO terms of PlasmoDB, which turned out to be significantly enriched in sporozoites (FDR = 0.004), but after manual examination, showed many discrepancies with the review by Shears *et al.*[50], and included a protein like PF3D7_0208500, a mitochondrial acyl carrier protein that is not in the apicoplast. We thus also used the protein list of Shears *et al.*[50] to examine enrichment of apicoplast FAS II in the sporozoites. Proteins for which no transcriptomic and proteomic data were available were left out as well. Manual examination of the Gene Set Enrichment Analysis results showed that some gene sets were significantly enriched in sporozoites only because they were depleted from the low scoring proteins. Therefore, we required for the significantly enriched processes in sporozoites that they actually contained proteins in the set of "sporozoite enriched proteins."
Sequencing of *P. falciparum* strains

NF54 originates from West Africa, and is isolated from a woman infected in the Netherlands near an airfield[51]. The NF135 clone is a clinical isolate that originates from Cambodia [52]. The NF166 clone is a clinical isolate from a child that visited Guinea[53]. Whole genome sequencing of the three strains was performed with Illumina NextSeq 500, resulting in raw paired-end fastq reads of 151 base pairs (bp).

Quality control and trimming

To examine the quality of the raw fastq reads, FastQC (version 0.11.5) was used[54]. The Nextera Transposase sequence contamination at the 3’ ends of the reads were trimmed off with a stringency of 12 bp, using Trim Galore (version 0.4.3)[55]. CleanNextSeq_paired was used to remove excess of G’s from 3’ ends of the reads after 100 bp[56]. Only reads with a minimal length of 35 base pairs were retained.

Alignment and variant calling

The *P. falciparum* 3D7 reference genome (v3.0, PlasmoDB, plasmodb.org, 14 chromosomes, mitochondrial genome and apicoplast genome) was indexed and the trimmed fastq reads were aligned to the reference genome using Bowtie2 (version 2.2.8) with the local alignment setting [57]. The SAM files obtained were converted to BAM files and subsequently sorted and indexed with SAMtools (version 1.4.1)[58]. SNPs and indels were called using SAMtools and BCFtools (version 1.4.1). Alignments were visually inspected with the Integrative Genomics Viewer (IGV, version 2.3.98)[58]. Coverage was calculated with BEDtools (version 2.26.0)[59].

Filtering of SNPs and indels

Filtering of SNPs and indels was performed with BCFtools (version 1.4.1)[58]. SNPs with a base Phred quality (Q) > 30 were used for further analysis. Furthermore, we required that the proportion of high quality bases (the DP4 scores in the VCF files) supporting the indel or the SNP in > 75% of the called bases to include them for further analysis.

Effect of SNPs and indels on protein sequences

The effect of the mutations on the predicted protein sequences was determined using the Genomic Ranges package in R [60]. Amino acid sequences of all proteins were compared to amino acid sequences of the reference genome and with each other. Any variation at the amino acid level between reference and the examined strain and among the examined strains was denoted.

Homology detection

Homology detection of *P. falciparum* proteins with unknown function was done using HHpred[11] with default settings (HHblits, 3 iterations). For orthology detection we used best bidirectional best hits at the level of sequence profiles[12].

Detecting a set of proteins that elicit antibodies in all chloroquine chemoprophylaxis (CPS) volunteers

We used a greedy “set cover” algorithm that in each step selects, from a list of evolutionary conserved sporozoite proteins (less than eight nonsynonymous SNPs per kb in PlasmoDB), the one that is immunogenic (elicits antibodies) in the highest numbers of volunteers subjected
to CPS for which no immunogenic protein was already in the set. In the analysis from Obiero et al. [13], some long proteins were split into separate peptides. Those were analyzed separately by the algorithm: i.e. as if they were separate proteins. When multiple proteins were immunogenic in the same number of volunteers for which no immunogenic protein had been selected yet, we chose from those the one that had the highest immunogenicity in all the volunteers.

We furthermore required genes to have maximally two non-synonymous SNPs in the combined NF135 and NF166 strains.

Results

Data integration

At least 26 studies, containing 48 data sets have been published of *P. falciparum*, *P. cynomolgi*, *P. vivax* and *P. yoelii* transcripts and proteins at various developmental stages, including 11 that were specific to the sporozoite stage (S1 Table). In order to optimally exploit those data to obtain sporozoite-enriched proteins we integrated them in a Bayesian manner (Methods). Integrating the data sets using the sets of 31 positive and 39 negative gold standard proteins (Tables 1 and S2) produced a list of all proteins in *P. falciparum* ranked according to their likelihood of being sporozoite-specific (S3 Table). The score distribution of the negative and positive gold standard proteins varied depending on using all available data sets, or proteomic or transcriptomic data separately (Fig 1). The Bayesian integration using only transcriptomic data sets resulted in a ranked list where 14 negative gold standard genes scored higher than the lowest scoring positive gold standard gene (Fig 1A). This overlap was lower when using only proteomic data sets (Fig 1B), but still contained 8 proteins. The least overlap between positive and negative gold standard members was observed when combining transcriptomic and proteomic data (Fig 1C). This also produced an outlier, STARP. Although this protein was identified as being a sporozoite protein [26], in our combined data sets it scored 42 times lower than the second to last scoring positive gold standard protein. It therewith does not appear specific to sporozoites in the available data. STARP was excluded from the score distribution of gold standard proteins that was used to define the sporozoite specific proteins (see below), but gold standard list and integration where not changed post hoc. Based on the observed overlaps, we decided to continue our research using the ranked list based on the combined proteomic and transcriptomic data sets.

Sporozoite specific proteins and sporozoite enriched proteins

Proteins were considered sporozoite-specific when ranking in the first quartile of the gold standard protein list i.e. scoring above 12.27 in the final integrated list of scores, which represents a factor of being at least $2^{12.37}$ to $2^{-5200}$ more likely to be specific to sporozoites than to any of the other stages. Do note that hereby we ignore the prior probability of a protein being sporozoite specific at all. Such a prior is standard in Bayesian data integration. Nevertheless, in this case a prior of e.g. 1/5, is given the high cut-off for sporozoite specific proteins that we used, not very relevant. As we did not consider STARP to be sporozoite specific (see above), we considered proteins that scored higher than the second to lowest positive gold standard protein (LIMP protein, score = -1.31) in the final integrated list of scores, to be enriched in sporozoites. Finally, the abundance of unique peptides by mass-spectrometry was assessed for each protein. Proteins were deemed present in sporozoites when identified in two independent studies or with more than 1 unique peptide in at least one study, rather than when having a higher score than a specific cut-off value. Our analysis thus identified 90 sporozoite-specific proteins, 975 sporozoite-enriched proteins (non-overlapping with the sporozoite specific proteins) and 2736 that were present in sporozoites (S3 Table). Out of the 90 sporozoite-specific proteins, 67 were
We validated our predictions by 5-fold cross validation (5-fold CV) by randomly skipping 1/5th of the gold standard proteins from the data integration and assessing their predicted sporozoite specificity based on the remaining data (Fig 1D). When we include a prior probability of 1/5 that a protein is enriched in sporozoites, the false discovery rate corrected for the prior (cFDR, Methods), is based on the ranking of gold standard proteins in the cross validation, 20% of the combined set of the sporozoite specific genes from data integration.
and the sporozoite enriched proteins. The high sensitivity and specificity indicated that novel sporozoite-specific proteins would also score higher than non-sporozoite specific proteins. We also compared the ranking of the gold standard proteins based on the integrated data with a ranking based on individual data sets of sporozoite RNAs and proteins. The cross validation separated the gold standard proteins better than individual data sets, supporting the integration of multiple data sets (Fig 1D).

**Function prediction of non-annotated proteins**

Many of the genes in the *P. falciparum* genome encode hypothetical proteins with unknown molecular function [61]. The fraction of unknowns that is specific for sporozoites (32 out of 90, 36%) is at least as high as in the rest of the genome (32%). To improve understanding of their potential functions, we examined the proteins for domains with known functions from any species using sensitive homology detection with HHpred [11], combined with manual examination of conserved residues. We compared sporozoite specific proteins with PFAM domains, the human proteome, and the proteome of *Toxoplasma gondii*, an apicomplexan related to *P. falciparum* that is present in the HHpred database. The sporozoite specific protein with the highest score that was not part of the gold standard, PF3D7_0104100, showed (barely) significant sequence similarity with the prominin family \((E = 10E^{-4})\) and low levels of sequence identity with e.g. human prominin-2 (12%). To cross-check the homology of PF3D7_0104100 with prominin we examined homology with *T. gondii* proteins. The sequence similarity between the *P. falciparum* protein and *T. gondii* TGME49_218910 \((E = 3.4e^{-44})\) and between the *T. gondii* protein and the human prominin-2 were highly significant \((E = 2.3e^{-21})\), indicating that PF3D7_0104100 is indeed member of the prominin family. PF3D7_0104100 has like the prominin family five (predicted) transmembrane regions with most of the protein localized outside the cell (Fig 2). Analysis of a sequence alignment with orthologs in other

![Fig 2. Predicted membrane topology of PF3D7_0104100, a sporozoite-specific protein that is homologous to the prominin/CD133 protein family. The level of polymorphisms among *P. falciparum* strains is indicated for the separate regions using the color scale below the cartoon of the protein as the average number of polymorphisms per nucleotide in the strains in PlasmoDB. PF3D7_0104100 has a high density of polymorphisms within *P. falciparum* strains that are concentrated in the second extracellular loop. Cysteines that are conserved among the homologs in Plasmodium species are indicated. The cysteines that are conserved also in human homologs are in bold. Note that the conserved cysteines occur in close proximity to each other, suggesting the formation of disulphide bonds.](https://doi.org/10.1371/journal.pcbi.1008067.g002)
Plasmodium species reveals the conservation of ten cysteine residues that are all predicted to be extracellular (Fig 2). Such extracellular cysteines can form disulphide bonds as has been observed for other extracellular Plasmodium protein domains [62] and has been suggested for human prominin[63]. Two pairs of the extracellular cysteines were conserved between the human prominin and PF3D7_0104100 (Figs 2 and S2). We were able to predict molecular functions of three other sporozoite specific proteins and 27 sporozoite enriched proteins using HHpred and best bidirectional hits with human proteins at the level of sequence profiles (S4 Table)[12].

Over-represented domains and pathways

We examined whether specific protein domains and pathways were over- or under-represented in the sporozoite proteins using Gene Set Enrichment Analysis[42]. For this we augmented the list of proteins involved in specific processes from PlasmoDB with the glideosome and glycosylphosphatidylinositol (GPI) anchor proteins that we considered specifically relevant to sporozoites (Methods)[42]. At the domain levels there was an over-representation of three protein domains: PF09175 (also named Plasmod_dom_1, unknown function), PF08373 (the RAP domain, putatively RNA binding) and PF12879 (the C-terminal domain of the SICA proteins that are associated with parasitic virulence). Two of these three domains are either unique to Plasmodium species (PF12879) or to P. falciparum (PF09715). At the level of pathways, we observe significant enrichment of the Type-II fatty acid synthesis (FAS-II, Fig 3) and

Fig 3. Apicoplast fatty acid synthesis proteins are enriched among sporozoites. The width of the arrows is determined by the Bayesian score reflecting the level of over representation of that enzyme in sporozoites, e.g. 16 for ACS2 and 8 for FabB/F (S3 Table). For PDH that consists of three proteins, the width of the arrow was determined by the average of those three. Most of FASII proteins are enriched in sporozoites, except PKII, FABZ and LipA. The scheme is a simplification of the pathway as depicted by Shears et al.[50], to which ACS2 was added as it is highly enriched in sporozoites and relevant for fatty acid synthesis.

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the GPI anchor biosynthesis pathways. The enzymes present in the FAS-II pathway are located in the apicoplast—a reduced plastid-like organelle that shares similarity with chloroplasts of algae and plants [64]. This over-representation is consistent with its essentiality for sporozoite development [65]. Interestingly, *P. falciparum* has fourteen Acyl CoA synthetases [66], of which the distribution over the various fatty acid metabolizing processes is largely unresolved. There is one Acyl CoA synthetase, ACS2, that is specific to sporozoites and that would be an interesting candidate to convert the fatty acids produced in the apicoplast to acyl-CoA (Fig 3).

Curiously, there was no enrichment in sporozoites of members of the acyl-ACP thioesterase family (PF3D7_1135400 and PF3D7_0217900). Acyl-ACP thioesterases play an important step in the pathway by hydrolyzing the acyl moiety from the ACP before it can be converted to acyl-CoA.

The second enriched pathway is GPI-anchor biosynthesis, with three proteins (GPI mannosyltransferases 1, 2 and 3: PF3D7_1210900, PF1247300 and PF3D7_1341600) enriched in sporozoites. GPI represents a class of glycolipids found as either free lipids or attached to proteins [67,68]. Surface parasite proteins (such as the merozoite surface proteins 1, 2 and 4) anchor to the parasite cell-membrane via GPI moieties [69]. Sporozoites shed surface proteins (such as circumsporozoite protein and thrombospondin related anonymous protein) when moving [70]. With GPI being used to anchor proteins to the parasite surface, it would have to be synthesized constantly to replace the surface proteins shed during motility. There was no enrichment of the glideosome or of GPI anchor proteins among proteins specific to the sporozoite. However, 39 of the 52 glideosome associated proteins are “present” in the sporozoite stage (75%), representing a slight overrepresentation when compared to the 2,736 of 5,447 proteins (50.2%) detected in sporozoites (Fisher’s exact, P = 0.0018). Similarly, there are 19 out of 28 GPI-anchor proteins identified in Gilson et al. [49] among the proteins present at the sporozoite stage, but they are not specifically enriched in sporozoites and hence are also expressed in other developmental stages. The fact that proteins involved in movement (such as glideosome and GPI) are enriched but not specific to sporozoites indicated that sporozoite share a common movement machinery with other the life-cycle stages.

**Protein functions of sporozoite-specific proteins in the context of sporozoite biology**

Apart from examining over represented pathways we also examined the individual sporozoite specific proteins with regard to their potential function in sporozoite biology. One of the interesting features of sporozoites is their inactivity in the sporozoite glands, which is among others maintained by translational repression with the Puf2 protein [71]. In view of the observation that the translational repression occurs in two waves [71], it is interesting that the sporozoite specific protein PF3D7_0411400 contains a Dead box helicase like its paralog of DOZI that is involved in translational repression in gametocytes of *Plasmodium berghei* [72]. From a gene expression regulatory perspective are furthermore interesting: three Zinc finger proteins (PF3D7_0615600, PF3D7_0521300, PF3D7_1008600), and the transcription factor AP2-04 that has mainly been implicated in ookinete [73]. With respect to the epigenetic regulation, the histone deacetylase sir2b (PF3D7_1451400) that is involved in epigenetic silencing of var gene expression [74] is sporozoite specific, in contrast to its paralog sir2a that is not enriched in sporozoites. Examining the sporozoite specific signaling proteins with respect to their potential function in maintaining the temporal inactivity of the parasite we noted the RAC-beta serine/threonine protein kinase PfAKT (PF3D7_1246900) and two 14-3-3 domain proteins (PF3D7_1422900 and PF3D7_1362100). The PfAKT that is involved in Artemisinin resistance [75] is specifically interesting in view of the role of its metazoan orthologs, AKT1/2/3, in
regulating cellular metabolism to support cell survival [76]. In human it phosphorylates a large number of targets, a number of which are after phosphorylation bound by 14-3-3 proteins. Two 14-3-3 proteins are sporozoite specific: Pf14-3-3I (PF3D7_1422900) and PF3D7_1362100. Pf14-3-3I has been shown to bind phosphorylated Histone H3 [77]. The Raf kinase inhibitor RKIP (PF3D7_1219700), which may be a substrate of the calcium-dependent protein kinase 1 [78], may also be involved in keeping sporozoites in an inactive state as its human ortholog in inhibits the MAPK pathway [79]. Furthermore, with respect to inactivity of sporozoites it is interesting to mention that Prominin-1/CD133, one of the two human orthologs of the Prominin gene (PF3D7_0104100), is known as a marker for dormant stem cells, e.g. in melanoma [80] or in Neural cells [81], and has been shown to activate the PI3K/AKT pathway [82]. Together, these data suggest that these proteins may also modulate sporozoite survival via a mammalian Akt-like pathway as previously shown in asexual blood stages.

Finally, the presence of the tubulin polymerization promoting protein p25 alpha (PF3D7_1236600) as well as thioredoxin-like protein (PF3D7_0919300) and thioredoxin-like associated protein 1 (PF3D7_1230100) whose orthologs are associated with tubulin in *T. gondii* [83] are worth noting in view of the essential role of the microtubules in the sporozoite stage [84]. In contrast to these proteins, the β-tubulin, the two α-tubulins and the other thioredoxin-like associated proteins associated with tubulin [83] are not enriched in sporozoites. Given the presence of β- and α-tubulins in all stages of the life-cycle, PF3D7_1236600, PF3D7_0919300 and PF3D7_1230100 may be crucial players involved in providing sporozoites with their characteristic, thin cylindrical shape.

**Under-represented pathways**

In contrast to the paucity of upregulated processes, there is significant under representation (FDR = < 0.001) of processes linked to splicing, translation, translation elongation, folding of proteins as well as proteolysis (S5 Table). These processes are primarily modulated by a number of sporozoite specific proteins involved in transcriptional silencing e.g. PF3D7_0411400 that contains a Dead box helicase domain [72] and PF3D7_1451400, a histone deacetylase involved in the epigenetic silencing of virulence (var) gene expression [74]. Furthermore, there is significant depletion of proteins involved in carbon metabolism: glycolysis and citric acid cycle (S5 Table).

**Selecting sporozoite proteins as targets or markers of past infection**

Antibodies that bind sporozoite proteins have been induced in 38 volunteers after chloroquine chemoprophylaxis with *P. falciparum* sporozoites (CPS) [13] (S6 Table). Induced antibody profiles represent a blue print of immunogenic proteins in sporozoites, liver stages and early blood stages. Here we focus on the set of sporozoite target proteins that may be used as markers of previous sporozoite exposure or may act as potential targets for vaccines. Minimal sequence variation between *Pf* strains would thereby strengthen the candidature of the proteins for epidemiological or clinical applications, however antibody eliciting proteins including CSP, show relatively high levels of polymorphisms among sequenced malaria strains (S3 Fig), and also sporozoite specific proteins show high levels of polymorphisms (S4 Fig). The selection of proteins that could serve as markers of exposure or vaccine candidates is a compromise between on the one hand protein sequence conservation and on the other hand the frequency of volunteers with antibodies to that protein. As the maximum level of sequence variation between candidate marker proteins we used 8 non-synonymous SNPs per kilobase, which is lower than the variation in for instance Pf64/45, a highly conserved gametocyte protein with 8.9 nonsynonymous SNP per kilobase. As the minimum number of people in which a protein
should elicit antibodies we chose six (out of the 38). Using a “greedy search algorithm” (Methods) we selected a set of nine proteins of which at least one elicited antibodies per volunteer (Table 1).

**Variation in sporozoite proteins among selected Pf strains NF135 and NF166**

Aside from the criteria that the proteins selected as markers for sporozoite exposure are conserved in sequenced *P. falciparum* strains in PlasmoDB, we also required them to be conserved in two strains that have been used in research into heterologous protection after CPS: NF135 and NF166. We therefore sequenced these strains, as well as NF54 (Methods). The mean coverage of mapped reads in the coding regions ranged from 28 for NF54 to 44 for NF166 (S7 Table). A Phred quality score cut-off of 30 was applied similar to other studies involving *Plasmodium vivax* and *P. falciparum* sequencing and variant calling, [85–88]. Manual examination of SNPs still uncovered SNPs with a high Phred score (> 100) that were polymorphic within a single, in principle haploid genome, possibly reflecting mapping issues in duplicated regions. Next to a Phred score of 30, we also introduced a required the presence of a variant nucleotide in at least 75% of the high quality bases. Both criteria were also applied to the indels. Numbers of called SNPs and indels were roughly similar for NF135 and NF166, reflecting their independent geographic origins. As expected, NF54 showed much lower numbers of called SNPs and indels than the other strains, as it is the parental line from which 3D7 is derived (S7 Table). As also observed in Plasmodb, proteins in NF166 and NF135 showed high levels of polymorphisms for antibody-binding proteins enriched in sporozoites (S4 Fig). Nevertheless, most of them had few or no variations in the set of proteins that we selected as markers for previous exposure (Table 2). Lowering the maximum allowed variation in these two strains further, e.g. to zero polymorphisms for all proteins, did not allow us to select a set of proteins that together elicited antibodies in all 38 volunteers.

**Discussion**

The sporozoites stage of the *Plasmodium* life cycle represents the parasite’s first interaction with the human immune system and can be used to effectively vaccinate against infection [13]. The set of genes and proteins expressed at this stage has been determined in at least 11 studies on *Plasmodium spp*, of which nine on *P. falciparum*, creating a conundrum of which dataset to use when studying sporozoite biology, and when deciding to use multiple datasets, how to

| Gene ID          | function            | No. (%) of volunteers with antibodies | Non-syn SNP/kb PlasmoDB | Non-syn SNPs |
|------------------|----------------------|--------------------------------------|-------------------------|--------------|
| PF3D7_0630600    | Conserved hyp        | 20 (52.6)                            | 2.8                     | 0            |
| PF3D7_0906500    | Arginase             | 19 (50.0)                            | 6.5                     | 1            |
| PF3D7_1456700    | Conserved hyp.       | 19 (50.0)                            | 2.9                     | 0            |
| PF3D7_0719700    | 40S ribosomal S10    | 15 (39.5)                            | 0                       | 0            |
| PF3D7_1219100    | Clathrin heavy chain | 12 (31.6)                            | 5.5                     | 0            |
| PF3D7_0301700    | Hypothetical exp.    | 10 (26.3)                            | 6.3                     | 0            |
| PF3D7_1122700    | Conserved hyp.       | 9 (23.7)                             | 7.8                     | 0            |
| PF3D7_1455800    | LCCL prot.           | 6 (15.8)                             | 4.1                     | 0            |

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integrate the datasets. The individual sporozoite studies did not focus on determining which expression patterns are specific to the sporozoite stage, rather they examined the presence of transcripts or proteins. Such data of course can be integrated by combining them in a relatively straightforward manner as has e.g. been done for sporozoite RNA expression[89], but that does not address how specific the expression of a gene is to sporozoites. By combining RNA and protein expression data measured across life stages with sets of proteins known to be present or absent from sporozoites in a Bayesian manner we have created a single list of proteins ranked by their overrepresentation in sporozoites relative to other stages. Such overrepresentation is relative, not absolute. Proteins scoring high on the list can still be expressed at other stages than sporozoites, albeit it at much lower levels. Despite translational repression, which is expected to reduce the correlation between mRNA and protein levels, including the mRNA levels led to a better performance at the protein level that only including proteomics data. Cross validation shows furthermore that the integrated list is better at separating the gold standard positive and negative data sets from each other than the individual data sets.

In this study, we did not separate datasets derived from oocyst sporozoites (the earliest form of this stage) and salivary gland sporozoites (the mature form). There may have been subtle difference in protein expression between sporozoites in the two differing host environments (midgut versus salivary gland) that were not detected. However, oocyst-sporozoite data represent a minority of the combined data set (2/26) and are highly correlated with salivary gland-derived sporozoite data (S1 Fig). A list of proteins with its own sporozoite specificity score will be a valuable resource for studying sporozoite biology and understanding novel protein function. Genetic manipulations of malaria parasites can only occur during blood stage development, which makes studying proteins that are essential for both blood and sporozoite stages difficult. Using our list in combination with the recently published piggyBac whole genome mutagenesis study [90], will allow researchers to determine the approach required for generating a knockout parasite i.e. whether an inducible (for essential blood stage and low sporozoite specificity score) or straight knockout (for a high sporozoite specificity score) system should be considered.

In our analysis, we found an enrichment in the proteins involved in type II fatty acid synthesis which consistent with literature [65]. An increased output of lipids from this pathway may feed into the production of GPI anchors that are made up of different sugar and lipid components. We did observe a slight enrichment in proteins involved in creating GPI anchors, i.e. the three GPI mannosyltransferases. Although there is currently no established relationship between type II fatty acid pathway and synthesis of GPI anchors[50], it may be interesting to pursue it for this stage of the life-cycle given the importance of CSP—the most abundant protein on sporozoites, that is anchored to the surface via a GPI anchor.

Processes involved in the production, folding and catabolism proteins were under-represented in the sporozoite specific and enriched list. Similarly, genes involved in metabolism such as those part of glycolysis and citric acid cycle were also under-represented, suggestive of sporozoites existing in a low metabolic state. Sporozoites are released in the mosquito’s circulation as early as day 12 post blood meal [3] and make their way to the salivary gland. Nevertheless they are generally less infectious for liver cells until after a period of maturation within the salivary gland[91]. Although the exact nature of this maturation is not known, there is evidence that these parasites remain in a latent state until ejected into the human host[4,92]. To understand how sporozoites exist in this state of inactivity, we have identified several interesting targets such as an ortholog of AKT1/2/3, two 14-3-3 proteins, an ortholog of the raf kinase inhibitor and an ortholog of the quiescent stem cell marker prominin/CD133.

Our integration prioritizes proteins based on their presence in datasets in which Gold Standard proteins are present and their absence from data sets in which negative Gold Standard
proteins are present. Those gold standards were deliberately based on other types of data than the ‘omics data that were integrated: western blot and immunofluorescent assay data. The cross validation results with respect to the gold standard proteins underscore the quality of gold standards. Nevertheless, for some of those proteins the evidence from those data is not consistent with the ‘omics data types, like the STARP and LIMP proteins that score low on the list of proteins ranked by their overrepresentation in sporozoites. One can then of course leave such proteins out from the gold standards and redo the analysis. We have decided not do that as it makes the procedure opaque and the runs the risk of circular arguments that inflate the results. Nevertheless, we have left out the STARP protein in deciding upon which proteins to call “enriched” in sporozoites.

Sequence variation among *Plasmodium* strains is pervasive [93], and is possibly responsible for the limited heterologous protection after CPS vaccination with NF54 and challenge with NF135 and NF166 [94]. Indeed as we have shown here both sporozoite specific proteins and proteins that elicit antibodies are highly polymorphic, and only a fraction of those are conserved between NF54, NF135 and NF166 (S4 and S5 Figs). The correlation between immunogenicity and level of sequence conservation suggest that antigenic drift plays a role in the sequence variation. It is not clear whether antigenic drift would also be responsible for high variation among sporozoite specific proteins, as we did not observe a correlation between the Bayesian score of sporozoite specificity and the immunogenicity. Nevertheless, among the large number of sporozoite proteins that elicit antibodies there are still proteins that show limited sequence variation and allow selecting of a small set of proteins that are well conserved. Antibodies generated against these targets could serve as markers of current or previous exposure to sporozoites and are potentially useful in epidemiological settings with low and/or waning transmission for instance in malaria elimination programs. Few of the identified conserved targets are (potentially) membrane bound and could serve as targets for sporozoite vaccines.

In summary, we show a set of previously unidentified sporozoite-specific proteins and assign functions potentially related to the enduring state of inactivity of the salivary gland sporozoite. We further identify sporozoite-directed humoral immune responses and their potential as functional or diagnostic responses that can be elucidated in future studies.

**Supporting information**

**S1 Table.** Overview of all datasets used for the Bayesian data integration.  
(DOCX)

**S2 Table.** Negative gold standard members. Evidence for expression in other life stages than sporozoites.  
(DOCX)

**S3 Table.** Ranked genes for their sporozoite-specificity. Genes in red are “sporozoite specific”, genes in green are “enriched in sporozoites”, blue genes encode proteins that are “present in sporozoites”.  
(XLSX)

**S4 Table.** Sporozoite enriched proteins annotated as “unknown function” in PlasmoDB for which orthology with human proteins could be detected using best bidirectional hits at the level of sequence profiles.  
(DOCX)

**S5 Table.** Biological processes enriched (top list) or depleted (bottom list) in the sporozoite. Enrichment based on the relative absence of the proteins involved at an FDR < = 0.01 and
an enrichment score > 0.20. The type II Fatty Acid Synthesis are the genes from Fig 3, of which the gold standard genes were left out in the GSEA analysis. The “translocation of peptides or proteins into hosts” GO category did not have any proteins among the sporozoite enriched proteins, and was left out of the description of the results. (N)ES: (normalized) enrichment score, FDR: false discovery rate.

S6 Table. Antibody responses in volunteers after CPS immunization. Proteins are from the study by Obiero et al [13]. Proteins that elicited antibodies in at least six subjects (out of 38 infected) are shown.

S7 Table. Levels of polymorphism in NF54, NF135 and NF166 relative of the reference strain 3D7. Information for all annotated Plasmodium falciparum proteins and the proteins considered immunogenic and sequencing depth.

S1 Fig. Correlations of all integrated data sets with each other, hierarchically clustered. Study code (two letters and year) as in S1 Table. Samples are coded as sporozoite (sporo), salivary gland sporozoite (SGS), oocyst derived sporozoite (ODS) or oocyst (OOC), liver stage (LS), blood stage (BS, ery, asexual, merozoite, schiz, ring) or other stages (gametocyte/gam, zygote, ookinete). Transcriptomics studies are given a percentile (percent) for each gene they detected and proteomics studies a score for each unique peptide (uniq_pept) per gene.

S2 Fig. Alignment of PF3D7_0104100 from P. falciparum, TGME49_218910 from Toxoplasma gondii and Prominin-2 from Homo sapiens. The in PF3D7_0104100 predicted transmembrane regions are underlined, the conserved cysteines are boxed. Do note that the fourth transmembrane region is relatively long. Predictions with other tools than TMHMM [95] like Phobius [96] indicate a shorter TM region, putting the cysteine that is located in that TM region in the extracellular space. The Toxoplasma protein was included because its sequence profile has significant sequence similarity against both the human protein profile (E = 2e-20) and the Plasmodium protein profile (E = 3.4e-44), while the similarity between the human and the Plasmodium protein is less significant (E = 0.0001).

S3 Fig. Number of people in whom antibodies against Plasmodium protein were detected by protein microarray after CPS immunization versus the density of non-synonymous polymorphisms. Antibody prevalence shows moderate correlation with the number of Non-synonymous SNPs per kb coding region of the respective gene (PlasmoDB).

S4 Fig. Combined levels of polymorphisms for strains NF135 and NF166 among stage-specific proteins. Sporozoite proteins, selected at various levels of stringency, gametocyte proteins, and the remaining proteins. Sporozoite enriched or sporozoite specific proteins show relatively high levels of polymorphisms, while gametocyte proteins are clearly depleted of polymorphisms. Furthermore, antigenic proteins of either stage are enriched in polymorphisms relative to non-antigenic proteins.

S5 Fig. Venn diagram with Plasmodium falciparum proteins that elicit antibody responses and have non-synonymous SNPs. SNPs in NF135 (blue) and NF166 (yellow) compared to the
reference in NF54/3D7. The grey shaded area contains proteins without any SNPs, they are hence identical to the 3D7 reference and NF54 strain in both NF135 and NF166. The overlap (light green) shows proteins that have SNPs in both NF135 and NF166, and 27 proteins (dark green) have the exact same SNPs in both strains.

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**References**

1. Amino R., Thiberge S., Martin B., Celli S., Shorte S., Frischknecht F., et al. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 2006; 12:220–4. https://doi.org/10.1038/nm1350 PMID: 16429144

2. Matuschewski K., Ross J., Brown S.M., Kaiser K., Nussenzweig V., Kappe S.H. Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. J Biol Chem 2002; 277:41948–53. https://doi.org/10.1074/jbc.M207315200 PMID: 12177071

3. Yang A.S.P., Lopaticki S., O’Neill M.T., Erickson S.M., Douglas D.N., Kneteman N.M., et al. AMA1 and MAEBL are important for Plasmodium falciparum sporozoite infection of the liver. Cell Microbiol 2017; 19. https://doi.org/10.1111/cmi.12745 PMID: 28371168

4. Porter R.J., Laird R.L., Dusseau E.M. Studies on malarial sporozoites. II. Effect of age and dosage of sporozoites on their infectiosity. Exp Parasitol 1954; 3:267–74. https://doi.org/10.1016/0014-4894(54)90026-0 PMID: 13161969

5. Le Roch K.G., Zhou Y., Blair P.L., Grainger M., Moch J.K., Haynes J.D., et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 2003; 301:1503–8. https://doi.org/10.1126/science.1087025 PMID: 12893887

6. Flores L., Washburn M.P., Raine J.D., Anthony R.M., Grainger M., Haynes J.D., et al. A proteomic view of the Plasmodium falciparum life cycle. Nature 2002; 419:520–6. https://doi.org/10.1038/nature01107 PMID: 12368866

7. Lindner S.E., Swearingen K.E., Harupa A., Vaughan A.M., Sinnis P., Moritz R.L., et al. Total and putative surface proteomics of malaria parasite salivary gland sporozoites. Mol Cell Proteomics 2013; 12:1127–43. https://doi.org/10.1074/mcp.M112.024505 PMID: 23325771

8. Bennink S.G. Pradel. The molecular machinery of translational control in malaria parasites. Mol Microbiol 2019; 112:1659–1673. https://doi.org/10.1111/mmi.14386 PMID: 31531994
9. Meerstein-Kessel L., van der Lee R., Stone W., Lanke K., Baker D.A., Alano P., et al. Probabilistic data integration identifies reliable gametocyte-specific proteins and transcripts in malaria parasites. Sci Rep 2018; 8:410. https://doi.org/10.1038/s41598-017-18840-7 PMID: 29323249

10. van Dam T.J.P., Kennedy J., van der Lee R., de Vrieze E., Wunderlich K.A., Rix S., et al. CiliaCarta: An integrated and validated compendium of ciliary genes. PLoS One 2019; 14:e0216705. https://doi.org/10.1371/journal.pone.0216705 PMID: 31095607

11. Zimmermann L., Stephens A., Nam S.Z., Rau D., KUBLER J., Loxajic M., et al. A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. J Mol Biol 2018; 430:2237–2243. https://doi.org/10.1016/j.jmb.2017.12.007 PMID: 29258817

12. Szklarczyk R., Wanschers B.F., Cyupers T.D., Esseling J.J., Riemsma R.A., ten Brink B.P., et al. Iterative orthology prediction uncovers new mitochondrial proteins and identifies C12orf62 as the human ortholog of COX14, a protein involved in the assembly of cytochrome c oxidase. Genome Biol 2012; 13:R2. https://doi.org/10.1038/gb.2012-13-2-r2 PMID: 22356826

13. Obiero J.M., Campo J.J., Scholten A., Randall A., Bijker E.M., Roestenberg M., et al. Antibody Biomarkers Associated with Sterile Protection Induced by Controlled Human Malaria Infection under Chloroquine Prophylaxis. mSphere 2019; 4. https://doi.org/10.1128/mSphereDirect.00027-19 PMID: 30787114

14. Aurrecoechea C., Brestelli J., Brunk B.P., Dommer J., Fischer S., Gajria B., et al. PlasmoDB: a functional genomic database for malaria parasites. Nucleic Acids Res 2009; 37:D359–43. https://doi.org/10.1093/nar/gkn814 PMID: 18957443

15. Labaied M., Camargo N., Kappe S.H.I. Depletion of the Plasmodium berghei thrombospondin-related sporozoite protein reveals a role in host cell entry by sporozoites. Molecular and Biochemical Parasitology 2007; 153:158–166. https://doi.org/10.1016/j.molbiopara.2007.03.001 PMID: 17418435

16. Matuszewski K., Ross J., Brown S.M., Kaiser K., Nussenzieg V., Kappe S.H.I. Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. Journal of Biological Chemistry 2002; 277:41548–41553. https://doi.org/10.1074/jbc.M207315200 PMID: 12177071

17. Arisue N., Hirai M., Arai M., Matsuoka H., Horii T. Phylogeny and evolution of the SERA multigene family in the genus Plasmodium. Journal of Molecular Evolution 2007; 65:82–91. https://doi.org/10.1007/s00239-006-0253-1 PMID: 17609844

18. Khater E.I., Sinden R.E., Dessens J.T. A malaria membrane skeletal protein is essential for normal motility and infectivity of sporozoites. Journal of Cell Biology 2004; 167:425–432. https://doi.org/10.1083/jcb.200406068 PMID: 15339999

19. Singh A.P., Buscaglia C.A., Wang Q., Levay A., Nussenzieg D.R., Walker J.R., et al. Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. Cell 2007; 131:492–504. https://doi.org/10.1016/j.cell.2007.09.013 PMID: 17981117

20. Arevalo-Pinzon G., Curtidor H., Munoz M., Patarroyo M.A., Patarroyo M.E. Synthetic peptides from two Pf sporozoite invasion-associated proteins specifically interact with HeLa and HepG2 cells. Peptides 2011; 32:1902–1908. https://doi.org/10.1016/j.peptides.2011.08.008 PMID: 21864602

21. Yang A.S.P., O’Neill M.T., Jennison C., Lopaticki S., Allison C.C., Armistead J.S., et al. Cell Traversal Activity Is Important for Plasmodium falciparum Liver Infection in Humanized Mice. Cell Reports 2017; 18:3105–3116. https://doi.org/10.1016/j.celrep.2017.03.017 PMID: 29355563

22. Gomes-Santos C.S.S., Braks J., Prudencio M., Carret C., Gomes A.R., Pain A., et al. Transition of Plasmodium Sporozoites into Liver Stage-Like Forms Is Regulated by the RNA Binding Protein Pumilio. Plos Pathogens 2011; 7:15. https://doi.org/10.1371/journal.ppat.1002046 PMID: 21625527

23. Schlarmann M.S., Roberts R.N., Kariuki M.M., LaCrue A.N., Ou R.G., Beemtsen B.T. PF0565w, a Plasmodium falciparum Protein Expressed in Salivary Gland Sporozoites. American Journal of Tropical Medicine and Hygiene 2012; 86:943–954. https://doi.org/10.4269/ajtmh.2012.11-0797 PMID: 22655589

24. Ganter M., Schuler H., Matuschewski K. Vital role for the Plasmodium actin capping protein (CP) beta-subunit in motility of malaria sporozoites. Molecular Microbiology 2009; 74:1356–1367. https://doi.org/10.1111/j.1365-2958.2009.06828.x PMID: 19682250

25. Vaughan A.M., O’Neill M.T., Tarun A.S., Camargo N., Phuong T.M., Aly A.S.I., et al. Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. Cellular Microbiology 2009; 11:506–520. https://doi.org/10.1111/j.1462-5822.2008.01270.x PMID: 19068099

26. Fidock D.A., Bottius E., Braham K., Moeans II, Akwah M., Konings R.N., et al. Cloning and characterization of a novel Plasmodium falciparum sporozoite surface antigen, STARP. Mol Biochem Parasitol 1994; 64:219–232. https://doi.org/10.1016/0166-6851(94)00012-3 PMID: 7935600

27. Zanghi G., Vembar S.S., Baumgarten S., Ding S., Guizetti J., Bryant J.M., et al. A Specific PIM1P Is Expressed in P. falciparum Sporozoites and Plays a Role in Hepatocyte Infection. Cell Reports 2018; 22:2951–2963. https://doi.org/10.1016/j.celrep.2018.02.075 PMID: 29539423
28. Harupa A., Sack B.K., Lakshmanan V., Arang N., Douglass A.N., Oliver B.G., et al. SSP3 Is a Novel Plasmodium yoelii Sporozoite Surface Protein with a Role in Gliding Motility. Infection and Immunity 2014; 82:4643–4653. https://doi.org/10.1128/IAI.01800-14 PMID: 25156733

29. Montagna G.N., Buscaglia C.A., Munter S., Goosmann C., Frischknecht F., Brinkmann V., et al. Critical Role for Heat Shock Protein 20 (HSP20) in Migration of Malarial Sporozoites. Journal of Biological Chemistry 2012; 287:2410–2422. https://doi.org/10.1074/jbc.M111.302109 PMID: 22139844

30. Klug D.F. Frischknecht. Motility precedes egress of malaria parasites from oocysts. Elife 2017; 6:32. https://doi.org/10.7554/eLife.19157 PMID: 28115054

31. Siau A., Silvie O., Franetich J.F., Yalaoui S., Marinach C., Hannoun L., et al. Temperature shift and host cell contact up-regulate sporozoite expression of Plasmodium falciparum genes involved in hepatocyte infection. Plos Pathogens 2008; 4:13. https://doi.org/10.1371/journal.ppat.1000121 PMID: 18688281

32. Al-Nihmi F.M.A., Kolli S.K., Reddy S.R., Mastan B.S., Togiri J., Maruthi M., et al. A Novel and Conserved Plasmodium Sporozoite Membrane Protein SPELD is Required for Maturation of Exo-erythrocytic Forms. Scientific Reports 2017; 7:14. https://doi.org/10.1038/s41598-017-00045-7 PMID: 28144039

33. Schlarman M.S., Roberts R.N., Kariuki M.M., LaCrue A.N., Ou R., Beerntsen B.T. Transcript and protein expression profile of PF11_0394, a Plasmodium falciparum expressed in salivary gland sporozoites. Malaria Journal 2011; 11:12. https://doi.org/10.1186/1475-2875-11-12 PMID: 22230294

34. Silvie O., Goetz K., Matuschewski K. A sporozoite asparagine-rich protein controls initiation of Plasmodium sporozoite membrane protein expression. Cell Microbiol 2012; 14:316–24. https://doi.org/10.1111/j.1462-5822.2011.01734.x PMID: 21098480

35. Santos J.M., Egarter S., Zuzarte-Luis V., Kumar H., Moreau C.A., Kehrer J., et al. Malaria parasite LIMP protein regulates sporozoite gliding motility and infectivity in mosquito and mammalian hosts. Elife 2017; 6:26. https://doi.org/10.7554/eLife.24108 PMID: 2852314

36. Douradinha B., Augustijn K.D., Moore S.G., Ramesar J., Mota M.M., Waters A.P., et al. Plasmodium Cysteine Repeat Modular Proteins 3 and 4 are essential for malaria parasite transmission from the mosquito to the host. Malaria Journal 2011; 10:9. https://doi.org/10.1186/1475-2875-10-9 PMID: 21235777

37. Bergmann-Leitner E.S., Legler P.M., Savranskaya T., Ockenhouse C.F., Angov E. Cellular and humoral immune effector mechanisms required for sterile protection against sporozoite challenge induced with the novel malaria vaccine candidate CellTOS. Vaccine 2011; 29:5940–5949. https://doi.org/10.1016/j.vaccine.2011.06.053 PMID: 21722682

38. Dessens Tremp, A.Z.T. Malaria IMC1 Membrane Skeleton Proteins Operate Autonomously and Participate in Motility Independently of Cell Shape. Journal of Biological Chemistry 2011; 286:5383–5391. https://doi.org/10.1074/jbc.M110.187195 PMID: 21098480

39. Eljtigi I., Ragheb D.R.T., Pino P., Coppi A., Bennett B.L., Soldati-Favre D., et al. Shedding of TRAP by a Rhomboid Protease from the Malaria Sporozoite Surface Is Essential for Gliding Motility and Sporozoite Infectivity. Plos Pathogens 2012; 8:17. https://doi.org/10.1371/journal.ppat.1002725 PMID: 22911675

40. Matuschewski Steinbuechel, M.K. Role for the Plasmodium sporozoite-specific transmembrane protein S6 in parasite motility and efficient malaria transmission. Cellular Microbiology 2009; 11:279–288. https://doi.org/10.1111/j.1462-5820.2008.01252.x PMID: 19016774

41. Meerstein-Kessel L., van der Lee R., Stone W., Lanke K., Baker D.A., Alano P., et al. Probabilistic data integration identifies reliable gametocyte-specific proteins and transcripts in malaria parasites. Scientific Reports 2018; 8:13. https://doi.org/10.1038/s41598-017-18467-8 PMID: 29311572

42. Subramanian A., Tamayo P., Mootha V.K., Mukherjee S., Ebert B.L., Gillette M.A., et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102:15545–50. https://doi.org/10.1073/pnas.0506580102 PMID: 16199517

43. Lindner S.E., Miller J.L., Kappe S.H. Malaria parasite pre-erythrocytic infection: preparation meets opportunity. Cell Microbiol 2012; 14:316–24. https://doi.org/10.1111/j.1462-5822.2011.01734.x PMID: 22191703

44. Dessens J.T., Beetsma A.L., Dimopoulos G., Wengelnik K., Crisanti A., Kafatos F.C., et al. CTRP is essential for mosquito infection by malaria oocystes. Embo Journal 1999; 18:6221–6227. https://doi.org/10.1093/emboj/18.22.6221 PMID: 10562534

45. Engelmann S., Silvie O., Matuschewski K. Disruption of Plasmodium Sporozoite Transmission by Depletion of Sporozoite Invasion-Associated Protein 1. Eukaryotic Cell 2009; 8:640–648. https://doi.org/10.1128/EC.00347-08 PMID: 19181869

46. Boucher L.E.J. Bosch. The apicomplexan glideosome and adhesins—Structures and function. J Struct Biol 2015; 190:33–114. https://doi.org/10.1016/j.jsb.2015.02.008 PMID: 25769498
47. Green J.L., Wall R.J., Vahokoski J., Yusuf N.A., Ridzuan M.A.M., Stanway R.R., et al. Compositional and expression analyses of the glideosome during the Plasmodium life cycle reveal an additional myosin light chain required for maximum motility. J Biol Chem 2017; 292:17857–17875. https://doi.org/10.1074/jbc.M117.802769 PMID: 28893907

48. Kumar V., Behl A., Kapoor P., Nayak B., Singh G., Singh A.P., et al. Inner membrane complex 11 protein of Plasmodium falciparum links membrane lipids with cytoskeletal element ‘actin’ and its associated motor ‘myosin’. Int J Biol Macromol 2019; 126:673–684. https://doi.org/10.1016/j.ijbiomac.2018.12.239 PMID: 30599160

49. Gilson P.R., Nebi T., Vukcevic D., Moritz R.L., Sargeant T., Speed T.P., et al. Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite Plasmodium falciparum. Mol Cell Proteomics 2006; 5:1286–99. https://doi.org/10.1074/mcp.M600035-MCP200 PMID: 16603579

50. Shears M.J., Botte C.Y., McFadden G.I. Fatty acid metabolism in the Plasmodium apicoplast: Drugs, doubts and knockouts. Mol Biochem Parasitol 2015; 199:34–50. https://doi.org/10.1016/j.molbiopara.2015.03.004 PMID: 25841762

51. Delemarre B.J.H.J. van der Kaay. [Tropical malaria contracted the natural way in the Netherlands]. Ned Tijdschr Geneeskd 1979; 123:1981–2. PMID: 390409

52. Terlinsk A.C., Roestenberg M., van de Vegte-Bolmer M., Scholzen A., Heinrichs M.J., Siebelink-Stoter R., et al. NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections. J Infect Dis 2013; 207:656–60. https://doi.org/10.1093/infdis/jits129 PMID: 23186785

53. McCall M.B.B., Wammes L.J., Langenberg M.C.C., van Gemert G.J., Walk J., Hermse C.C., et al. Infectivity of Plasmodium falciparum sporozoites determines emerging parasitemia in infected volunteers. Sci Transl Med 2017; 9. https://doi.org/10.1126/scitranslmed.aag2490 PMID: 28637923

54. Andrews S., Kellis M., Eichler E.E. A new Plasmodium falciparum clone for controlled human malaria infections. J Infect Dis 2013; 207:656–60. https://doi.org/10.1093/infdis/jits129 PMID: 23186785

55. Weigmann A., Corbeil D., Hellwig A., Huttner W.B. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci U S A 2012; 109:6692–7. https://doi.org/10.1073/pnas.1204363109 PMID: 22493233

56. Lawrence M., Huber W., Pages H., Abcyoun P., Carlson M., Gentleman R., et al. Software for computing and annotating genomic ranges. PLoS Comput Biol 2013; 9:e1003118. https://doi.org/10.1371/journal.pcbi.1003118 PMID: 23950696

57. Gardner M.J., Hall N., Fung E., White O., Berriman M., Hyman R.W., et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 2002; 419:498–511. https://doi.org/10.1038/nature01097 PMID: 12368684

58. Arredondo S.A., Ca M., Takayama Y., MacDonald N.J., Anderson D.E., Aravind L., et al. Structure of the Plasmodium 6-cysline e48/45 domain. Proc Natl Acad Sci U S A 2012; 109:6692–7. https://doi.org/10.1073/pnas.1204363109 PMID: 22493233

59. Coolen, J., CleanNextSeqpaired. 2017.

60. Salzberg S.L., Shentu H., Smoot M.D., Wagner L., Wetteroth E.C., et al. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9:357–9. https://doi.org/10.1038/nmeth.1923 PMID: 23388286

61. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009; 25:2078–9. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19505943

62. Hall Quinlan, A.R.I. M. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 2010; 26:841–2. https://doi.org/10.1093/bioinformatics/btp339 PMID: 20110278

63. Gardner M., Hall N., Fung E., White O., Berriman M., Hyman R.W., et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 2002; 419:498–511. https://doi.org/10.1038/nature01097 PMID: 12368684

64. van Dooren G.G.B. Striepen. The algal past and parasite present of the apicoplast. Annu Rev Microbiol 2013; 67:271–89. https://doi.org/10.1146/annurev-micro-092412-155741 PMID: 23808340

65. van Schaijk B.C., Kumar T.R., Vos M.W., Richman A., van Gemert G.J., Li T., et al. Type II fatty acid biosynthesis is essential for Plasmodium falciparum sporozoite development in the midgut of Anopheles mosquitoes. Eukaryot Cell 2014; 13:550–9. https://doi.org/10.1128/EC.00264-13 PMID: 24297444

66. Bethke L.L., Zillversmit M., Nielsen K., Daily J., Volkman S.K., Ndiaye D., et al. Duplication, gene conversion, and genetic diversity in the species-specific acyl-CoA synthetase gene family of Plasmodium falciparum. Mol Biochem Parasitol 2006; 150:10–24. https://doi.org/10.1016/j.molbiopara.2006.06.004 PMID: 16860410

67. Ferguson McConville, M.J.M.A. The structure, biosynthesis and function of glycosylated phosphatidylinositol in the parasitic protozoa and higher eukaryotes. Biochem J 1993; 294 (Pt 2):305–24. https://doi.org/10.1042/bj2940305 PMID: 8373346
68. Englund P.T. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu Rev Biochem 1993; 62:121–38. https://doi.org/10.1146/annurev.bi.62.070193.001005 PMID: 8352586

69. Halder K., Henderson C.L., Cross G.A. Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. Proc Natl Acad Sci U S A 1986; 83:8565–9. https://doi.org/10.1073/pnas.83.22.8565 PMID: 3534892

70. Ejigiri I., Ragheb D.R., Pino P., Coppi A., Bennett B.L., Soldati-Favre D., et al. Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. PLoS Pathog 2012; 8:e1002725. https://doi.org/10.1371/journal.ppat.1002725 PMID: 22911675

71. Lindner S.E., Swearingen K.E., Shears M.J., Walker M.P., Vrana E.N., Hart K.J., et al. Transcriptomics and proteomics reveal two waves of translational repression during the maturation of malaria parasite sporozoites. Nat Commun 2019; 10:4964. https://doi.org/10.1038/s41467-019-12396-6 PMID: 31673027

72. Mair G.R., Braks J.A., Garver L.S., Wiegant J.C., Hall N., Dirks R.W., et al. Regulation of sexual development of Plasmodium by translational repression. Science 2006; 313:667–9. https://doi.org/10.1126/ science.1125129 PMID: 16888139

73. Modrzynska K., Pfander C., Chappell L., Yu L., Suarez C., Dundas K., et al. A Knockout Screen of ApiAP2 Genes Reveals Networks of Interacting Transcriptional Regulators Controlling the Plasmodium Life Cycle. Cell Host Microbe 2017; 21:11–22. https://doi.org/10.1016/j.chom.2016.12.003 PMID: 28081440

74. Petter M., Lee C.C., Byrne T.J., Boysen K.E., Volz J., Ralph S.A., et al. Expression of P. falciparum var genes involves exchange of the histone variant H2A.Z at the promoter. PLoS Pathog 2011; 7: e1001292. https://doi.org/10.1371/journal.ppat.1001292 PMID: 2193942

75. Mbengue A., Bhattacharjee S., Pandharkar T., Liu H., Estiu G., Stahelin R.V., et al. Raf kinase inhibitor protein affects activity of Plasmodium falciparum calcium-dependent protein kinase 1. Mol Biochem Parasitol 2007; 151:111–7. https://doi.org/10.1016/j.molbiopara.2006.10.012 PMID: 17123645

76. Vandersmissen D., Winter D., Pluckhahn K., Lehmann W.D., Kappes B. Raf kinase inhibitor protein affects activity of Plasmodium falciparum calcium-dependent protein kinase 1. Mol Biochem Parasitol 2007; 151:111–7. https://doi.org/10.1016/j.molbiopara.2006.10.012 PMID: 17123645

77. Vandersmissen D., Winter D., Pluckhahn K., Lehmann W.D., Kappes B. Raf kinase inhibitor protein affects activity of Plasmodium falciparum calcium-dependent protein kinase 1. Mol Biochem Parasitol 2007; 151:111–7. https://doi.org/10.1016/j.molbiopara.2006.10.012 PMID: 17123645

78. Petter M., Lee C.C., Byrne T.J., Boysen K.E., Volz J., Ralph S.A., et al. Expression of P. falciparum var genes involves exchange of the histone variant H2A.Z at the promoter. PLoS Pathog 2011; 7: e1001292. https://doi.org/10.1371/journal.ppat.1001292 PMID: 2193942

79. Vandersmissen D., Winter D., Pluckhahn K., Lehmann W.D., Kappes B. Raf kinase inhibitor protein affects activity of Plasmodium falciparum calcium-dependent protein kinase 1. Mol Biochem Parasitol 2007; 151:111–7. https://doi.org/10.1016/j.molbiopara.2006.10.012 PMID: 17123645

80. Flores-Guzman F., Utikal J., Umansky V. Dormant tumor cells interact with memory CD8(+) T cells in vivo. Cancer Lett 2020; 474:74–81. https://doi.org/10.1016/j.canlet.2020.01.016 PMID: 31923142

81. Luo Y., Coskun V., Liang A., Yu J., Cheng L., Ge W., et al. Single-cell transcriptome analyses reveal signals to activate dormant neural stem cells. Cell 2015; 161:1175–1186. https://doi.org/10.1016/j.cell.2015.04.001 PMID: 26000486

82. Wei Y., Jiang Y., Zou F., Liu Y., Wang S., Xu N., et al. Activation of PI3K/Akt pathway by CD133-p85 interaction promotes tumorigenic capacity of glioma stem cells. Proc Natl Acad Sci U S A 2013; 110:6829–34. https://doi.org/10.1073/pnas.1217002110 PMID: 23569237

83. Liu J., Wetzel L., Zhang Y., Nagayasu E., Ems-McClung S., Flores L., et al. Novel thioredoxin-like proteins are components of a protein complex coating the cortical microtubules of Toxoplasma gondii. Eukaryot Cell 2013; 12:1588–99. https://doi.org/10.1128/EC.00082-13 PMID: 23873863

84. Spreng B., Fleckenstein H., Kubler P., Di Biagio C., Benz M., Patra P., et al. Microtubule number and length determine cellular shape and function in Plasmodium. EMBO J 2019; 38:e100984. https://doi.org/10.15252/embj.201900984 PMID: 31368598

85. Diez Benavente E., Ward Z., Chan W., Mohareb F.R., Sutherland C.J., Roper C., et al. Genomic variation in Plasmodium vivax malaria reveals regions under selective pressure. PLoS One 2017; 12: e0177134. https://doi.org/10.1371/journal.pone.0177134 PMID: 28493919

86. Shen H.M., Chen S.B., Wang Y., Xu B., Abe E.M., Chen J.H. Genome-wide scans for the identification of Plasmodium vivax genes under positive selection. Malar J 2017; 16:238. https://doi.org/10.1186/s12936-017-1882-0 PMID: 28587615
87. Campino S., Benavente E.D., Assefa S., Thompson E., Drought L.G., Taylor C.J., et al. Genomic variation in two gametocyte non-producing Plasmodium falciparum clonal lines. Malar J 2016; 15:229. https://doi.org/10.1186/s12936-016-1254-1 PMID: 27098483

88. Ocholla H., Preston M.D., Mipando M., Jensen A.T., Campino S., MacInnis B., et al. Whole-genome scans provide evidence of adaptive evolution in Malawian Plasmodium falciparum isolates. J Infect Dis 2014; 210:1991–2000. https://doi.org/10.1093/infdis/jiu349 PMID: 24948693

89. Gomez-Diaz Ruiz, J.L.E. The second life of Plasmodium in the mosquito host: gene regulation on the move. Brief Funct Genomics 2019; 18:313–357. https://doi.org/10.1093/bfgp/elz007 PMID: 31058281

90. Zhang M., Wang C., Otto T.D., Oberstaller J., Liao X., Adapa S.R., et al. Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. Science 2018;360. https://doi.org/10.1126/science.aap7847 PMID: 29724925

91. Ghosh A.K.M. Plasmodium sporozoite invasion of the mosquito salivary gland. Curr Opin Microbiol 2009; 12:394–400. https://doi.org/10.1016/j.mib.2009.06.010 PMID: 19608457

92. Beier J.C. Malaria parasite development in mosquitoes. Annu Rev Entomol 1998; 43:519–43. https://doi.org/10.1146/annurev.ento.43.1.519 PMID: 9444756

93. Amambua-Ngwa A., Amenga-Etego L., Kamau E., Amato R., Ghansah A., Golassa L., et al. Major sub-populations of Plasmodium falciparum in sub-Saharan Africa. Science 2019; 365:813–816. https://doi.org/10.1126/science.aav5427 PMID: 31439796

94. Walk J., Reuling I.J., Behet M.C., Meerstein-Kessel L., Graumans W., van Gemert G.J., et al. Modest heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial. BMC Med 2017; 15:168. https://doi.org/10.1186/s12916-017-0923-4 PMID: 28903777

95. Sonnhammer E.L., von Heijne G., Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 1998; 6:175–82. PMID: 9783223

96. Kall L., Krogh A., Sonnhammer E.L. A combined transmembrane topology and signal peptide prediction method. J Mol Biol 2004; 338:1027–36. https://doi.org/10.1016/j.ymb.2004.03.016 PMID: 1511065