Chapter

Systems Biology Approaches towards Immunity against *Plasmodium*

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Abstract

Malaria is one of the most devastating infectious diseases known to humans. It is caused by unicellular protozoan parasites belonging to the genus *Plasmodium*. Till date, over 200 species of *Plasmodium* have been formally described, and each species infects a certain range of hosts. However, the human infection is limited to only five of the species, of which *P. falciparum* is the most responsible. Due to the emergence of parasite resistance to frontline chemotherapies and mosquito resistance to current insecticides which threaten the control programmes, new antimalarial therapeutics or approaches capable of predicting useful models of how different cells of the innate immune system function, is the need of the hour. Systems Immunology is a relatively recent discipline under Systems Biology to understand the structure and function of the immune system and how the components of the immune system work together as a whole. Thus, this chapter aims to give insight into the approaches of Systems Biology for investigating the immune factors that are formed during *Plasmodium falciparum* infection in the human body. Here, the numerous experimental and computational works with the ongoing methodologies using Systems Biology approaches along with the interactions of host and pathogen will be discussed.

Keywords: omics, *Plasmodium falciparum*, systems immunology, Systems Biology

1. Introduction

Plasmodium falciparum is a protozoan parasite that causes malaria in humans and is transmitted through an insect vector, the female *Anopheles* mosquito. The species' malaria (also called malignant malaria or *falciparum* malaria) is the most deadly type of malaria with the highest incidence of complication and mortality rates. Malaria is a world-wide infectious illness that continues to be a leading source of morbidity and mortality in developing countries. Malaria is an ancient disease, with references to what was nearly actually a protozoan ailment believed to be malaria, appearing in a Chinese document from around 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC, and Hindu scriptures from the sixth century BC. For over 2500 years the thought that malaria fevers were caused by miasmas

rising from marshes for many years and it is usually believed that held that the word malaria comes from the Italian *mal'aria,* which spoiled air; however, this is controversial. The origins of the *Plasmodium* parasites infecting humans have long been a source of fascination. Ancient scriptures from China, India, the Middle East, Africa, and the European continent, contain descriptions of malaria-like illnesses indicating that humans have been fighting *Plasmodium* infections throughout our recorded history [1, 2]. Scientific studies were only possible after the discovery of the parasites themselves by Charles Louis Alphonse Laveran in 1880 and the incrimination of mosquitoes as vectors, first for avian malaria by Ronald Ross in 1897 and then for human malaria by Amico Bignami, Angelo Celli, Camillo Golgi, Ettore Marchiafava, Giovanni Battista Grassi and Giuseppe Bastianelli between 1898 and 1900.

Malaria is a world-wide infectious disease that continues to be a major cause of morbidity and mortality in developing countries. Malaria is found in more than 90 countries and affects over 40% of the world's population. Out of four species of malaria, *Plasmodium falciparum* is the lethal one. *P. falciparum* is responsible for more than 90% of the global malaria death and hence continues to be a major public health concern on a global scale. According to WHO, World Malaria report 2020, there are 241 million cases of malaria world-wide, resulting in 627,000 fatalities world-wide. In non-malarial nations such as North America and Europe, there are a considerable number of instances of imported malaria and local transmission following importation [2].

Variants in the human genome linked with resistance to *Plasmodium* infection and malaria-related illness are thought to be thousands of years old [3]. One long-held theory proposed that humans and chimpanzees both acquired *P. falciparum*-like infections from their common ancestor and that these parasites co-evolved for millions of years with their respective host species. *P. vivax,* on the other hand, is thought to have emerged hundreds of thousands of years ago, when the cross-species transmission of a parasite from a macaque occurred in South-eastern Asia [4, 5]. However, both of these notions have recently been debunked following the characterisation of a large number of new *Plasmodium* parasites from African apes. *P. falciparum* infection is now known to be relatively new for humans, having emerged after the acquisition of a parasite from a gorilla, most likely within the last 10,000 years [6, 7]. Characterisation of the numerous ape *Laverania* spp. discovered a parasite lineage in western gorillas with parasites that were nearly identical to *P. falciparum* [5, 7]. This was first misinterpreted that gorillas can be infected by human parasites [5]. However, after analysing the mtDNA sequences from significant numbers of additional wild-living gorillas, it was discovered that all extant *P. falciparum* strains from humans fall within the radius of these gorilla parasites [7]. *P. praefalciparum* is the name given to this gorilla parasite lineage to indicate its role in the origin of *P. falciparum*.

When compared to viruses and bacteria, eukaryotic protozoans present a larger genome and have a complex biology, which hinders the development of vaccines. Even though malaria is a curable disease, there are currently no established vaccines. Quinine and artemisinin, both extracted from the bark of the Peruvian Cinchona Succirubra tree and the Chinese herb *Artemisia annua*, are now the most potent antimalarials available. Artemisinin-based Combination Therapies (ACTs), which have just recently been accepted as a last option in the fight against malaria, are already being tested by ACT-resistant strains in Southeast Asia. With parasite resistance to all current antimalarial medications spreading, successful control and eradication will need the development of new tools and cost-effective antimalarial tactics [8]. The complex biology of *Plasmodium* poses a hindrance in the detailed understanding of

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the mechanisms that control malarial infection, thus giving rise to technical challenges in the eradication of malaria. New approaches to elucidate key host–parasite interactions, and predict how the parasites will respond in various biological settings, could dramatically enhance the efficacy of intervention strategies [9]. Advances in the field of Systems Biology are well poised to meet these challenges.

2. Immune response to *Plasmodium falciparum* using systems biology approaches

The immune response involves components from both innate and adaptive immune system molecules. During *Plasmodium* infection, an innate immune response is generated as the first line of defence, followed by an adaptive immunological response that comprises T-cells, B-cells, and antibodies. The sporozoites remain active for several hours after being inoculated into the host's skin. Systems Immunology is a part of Systems Biology that aims to better understand the structure and function of the immune system, and how its various components interact [10]. In contrast to a reductionist approach focused on limited subsets of a class of biomolecules, Systems Biology approaches encompass the study of biological systems through a near-comprehensive investigation of specific classes of biomolecules. Because of the complexity of the system under consideration and the vast information created by experimental approaches connected with Systems Biology, computational modelling and analysis are also essential parts of Systems Biology [11]. **Figure 1** illustrates the life cycle of *P. falciparum* alongwith the immune responses to *P. falciparum* and how Systems Biology can be incorporated into it.

Beyond the aforementioned computational models and analysis, Systems Biology ideally involves mechanistic mathematical models of a system, allowing biological understanding and the ability to forecast system behaviour [12]. In the realm of malaria research, mathematical modelling is most typically employed in population modelling to track and predict malaria transmission via host- parasite populations.

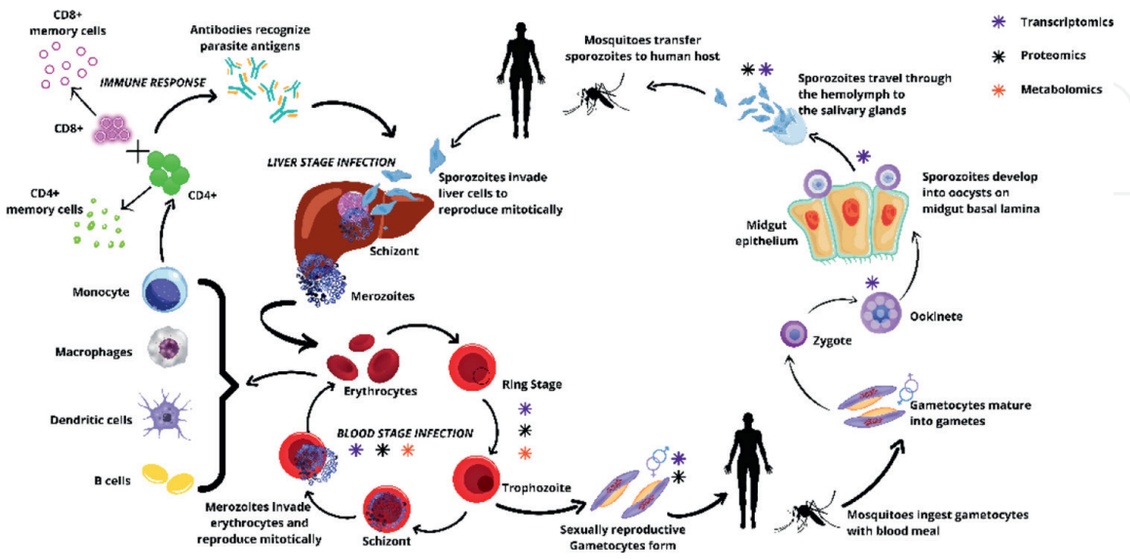

Figure 1.
Life cycle of P. falciparum *and the different stages of immune responses to it. The different systems biology approaches, i.e. Transcriptomics, proteomics and metabolomics, to study the different stages of the life cycle has been shown using asterics.*

Using modelling approaches and mathematical modelling at system-scale data, specific phases of the immune response along with the distribution and timing of parasite sequestration in different tissues of the body have been recently studied in malaria host-pathogen interactions effectively and also in other diseases, such as cancer. As a matter of fact, prevalence of system-scale data has been generated in-vitro rather than in-vivo in the literature to date [13–16].

The advanced analytical approaches are required to generate the large, systems-scale experimental datasets that are distinctive of Systems Biology, which is frequently referred to as "Omics" collectively and generically. Omics datasets, a biological system of transitions from one state to another, are the starting point for Systems Biology. They are systematic and complete identifications and quantifications of molecules. Several researchers have generated Omics data during the malaria life cycle transitions. Omics data include genomics, transcriptomics, proteomics, metabolomics, and translational repression/de-repression of transcripts as the parasite transitions throughout the malaria life cycle stages [17]. Transcriptomics implies the use of RNA-sequencing and microarrays for the investigation of gene expression at the systems level. Protein levels, proteomics, and metabolites levels for metabolomics are typically measured using mass spectrometry or nuclear magnetic resonance spectroscopy, respectively. Omics technologies are being increasingly employed to investigate how *Plasmodium* parasites impact their hosts and how the parasite affects the host environment [13]. The range of computational approaches used to analyse and understand such large datasets is crucial to turning them into biological knowledge. Computational methodologies such as network modelling, ontology analysis, and phenotypic association are utilised to analyse and interpret these data in addition to classic statistical analyses. The use of a structural or graphical model to depict relationships between constituent pieces of a dataset is known as network modelling. These connections could arise from the experimental dataset itself, such as a substantial correlation or mutual information between two measured variables, or from other sources, such as sequence data or previously published links. Individual measured variables are associated with groups, sets, or classes to which they belong, and statistical trends, such as enrichment in significantly changing variables for each class, are assessed based on the dataset. Identification of correlations between abundances of a biomolecule, such as a protein or RNA product, and a trait of interest in order to determine which biomolecules may impact the characteristics is referred to as phenotype association. These computational techniques, when combined, can lead to a systems-scale knowledge of host–parasite relationships, which will be critical for disrupting them through the development of novel treatments and vaccines in the battle against malaria.

Recent research shows that the immune response of the host may potentially play a role in the pathogenesis of the illness in humans. Human investigations of the immune response to malaria parasites have yielded a lot of information about the cells and cytokines involved in the pathophysiology of survival and death in severe infections [18, 19]. This chapter will look at a variety of experimental and computational activities that are currently being done utilising Systems Biology techniques, as well as the methodology that is being used.

3. Experimental approaches

To solve biological questions, Systems Biology techniques use both experimental and computational frameworks. We discussed the newly evaluated continuing

techniques of omics datasets in this chapter in order to better understand the mechanisms of underlying illnesses and to learn more about target medications for enhanced malaria therapeutics. Genes, mRNAs, proteins, and metabolites supply a wealth of information through omics technology. The omics revolution has accelerated the data-intensive approach to biology, which stresses large-scale, aggregate investigations employing high-throughput methods. Despite the fact that these treatments were time-consuming and costly, they were now inexpensive and widely available because of technological advancements. During the experimental strategies, a variety of methodologies are used. Microarray Techniques, Shotgun Proteomics (LC-MS), Conditional Gene Knockout, Conditional Knockdown of Translation and Conditional Knockdown of Protein Function may be summarized.

3.1 Microarray technique

Microarray techniques are the developing techniques to study gene expression all at once. Transcriptomics studies involve microarray techniques to study gene expression and are also involved in immune response and play a significant role. Transcriptomics is a commonly used approach in malaria for omics studies used by collecting blood samples [13, 18, 19]. In 2003, the first high-resolution *P. falciparum* blood-stage transcriptome was published for studying various gene expressions to achieve diverse parasite phenotypes, immune evasion, adaptations, reproduction, and transmission. To study more about the *Plasmodium falciparum* gene functions, shotgun DNA microarray is implied. The shotgun DNA microarray by Rhian *et al.* studies variation of gene expression during the parasite life cycle, and the array is probed with differently labelled cDNAs prepared from the total RNA isolated cells from the defined developmental stage. Yatsushiro *et al.*, put forward a cell microarray chip system for rapid and accurate diagnosis for malaria, which included a push column for recovery of erythrocytes and a fluorescence detector [20]. The malarial shotgun microarray is constructed by printing PCR-amplified inserts from the *P. falciparum* library. The shotgun microarray is useful for analysing malarial transcriptomics programs for comparing gene expression of *Plasmodium*. But to date, in *P. falciparum,* only a few proteins have been identified in the sexual stage of the life-cycle [21]. Microarray techniques have answered many fundamental questions but due to limited cost and comparatively large amount of biological materials required, which is limited in clinical isolates [19].

3.2 Shotgun proteomics

Shotgun proteomics refers to bottom-up proteomics techniques to identify proteins that are detected during the malaria life cycle using Liquid Chromatography (LC) coupled to tandem Mass spectrometry (MS). Proteomics studies involve understanding multiprotein systems in an organism for identifying, characterising, or quantifying proteins in the field of Systems Biology. In this LC–MS method, the peptides are loaded onto the reserved phase column using C18 and then separated according to hydrophobicity through the mobile phase medium, which consists of water and elongated percentage acetonitrile. In this method, at first, the proteins are extracted from a given sample, followed by removal of contaminants and protein of no interest, followed by digestion of proteins into peptides, followed by separation of post digestions to obtain a more homogeneous mixture of peptides, and lastly analysis by MS [22, 23]. A study by Briquet *et al*.,

using shotgun proteomics tools, has explored the nuclear proteins of *P. falciparum* extracted from erythrocytes [24]. To identify novel drug targets, there is a significant challenge which lies in identifying genes that are probably essential to parasite viability. Despite the progress in proteomics techniques, these techniques remain limited in the clinical field due to the time-consuming and huge cost of equipment.

3.3 Conditional gene knockout

The *P. falciparum* genome is haploid for the majority of its life cycle, thus preventing the generation of random mutants except under exceptional circumstances. This prevents the use of random mutagenesis screens to identify biological mechanisms behind the parasitic pathways in most cases. *Plasmodium* is evolutionarily distant from model organisms that have been thoroughly studied. A large percentage of parasitic genes lack homology outside of the phylum, thus suggesting that while some protein functions are conserved, the complex biological machinery of the life cycle of *Plasmodium* spp. prevents direct comparison with the established biological function of conserved proteins from work on model systems [25]. The development of conditional gene or protein expression systems that allow disruption of the spatio-temporal protein function has aided in transcending the roadblocks set by the biology of the parasite. The ability to conditionally disrupt essential protein function is critical for understanding parasite biology and identifying new drug targets. This was further advanced by the important technological tool of CRISPR/Cas-9 gene editing that enabled the implementation of these conditional tools to be widely applied in *Plasmodium falciparum*. Conditional gene/protein systems are present at various levels of the genomic system. This allows researchers to modify the parasitic genome. Modifications include disruption of translation or direct inhibition of protein function by adding or removing small molecules. While some systems have been adapted for use from other organisms, e.g., the Cre recombinase, few systems like the P*f*DOZI-TetR aptamer system have been developed specifically for *P. falciparum*. These systems have been widely used to study the clinical manifestations caused by the blood stages of *P. falciparum*, albeit a few exceptions.

Conditional gene knockout involves the perturbation of transcription by completely removing DNA sequences by activating a site-specific recombinase expressed in the parasite. A major advantage of this approach is that translation can be completely prevented in the parasites that excise the DNA, although the knockout may not be 100% penetrant within the population. The use of site-specific recombinases in *P. falciparum* has become a powerful tool to study the function of essential genes in the parasite in the last few decades. Two site-specific recombinases, Cre and Flippase recombination enzyme (FLP), have been successfully utilised to modify the parasitic genome [26]. The DiCre system is another tool that has been put forward to conditionally knockout genes. This system was adapted for *P. falciparum* blood stages to introduce conditional control over DNA excision. The majority of current conditional systems have been adapted to *Plasmodium falciparum* strains that are not mosquito-infectious. This makes it impossible to study protein function during the parasite's transmission stages. To overcome this challenge, a DiCre-expressing line was recently generated using the mosquito-infective *P. falciparum* NF54 strain. The DiCre-expressing parasites have been recently used to characterise the entire FIKK kinase family in the asexual stage, identifying essential kinases for virulence, growth, and host cell remodelling [27].

3.4 Conditional knockdown of translation

Introducing protein knockdown by targeting mRNA translation has been a valuable strategy for studying protein functions in many organisms. This involves the introduction of double-stranded RNA into cells, which binds homologous mRNA within the cell, leading to the degradation of the RNA transcripts. However, *Plasmodium* parasites lack a functional RNAi pathway, leading to the development of other RNA-based knockdown systems for use in the parasites. The *glmS* and *Pf*DOZI-TetR systems are mRNA-targeting knockdown systems that have been used to study the *P. falciparum* blood stages. The *glmS* ribozyme sequence is inserted into the gene of interest after the stop codon. The transcribed mRNA containing the ribozyme is activated by glucosamine-6-phosphate to cleave its associated mRNA, thus leading to transcript instability and degradation. Modifying the parasite genome to introduce the ribozyme is a relatively uncomplicated protocol, with the simultaneous introduction of an epitope tag sequence at the end of the ORF, a stop codon, then the ribozyme sequence, being a common approach. The culture medium is supplemented with glucosamine to activate the ribozyme in *P. falciparum*. The parasite converts glucosamine to glucosamine-6-phosphate. An advantage of this system is the availability of an inactive "M9" version of the ribozyme containing a single point mutation rendering it incapable of cleaving mRNA. One significant concern associated with the *glmS* system is whether the knockdown achieved will result in a definite observable phenotype. For example, the use of the *glmS* system to determine the essentiality of the proteases *Pf*PMV and *Pf*ClpP showed no growth defects after the knockout of the respective proteases. Both *Pf*PMV and *Pf*ClpP proteases have been reported to be essential via different methods. These observations suggest that while the *glmS* system has been extensively utilised to study parasite biology, it may prove insufficient to establish the utility of high expression protein systems [28].

Another conditional approach for controlling protein expression via translation is *Pf*DOZI-TetR. Although its mode of function and use is more complex than the above-mentioned glmS system, the *Pf*DOZI-TetR system has become a go-to in studying asexual parasite biology. Following the stop codon of the gene of interest, ten copies of a TetR-binding aptamer sequence are introduced in this conditional system [29]. The TetR protein, which is linked to *Pf*DOZI, a protein that localises to mRNA sequestration sites known as P-bodies, recognises the aptamers in the mRNA. *Pf*DOZ-TetRI binds to the mRNA, causing it to relocalize to P-bodies and suppress translation. The *Pf*DOZI-TetR regulatory fusion protein is introduced into the parasite genome using CRISPR/Cas-9 technology, which includes a linearized repair template with homology regions to the gene of interest, a drug selection marker, 10x aptamer sequences, an epitope tag, and a cassette to express the *Pf*DOZI-TetR regulatory fusion protein. Tang and colleagues utilised the *Pf*DOZI-TetR system to validate results from a forward genetic screen aimed at uncovering genes essential for apicoplast biogenesis. Their work demonstrates the importance of having conditional knockdown tools for researching druggable pathways in the parasites [25].

An important feature of the *glmS* system is its ease of use, simple molecular cloning, and addition of a molecule to induce knockdown. However, research shows that the *Pf*DOZI-TetR approach accomplished a better knockdown than the glmS technique when used to study *Pf*ClpP or the apicoplast resident protease. This demonstrates that the same protein may respond distinctly to different conditional systems. Unlike the more direct molecular cloning used to make glmS mutant parasites, the plasmids used to make *Pf*DOZI-TetR mutants are significantly bigger, making them

more susceptible to recombination. However, this particular issue may be combated by growing bacteria containing the plasmids at 30°C. It has been reported that the loss of aptamer copies can also occur in clonal parasites that originally possessed all ten aptamer copies, leading to loss of knockdown efficiency. In light of this, a newer, optimised version of the TetR system has been developed that is less prone to loss of the aptamer copies via recombination [30]. Another negative feature of the *glmS* system is that the drug used to induce knockdown, glucosamine, can be toxic to the parasites at certain concentration levels. However, both systems continue to be widely used to research *P. falciparum* biology [31].

3.5 Conditional knockdown of protein function

Knockdown atprotein level is advantageous because the protein of interest is directly targeted instead of target transcription or translation. The FK506 binding protein destabilisation domain (FKBP-DD) is one of the first systems adapted to modify the protein levels in *P. falciparum*. FKBP-DD can be appended to the N or carboxyl terminus of the protein of interest to initiate its degradation via the proteasome [32]. Another similar protein degradation system was applied to *P. falciparum* through the use of dihydrofolate reductase (DHFR) domain derived from *Escherichia coli*. The DHFR degradation domain (DDD), contains mutations that cause the unfolding of the domain. This unfolded domain is then targeted for degradation in the absence of trimethoprim (TMP). This technique was first employed in *P. falciparum* asexual parasites to demonstrate the ability to degrade yellow fluorescent protein-tagged with the degradation domain and to establish the requirement of the proteasome subunit RPN6 [33]. The TMP concentrations involved in stabilising the DDD domain are lethal to the parasite. To combat this particular problem, DDD-mediated protein knockdowns are performed in a TMP-resistant parasite line that has the human DHFR cassette integrated into the nonessential gene, plasmepsin I. This system has also been widely used to study chaperone functions in the asexual stages of *P. falciparum*. DDD tagging of a cytoplasmic chaperone (HSP110) reveals that chaperones are not targeted for degradation in the absence of TMP. Instead, inhibition was achieved through the chaperone binding to the unfolded domain, thereby preventing interactions with client proteins [34]. However, there are drawbacks to both the FKBP-DD and DDD methods. Both necessitate the addition of domains to the protein's N-terminus or carboxyl terminus, which may affect the protein's function or localization. Furthermore, both systems necessitate the ongoing supply of a stabilising ligand, such as TMP or Shld1, to the parasite in order to sustain steady expression of the protein of interest. Shld1 is toxic to asexual parasite development at high concentrations, while TMP is lethal to parasites unless used in a parasite line that expresses the hDHFR gene [35].

One other option which does not utilise an unfolded domain is the knock sideways (KS) system. This system relies on subcellular relocalisation of the protein of interest to disable the protein functionality. A KS system was recently established in *P. falciparum*, using the rapamycin-dimerizable domains FRB and FKBP. In a parasite line expressing a "mislocalizer" fusion protein, the protein of interest is fused to two copies of the FKBP domain. This mislocalizer is made up of the FRB domain, which has been engineered to localise to the nucleus and/or the plasma membrane, causing the protein of interest to relocalize to one of these places when rapamycin is added. As with the previously discussed degradation based system, the KS system likely has similar limitations. One such limitation being that the the protein of interest must be amenable

to fusion with larger domains. Also, it must have access to the mislocalizer protein. Exported proteins may be expected to have limited utility. This may be countered by expressing and exporting a new mislocalizer protein in addition to the previously exported proteins [36].

4. Need for new antimalarial strategies

Artemisinin-based combination treatments (ACTs), which have only lately been chosen as the last choice in the fight against malaria, are already being challenged by ACT-resistant strains discovered in Southeast Asia. With parasite resistance to all currently available antimalarial medications spreading, successful control and eradication will require the development of more efficient tools and cost-effective antimalarial methods.

Chloroquine-resistant transporter (crt), Multidrug-resistant protein (mdr1), Kelch13(k13), Bifunctional dihydrofolate reductase (dhfr), Dihydropteroate synthetase(dhps), and cytochrome b are the drug resistance genes of *P. falciparum*. Drug resistance is mostly linked to single nucleotide polymorphisms. To discover the probable genetic alterations that result in drug resistance, next-generation sequencing technologies such as end-to-end Illumina targeted amplicon deep sequencing (TADS), and Malaria Resistance Surveillance (MaRS) was created [37].

5. Methods to identify the drug resistant gene of *P. falciparum*

Over the last few decades, a number of molecular genotyping approaches for tracking antibiotic resistance in clinical isolates have been developed and implemented. RFLP analysis [38], molecular beacons [39], real-time PCR [40, 41], dot blot probe hybridization [42], and single-nucleotide primer extension [43] are all examples of traditional approaches. High-resolution DNA melting (HRM) [44, 45], and SNP-based custom genotyping assay [46, 47], TaqMan allelic discrimination assay [48], mass spectrometry-based SNP genotyping [49], ligase detection reaction fluorescent microsphere (LDR-FM) assay [50], and loop-mediated isothermal amplification (LAMP) [51] are some of the more recent high-throughput methods. While these techniques allow for the detection of known drug resistance alleles of a given gene, they do not allow the discovery of novel genetic polymorphisms involved in drug resistance [52].

5.1 Genomics study

Sanger sequencing and Next-generation sequencing (NGS) are the methods that allow fragment sequencing, where NGS is appropriate for high throughput methodologies.

5.1.1 Genomics study

Sanger sequencing, or the chain termination method, is used to sequence DNA regions up to 900 base pairs in length. Fred Sanger, a British biochemist, and his collaborators invented Sanger sequencing in 1977. Sanger sequencing is still widely used for the sequencing of individual pieces of DNA, such as fragments used in DNA

cloning or generated through a polymerase chain reaction. Sanger sequencing is still commonly used for sequencing specific pieces of DNA, such as fragments used in DNA cloning or created through polymerase chain reaction (PCR), despite the fact that genomes are now generally sequenced using alternative faster and less expensive methods. Compared to the Sanger sequencing approach, NGS detected a much larger number of mutant alleles. Sanger's method of DNA sequencing is less sensitive than NGS [53].

5.1.2 Next-generation sequencing

Next-generation sequencing (NGS) is a massively parallel sequencing technology with extremely high throughput, scalability, and speed. This approach has been applied to determine the order of nucleotides in entire genomes or individual DNA or RNA segments. NGS has changed biological sciences, allowing labs to conduct a wide range of experiments and investigate biological systems on a scale that was previously unattainable. The methods for fragment sequencing are Sanger sequencing and next-generation sequencing (NGS), with NGS being more ideal for high-throughput procedures. Targeted Amplicon Deep Sequencing (TADS) and Malaria Resistance Surveillance (MaRS) are two of the most sensitive NGS-based technologies of relevance for antimalarial drug resistance surveillance [38]. The Roche 454, the Applied Biosystems SOLiD, and eventually the Illumina® (formerly known as Solexa) Genome Analyser and Hi-Seq platforms have been commercialised in the last few five years. Illumina® sequencers can presently produce up to 2 billion reads each run, with a recommended read length of 35–100 bp [54]. This value is constantly increasing due to steady advances in reagents and consumables.

6. Protein–protein interaction study

Artemisinin-resistant malaria parasites are emerging, as evidenced by prior reports of resistance to other antimalarial medicines. A greater understanding of the parasite's biology is essential for unravelling the mechanisms behind the emergence of drug-resistant strains and identifying better pharmacological targets. In the late 1990s, a ground-breaking effort to sequence the genome of *P. falciparum* was launched. *P. falciparum* 3D7's 22.8 megabase genome was sequenced and annotated in 2002 [17, 55]. Compared to other known eukaryotic species, the malaria proteome is projected to contain fewer enzymes and transporters and more proteins related to cell adhesion and immune system evasion. Along with publishing the genome, the first high-throughput proteomics experiments, which provided a life cycle stage-specific perspective of the malaria proteome [56, 57], marked another major step forward in malaria genetics. Understanding an organism at the molecular level is difficult due to the highly dynamic nature of cellular machinery and the complicated interactions that all proteins have with one another. The first-generation malaria protein interaction network was revealed using high-throughput yeast two-hybrid (Y2H) systems [58].

Other high-throughput methods for inferring cellular networks, such as RNA expression profiles, genetic interaction data, and mass spectrometry analysis of protein complexes, have intrinsic limits that affect the network's coverage and accuracy. However, combining these disparate pieces of evidence into a single PPI

Dataset used for integration	Integrative algorithm	Data source for validation	Algorithm	Nodes (protein)	Edges (Interaction)	Publication
Phylogenetic profiles, Rosetta stone fusion protein dataset, Microarray expression datasets	Bayesian approach	Gene ontology (GO assignment) KEGG database	High confidence links (confidence score > 14)	3667	388,969	Date et al. [65]
Orthologs datasets, Y2H dataset, Pfam DDI dataset	Hypergeometric distribution	Microarray expression datasets, GO functional annotation	Markov-Cluster algorithm	2321	19,979	Wuchty et al. [66]
Orthologs datasets (Chaperones only), Y2H dataset				212 (chaperones)	344	Pavithra et al. [68]
Orthologs datasets, Y2H dataset		Yeast protein complexes, microarray expression datasets	Clique-percolation algorithm	1872	4918	Wuchty et al. [64]
Y2H dataset, Microarray expression datasets (Stage specific), Orthologs datasets, Metabolic pathways	Bayesian approach	GO functional annotation				Mitrofanova et al. [69]
Y2H dataset, Microarray expression datasets	Weighted Interaction Grap	GO functional annotation	Linear time algorithm			Oyelade et al. [70]

Table 1.
An outline of the malaria PPI network's computational inference and analysis to date. (microarray expression datasets — Transcriptome data; Y2H dataset — Yeast two-hybrid generated interaction).

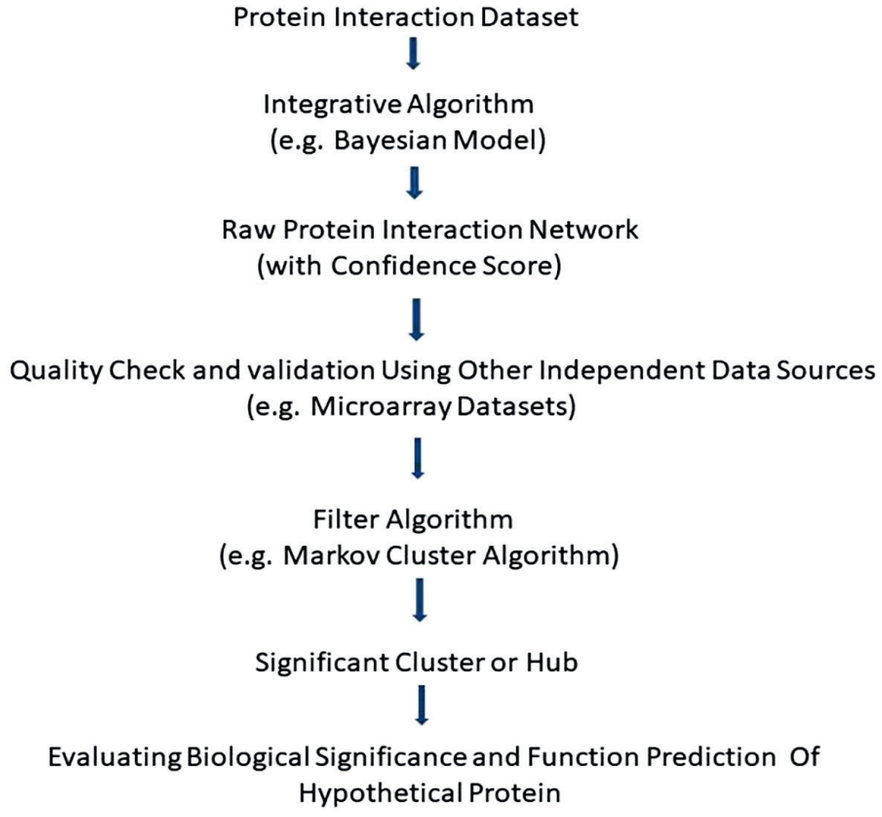

Figure 2.
Methods to construct PPI network of P. falciparum.

network improves accuracy and coverage. Such integrations can be done in software, and they have been used to predict the PPI network in yeast [59, 60]. A Y2H data collection [58], microarray expression profiles [61, 62], and more recently, an RNA-Seq dataset [63] are the experimentally determined datasets available for building malaria protein interaction networks (**Table 1**). Various computationally created datasets, such as the ortholog dataset [64], phylogenetic profiles [65], and P*fam* domain-domain interactions (DDI) dataset [66], have been utilised to supplement these datasets. These derived datasets are based on homology searches, with the only difference being the data range for which the search is conducted. Interlogs and protein–protein interactions are predicted using these databases. These datasets are collected from independent sources, and the linkages are measured by combining different parameters. Integrating the various parameters would necessitate a data integration Framework or algorithm. For example, a Bayesian framework is an integrative algorithm. **Figure 2** depicts the methods used to construct PPI network of *P. falciparum*.

Agamah *et al.,* curated data of pathogen and host selective genes, protein–protein interaction datasets, and data from literature and databases to perform network-based analysis of human host and *P. falciparum*. The study revealed 8 hub protein targets essential for parasite and human host-directed malaria drug therapy. In a semantic similarity approach, 26 potential repurposable anti-inflammatory drugs inhibiting residual malaria infection were put forward that can be appropriated for malaria treatment. Further analysis of host–pathogen network shortest paths enabled the prediction of immune-related biological processes and pathways impaired by *P. falciparum* to increase its survivability within the host [67].

7. Combined computer-aided drug designing (CADD) and virtual screening

Computer-aided drug discovery (CADD) can be described as utilising computer technology and software to elevate drug discovery efforts. It is also known as *in silico* drug discovery and is the application of computational methods to guide and systemise the process of drug discovery and its various stages. The two most commonly reported computational approaches to drug discovery are structure-based methods (primarily molecular docking studies) and ligand-based approaches such as ligand-based pharmacophore methods and three-dimensional quantitative structure–activity relationship (3D QSAR) models. Many therapeutically relevant malarial proteins have been identified and characterised, from receptors and transporters involved in membrane transport and signalling to enzymes used in biosynthesis. Many structure-based antimalarial drug development studies have been predicated on this diversity of protein targets [68].

Structure-based drug discovery (SBDD) searches for novel compounds that work against a specific target using information from the target's structure. The crystal structure of the drug target, preferably co-crystallised with a known ligand, can be used to extract the 3D structural information. It aids in the discovery of new ligands by identifying them from virtual chemical libraries or directing the design of novel compounds. The most commonly used SBDD approach is molecular docking, whereby computer software simulates ligand-target binding and predicts binding conformations and molecular interactions between ligands and target macromolecules. In the absence of a 3D crystal structure or a realistic homology model of the therapeutic target is unavailable, a ligand-based drug design (LBDD) technique can be useful. In research when the active inhibitors/ligands are known, ligand-based approaches are a potential alternative. Ligand-based pharmacophores are constructed using molecular descriptors of known active ligands. These models identify the ligand characteristics that are required for ligand-target compatibility, such as H-bond acceptors/donors, hydrophobicity, ionizable groups, and so on, making it easier to run comparison searches of huge compound datasets [68].

Drug development attempts for antiplasmodial drugs commonly use structure-based virtual screening techniques. Dahlgren et al. used structure-based screening of the Maybridge and ZINC drug-like databases to find four small-molecule inhibitors of *Plasmodium falciparum* macrophage migratory inhibitory factor (*Pf*MIF; PDB: 2WKF) [69]. In another study by Carrasco et al., 4 active compounds were identified against the chloroquine-resistant W2 strain *of P. falciparum*. For their study, they used the structure-based virtual screening a drug-like database included in the MOE package [70]. Similarly, a potential antiplasmodial against the *Plasmodium falciparum* FKBD35 protein was identified by Harikishore *et al*. by screening the database, ChemDiv, applying the same approaches. A structure-based pharmacophore model was developed 1st which was based on the *P. falciparum* FKBD35-FK506 X-ray crystal structure. As a result of pharmacophore-based screening of the ChemDiv library, a condensed library of 13,000 compounds was presented. ADME (absorption, distribution, metabolism, and excretion) filters were used to refine the library, yielding 2600 compounds. In docking investigations of the focused library into the active site of *P. falciparum* FKBP35, the docking software GOLD was used [71].

Lima *et al*. utilised a combi-QSAR approach, combining 2D- and 3D-QSAR models, in a virtual screening study of the ChemBridge database for a selection of

new antimalarial virtual hits. This was followed by in vitro experimental tests of the potential *Pf*dUTPase inhibitors against chloroquine-sensitive and multidrug-resistant strains of *P. falciparum*, in an effort to identify new potential and selective antimalarial hits [72].

CADD approaches represent the best opportunity so far to enhance productivity pipelines not only in malaria, but also in various other diseases [73]. CADD is a very appealing venture for improved and efficient drug discovery for not only tropical infections but also for non-tropical diseases, due to the easy availability of many diverse chemical libraries in pharmaceutical companies, virtual discovery organisations, and academic institutions, as well as the increased genomic information that facilitates computational predictions [74–76].

8. Conclusions

Malaria is one of the deadliest infectious diseases in the world with no certified vaccine against malaria pathogens. Artemisinin and quinine are two most efficacious drugs that prevail. Unfortunately, ACT (Artemisinin-based combination therapies) resistant strain has been discovered in Southeast Asia. *P. falciparum* has developed drug resistance through single nucleotide polymorphism (SNP) in its genome. Traditional methods have been utilised to identify SNPs, however, with the advancement of NGS technologies, new possibilities were being opened up in the malarial research development as well as in the primary and applied field. As the new genomic data revealed a large number of processes, which can transform it into new therapeutic strategies. New quantitative analysis methods are continually being developed and tested for the most accurate analytical approach in the case of the *Plasmodium* genome. Studies in genomics and Systems Biology have made a huge contribution towards a better understanding of the *P. falciparum* parasite. Most significantly, they have built an impressive pipeline of novel therapeutic targets and several potent vaccine candidates. Along with the malarial parasite, the genomic study has also contributed to the genetic factor of humans that have a significant vulnerability and response against both malaria and its potent drug or vaccines. Beyond new drug and vaccine candidates, genomics has also contributed towards the drug mechanism of action. Thus, integration of both the genomic and system can lead to a better eradication of malaria strategies.

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Conflict of interest

The authors declare no conflict of interest.

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