Omics: Approaches and applications related to diagnosis, treatment, and control of parasitic diseases. Part I: *Plasmodium* spp.

Review Article

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ABSTRACT

Over the last two decades, omics studies provide a revolutionary advance of datasets in the field of Medical Parasitology for understanding parasite system biology, host-parasite interactions, and phylogenetic analyses; i.e., genomics, transcriptomes, proteomics, metabolomics. Together with bioinformatics, genome-wide associated studies (GWASs) enabled scientists to identify diagnostic biomarkers, promising drug targets, and potential vaccine candidates for diagnosis, treatment, and protection against several neglected tropical diseases. Omics approaches are either structural (genomics) or functional (post-genomics). To survive, *Plasmodium* spp. are able to delete certain genes unessential for their survival and growth, enabling them to evade host immune response. In addition, they undergo antigenic variations that lead to gene mutations in enzymes controlling drug uptake. Previously unattainable goals, e.g., host immunoevasion, susceptibility or resistance to infection, drug resistance, novel drugs as well as prevention and control were achieved by omics studies powered by bioinformatics tools. This part of the present review aims to shed light on omics application outcomes regarding *Plasmodium* spp.

Keywords: bioinformatics, diagnostic biomarkers, drug target, malaria, novel drugs, omics, *Plasmodium* spp., vaccine candidates.

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Abbreviations: GWAS: Genome-wide associated study; **NGS:** Next generation sequencing; **PKs:** Protein kinases; **PTM:** Post-translational modification; **RNAi:** RNA interference; **SNP:** Single nucleotide polymorphism.

The primary objective of the present part is to highlight major applicable outcomes from omics studies in *Plasmodium*, i.e., diagnostic, therapeutic, as well as prevention and control with subsequent elimination of malaria. Secondary objectives include other applications related to molecular epidemiology, drug resistance, as well as host resistance and susceptibility to malaria. The review based on PubMed research deals also with approaches and methods utilized in omics studies.

Parasite omics provides unlimited datasets with revolutionary applications in several fields of molecular epidemiology, diagnostic biomarkers, drug resistance, and potential therapeutic targets or vaccine candidates. It is a comprehensive terminology involving genomic knowledge including several disciplinary biological fields, i.e., sequencing, function, evolution, and editing. Genetic mapping by linkage, also known as genome-wide associated studies (GWASs), are performed by selection of genomic regions under strong recent selection, e.g., candidate resistance loci, followed by genetic manipulations to demonstrate causality or wide expression analysis to identify genetic variation mapping[1]. Both functional omics studies (transcriptomics, and proteomics) refer to RNA transcription, and expressed proteins, respectively. They are performed to identify gene transcription with its protein expression that has an essential impact on the parasite survival, growth, and/or virulence[2]. Epigenomics, also GWAS, are performed to study the complete set of epigenetic modifications, i.e., stable changes regulating gene expression. Epigenetic landscape plays a fundamental role in understanding the biological processes that involve DNA manipulation and expression[3].

It is worth mentioning that functional genomics yielded several terms such as metabolomics, immunomics, phosphoproteomics, chemogenomics, kinomics, and secretomics. Metabolomics identify the functional metabolite profile expressed in different life cycle stages, i.e., metabolic enzymes and co-factors maintaining the parasite cellular structures and their biological functions. Such studies strengthen knowledge of host-parasite interactions in each stage with relevant outcome in identifying novel potential drug targets[4]. While immunomics identify parasite

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epitopes recognized by host's immune system at the molecular level. Simultaneous determination of the interaction between a specific molecule and chemical compounds is referred to as chemogenomics[5]. The latter is an efficient approach for drug design. While kinomics refers to protein kinases (PKs) encoded in its genome, phosphoproteomics identify, catalog, and characterize proteins containing a phosphate group as a post-translational modification (PTM). Required for protein phosphorylation, PKs are essentially involved in signaling pathways that transmit cellular signals in all eukaryotes. In such a process, they regulate protein functions involved in the parasite survival, growth, differentiation, and virulence[6]. Phosphorylation is a key reversible modification that regulates protein function, subcellular localization, complex formation, degradation of proteins and therefore cell signaling networks. It was estimated that between 30-65% of proteins were phosphorylated[7]. Finally, secretomics refer to all the secreted proteins by a cell, tissue, or organism, explaining their role(s) in diseases pathology and immunomodulation of host immune response[8].

On the other hand, bioinformatics (omics backbone) constitutes an interdisciplinary digital approach with computational, mathematical, statistical, and physical analyses necessary to analyze sequencing and functional data obtained from genomic and post-genomic studies, with subsequent conversion to useful information for further applicable outcomes[9], such as:

1. Understanding the molecular mechanisms of integrative parasite systems biology, and host-parasite interactions might lead to discovery of new efficient candidates for diagnosis, as well as drug and vaccine design development[10].
2. Due to host-parasite interactions, there are changes in specific amino acids that affect their chemical properties, e.g., PTMs such as phosphorylation, acetylation, deacetylation, methylation, and glycosylation. In this regard, PTMs are metabolic fingerprints (biosensors) utilized in diagnosis. Mobile ultrasensitive protein detection devices are developed for use as point-of-contact devices for detection of specific biomarkers[11].
3. Phylogenetic tree construction: Recent phylogenetic analyses rely on sequencing of multiple genes from different genomic sources, or genes expected to evolve under different selective conditions. This is preferred to eliminate false homology resulted from natural selection[12].
4. Molecular epidemiology studies become valuable in waterborne outbreaks to identify the causative pathogen, species and source of infection[13-15].
5. Nowadays, it is possible to perform simultaneous high throughput screening in parallel DNA sequencing in several parasites in multiple samples, whether biological or environmental, i.e., metabarcoding. This allows identification of barcodes or short DNA sequences of a gene or multiple genes[16].
6. Landscape genetics is an interdisciplinary field correlating population genetics and landscape ecology. Utilizing bioinformatics, data obtained from landscape community genetics reveal how ecological variations can influence the genetic structure and in-turn explains the basis of resistance and susceptibility to diseases[17]. In this context, understanding the potential role of epigenetic factors and the molecular mechanisms occurring in the resistance and susceptibility to some parasitic diseases would explore novel strategies in control measures and prophylaxis regimens[3].

Approaches

Relative to microarrays, next-generation sequencing (NGS) becomes an option for genome or transcriptome or proteome sequencing since it provides more complete and accurate data in addition to its low cost. Moreover, it becomes easy to produce and sequence a great number of DNA fragments in parallel, without the need for bacterial cloning, in contrast to the other technologies previously used[18]. Rapidly promoted by NGS, transcriptome sequencing (RNA-seq) allowed the investigators to reconstruct the entire transcriptome in a selected species of interest and generate quantitative expression scores for each transcript. Analysis of results obtained from RNA-seq provide quantitative survey of RNA expression patterns in comparative genomic-levels and identification of more molecular markers[1]. Spatial transcriptomics is a recent technology enabling biologists development of *in situ* single cell RNA sequencing, i.e., within a tissue or organ[19]. This is accomplished using either sequential-fluorescent *in situ* hybridization, or fluorescent-labeled cDNA. Such applications allowed investigators to discover novel anti-parasitic agents that target parasite crucial for its survival and virulence[20,21].

Interference RNA (RNAi) or double stranded-RNA (dsRNA) treatment are the major methods utilized for functional genomics, i.e., studying gene impact on parasite phenotype by experimental gene knocking down experiments[22]. Generating new gene function (knock-in), gene editing, is nowadays performed by the recent evolutionary technology "clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9)". In such technology, Cas9 enzyme is bound experimentally with a short RNA guide sequence that targets a specific sequence of genomic DNA. Using CRISPR-Cas9, precise changes in the eukaryotic genome facilitates removal of genetic material or addition of a customized genome sequence[23,24]. In a recent review, Jacinto *et al*.,[25] discussed advances in genetic engineering to modify CRISPR-Cas9. The CRISPR-Cas9 system in bacteria was developed in which bacteria slices-DNA from invading bacteriophages to create DNA segments (CRISPR arrays) that recognize bacteriophages DNA on reinfection, i.e., bacterial infection by phages[25].

Other technologies were also used in omics studies. Among them was RNA sequencing-based studies that identified the role of noncoding protein RNA (ncRNA) transcriptomes that play an essential role in parasite virulence[26]. It is worth mentioning that ncRNAs constitute heterogeneous RNA types that play crucial biological functions in eukaryotes, but not coding for protein synthesis. They include ribosomal RNA, transfer RNA, small nucleolar RNA, and long noncoding RNA[27].

High-resolution mass spectrometry was utilized in metabolomics studies to provide a chemical fingerprint of thousands of metabolites present in cells, tissues, or body fluids. Such metabolic phenotyping was successfully used to study various biologic processes (drug susceptibility or resistance) and disease states (subclinical, acute and chronic)[4]. Genome wide-single nucleotide polymorphism (SNP) analyses that involves germline substitution of a single nucleotide in the parasite genome to pinpoint gene mutation linked to drug resistance, was recently reviewed[28].

Two additional methodological strategies emerged in the field of bioinformatics, aptamers, and cell-free expression systems. Aptamers are DNA or RNA oligonucleotides or peptides with protein-binding characteristics that interact with high affinity, to a specific target; either diagnostic biomarker, or drug target or vaccine candidate. Escort aptamers are new delivery systems carrying therapeutic agents or fluorescent particles targeting specific cell or pathogen, whereas systemic evolution of ligands by exponential enrichment (SELEX) technology is applied for selection and isolation of aptamers that target specific pathogens (aptasensors). It constitutes repeated cycles of oligonucleotides binding and amplification to reach highest affinity interactions[29]. On the other hand, absence of a cell wall and membrane provides an open environment allowing direct gene translation from linear DNA fragments without requiring DNA cloning into vectors, i.e., recombinant form. *Escherichia coli* or wheat germ cell-free is used as *in vitro* transcription and translation system. These cell-free expression systems, used in protein synthesis, are highly throughput, rapid, and consume less time in gene cloning by synthesizing proteins directly from PCR products[30].

Furthermore, there are several supportive websites, widely utilized by researchers across the world, for automated systems that enable storage and analysis of knowledge related to molecular biology, gene sequencing, and bioinformatics. Among them, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih), GenBank (http://www.ncbi.nlm.nih.gov), and 70 best Bioinformatics Blogs and Websites (https://www.blog.feedspot.com/bioinformatics_blogs/). Regarding malaria, a consortium of 15 leading scientific laboratories established an association termed 'The Malaria Drug Accelerator (MalDA)' and created a website (https://www.malariada.org/). Its main objective is to eliminate the structural barriers often encountered in approaches for drugs discovery, i.e., improving and accelerating antimalarial drugs development. To achieve this, it provides recent strategies to identify novel essential druggable targets; it seeks to produce early lead inhibitors for further preclinical development and subsequent clinical trials; and provides resources for researchers, e.g. expertise, knowledge, materials, and reagents. Moreover, malaria genomics has several central data repositories, e.g., PlasmoDB (http://www.plasmodb.org) and the Pf3K project (www.malariagen.net/projects/ parasite/Pf3k) for parasite data, as well as VectorBase (vectorbase.org) and the Ag1000g project (www.malariagen.net/projects/ vector/ag1000g) for vector data.

Applications

Potential drug targets in apicomplexans

Most apicomplexans lack transcription factors commonly detected in all eukaryotes, and this explains the expansion of new and specific transcription factors such as the apicomplexan enzyme, integrase DNA with a binding domain (AP2) that has high affinity primary and secondary binding motifs[31]. This complexity of expansion played a crucial role in chromosome biology, gametogenesis, stage-to-stage differentiation, invasion and egress cascade, and virulence[32]. On the other hand, synthesis of DNA and RNA copies is a multi-step process that requires tight regulation of translation initiation factors. It is carried out through either cap-dependent or cap-independent processes, i.e., in stress conditions. In *P. falciparum* and *T. gondii*, upstream open reading frame (UORF) pathway takes place to initiate gene expression in response to stress. Indian reviewers discussed UORFs involved in the majority of transcripts in both parasites, and concluded their essentiality in translational regulation of gene expression, i.e. promising drug targets[33].

In addition, apicomplexans mitochondria have an essential role in several biological functions, e.g., energy metabolism, respiration, iron-sulfur clustering, calcium homeostasis, phospholipid biosynthesis and apoptosis. Therefore, two wide-genomic studies demonstrated that apicomplexan mitochondria were a rich potential source for drug targets[34,35]. In contrast, *Cryptosporidium* spp. represent an exception where the classical double-membrane mitochondrion is absent and instead, a reduced form of the organelle is present (a mitosome) with few retained functions[36].

Besides, PTMs are essential for viability and growth, developmental transformation, asexual reproduction, invasion and egress cascade, as well as pathogenesis, and virulence[37]. These PTMs are highly noticed in the molecules comprising the gliding motility in apicomplexans. Phosphoproteomic studies revealed

three issues. First, PTMs modified by methylation or phosphorylation enhanced invasion and egress cascade[38]. Second, calcium dependent PK (CDPK) was involved in phosphorylation essentially required for gliding motility in *P. falciparum* (*Pf*CDPK1)[38], and *T. gondii* (*Tg*CDPK3)[39]. Third, marking of the target protein by ubiquitin molecule for degradation was linked in cytoskeletal rearrangement of *Toxoplasma* gliding motility[39].

A group of Ras superfamily, Rab cell membrane-bound proteins, control the surface proteins trafficking and fusion of transport vesicles. They are small guanosine triphosphate (GTP)-binding membrane proteins, amd their encoding genes are of particular interest in apicomplexans. The Rab proteins act as molecular intracellular switches through regulation of trafficking between secretory organelles and cellular pathways. They switch between active and inactive forms, where the former binds with GTP, and the latter with guanosine diphosphate (GDP). To activate Rab proteins, Rabs A guanine nucleotide exchange factor catalyzes the conversion from both forms[40].

Plasmodium spp.

Host parasite interactions

- A study analyzing the transcriptome of *P. falciparum* intra-erythrocytic stages, proved that *Plasmodium* spp. possessed a specialized mode of transcriptional regulation facilitating RBCs invasion. They demonstrated that some regions of *Plasmodium* chromosomes varied between different *Plasmodium* strains and played key role in enabling erythrocytic stages transcriptomes, i.e., evidence of their ability for instantaneous on and off strategy according to erythrocytic cycle differentiation[41]. Later, similar results were obtained when British investigators conducted a single cell transcriptomic study that facilitated analysis of cell-cell transcriptomic variability. The investigators demonstrated two genes (*var* and *pir*) acting independently by switching on and off in each individual erythrocytic stage. They proved that both genes were involved in RBCs sequestration, chronic infection, and host immunoevasion. Moreover, sex-specific expression of *var* and *pir* genes suggested their role in microgametocytes fertility. Accordingly, both genes were suggested as novel potential therapeutic targets and vaccine candidates[42].
- Utilizing a proteome-reverse genetic approach, American investigators conducted a study in the mosquito vector. They observed differences in proteins mastering two types of sporozoites collected from salivary glands and developed oocysts. Proteins, assigned for hepatocytes invasion, predominated in the former such as sporozoite microneme protein, apical membrane antigen and surface protein P36. In contrast, a membrane antigen erythrocyte binding-like protein, important for salivary glands invasion, was detected in sporozites in developed oocysts. Other proteins, e.g. thrombospondin-related protein and circumsporozoite surface protein were observed in both sporozoites, suggestive of sporozoite development[43].
- Utilizing RNA interference (RNAi) knockdown, the investigators demonstrated that *P. falciparum* egress from hepatocytes and erythrocytes were dependent on a host signaling cascade in which guanine nucleotide-binding protein subunit alpha q (GNAQ) was the key factor. The latter activates intracellular signaling pathways in response to activation of cell surface G protein-coupled receptors (GPCRs)[44]. Host signaling cascade was stimulated by *P. falciparum* GPCR ligands and PK C activation with subsequent calcium influx and stimulation of host cysteine protease (calpain 1). Its increased expression resulted in weakening and digestion of host cells cytoskeleton with subsequent egress[45]. Later, an omics study attributed merozoites egress from hepatocytes to merosomes formation carrying egressed merozoites into the blood stream and infecting erythrocytes, i.e., parasitophorous vacuole (PV) rupture without lysis of host cell membrane. However, merozoites egress from the infected erythrocytes is by simultaneous lysis of both PV membrane and host cell membrane[46]. Utilizing CRISPR-Cas9 technology to produce GNAQ-deficient *HeLa* cells, the investigators recently confirmed the previous hypothesis (merosomes formation). Egress of *P. berghei* hepatic stages from *HeLa* cells was was not dependent on GNAQ in contrast to the erythrocytic stages[47].
- It was documented that *Plasmodium* spp. release exported proteins into the host cells that have a crucial role in host-parasite interactions and parasite development within the host. A recent omics study showed *Plasmodium* utilization of host specific pathways in order to survive and develop. To investigate the role of the host trafficking processes in extra-erythrocytic liver-stages development, and hepatocytes invasion, American investigators used a systematic RNAi screen of *P. berghei* genome. The most common were genes involved in protein transport between Golgi network and endosomal system, as well as genes encoding lipid endocytosis (clarthin-mediated trafficking). The latter facilitates lipids trafficking internalized across the plasma membrane into the mitochondria. This translates the subversion of host vesicular trafficking pathways by *P. berghei* genes for host hepatocytes invasion[48].

Pathogenesis

In *P. falciparum*, rigidity of infected RBCs and their adherence to the capillary blood vessels was attributed, in part, to increased RBC phospholipid levels with increased ratio of unsaturated to saturated fatty acids. In addition, production of micro-vesicles blebs out of the lipid rafts of the cell membrane of infected RBCs promote cellular signaling for gametocyte development[49,50].

Diagnosis and molecular barcoding

- Since *P. falciparum* metabolomics were extensively investigated, a study conducted in 2015 demonstrated a valuable approach to assess and predict the course of acute *falciparum* malaria in children. The investigators conducted a metabolomics study on *P. berghei*-experimentally infected mice to determine parasite metabolic profile during acute infection. For determination of several metabolites, blood samples were collected from infected children categorized into mild, moderate and severe according to their clinical conditions, as well as from controls. A multi-variant correlation analysis demonstrated usefulness of metabolic profile in categorization of malaria without depending on the clinical examination. Besides, it guided clinicians to assess disease progression, and evaluate therapy efficacy[51].
- Molecular barcoding of *P. falciparum* showed 24 SNPs that in combination created a fingerprint or signature for its genome. Characteristics of SNPs enabled the clinicians and health authorities to 1) distinguish recrudescence from re-infection in field trials, 2) monitor *P. falciparum* frequency and distribution in a patient population undergoing drug treatment, and 3) identify geographic origin of *P. falciparum* strains to regional level[52].
- Recently, a study developed a novel low cost, high throughput, automated method to identify mixed infections of *P. vivax* and *P. falciparum* with the exact proportion of each species in infected patients from Pakistan and Afghanistan. Utilizing metabarcoded DNA sequence of 18 subunit ribosomal DNA (18S rDNA), the investigators demonstrated the differential expression of certain parts of the genome in different stages of infection in humans. This molecular barcoding approach enabled the investigators to identify the causative *Plasmodium* spp., and its evolution during the cycle of infection. Besides, it allowed monitoring intra-species differences according to the geographical area[53].

Drug resistance

The worldwide antimalarial resistance network (wwarn.org), a global network of academic experts, was established aiming to identify and track the global spread of malaria drug resistance. As previously reviewed[28], anti-malarial drugs resistance are associated with established definitive genetic markers. *In vitro* evolution and whole-genomic analysis is nowadays a new approach to identify the molecular mechanisms of anti-malarial drugs resistance, i.e., *Plasmodium* resistome[54]. Therefore, identification of molecular markers is a valuable tool for strengthening resistance monitoring, confirming resistance, and providing an early warning signal, and for assessing resistance trends[55]. To demonstrate the resistance mechanism, a study conducted against a synthesized endoperoxide compound (N89), showed anti-malarial potency *in vitro*, and *in vivo*[56]. Using whole-genome sequencing, the investigators compared and described gene mutations in the susceptible and emerging resistant strains. Among the detected seven genes with mutations, only the gene encoding multidrug resistance protein 2 showed the highest mutation in N89 *P. falciparum* resistant strains, while the gene encoding endoplasmic reticulum-resident calcium binding protein (*Pf*ERC), N89 drug target showed similar gene mutations in both strains[56].

Potential drug targets

Several omics studies were conducted and revealed molecules essential for *Plasmodium* survival and some of them were established virulence factors.

- In 2004, British investigators[57] used a variety of bioinformatics tools to identify and characterize 65 *Plasmodium* eukaryotic PKs (ePKs) sequences aiming to classify them according to the established ePKs groups. Unfortunately, results revealed that several ePKs sequences did not cluster within any ePKs group. However, the highest number of ePKs involved in proliferation of erythrocytic stages included mitogen-activated PK (MAPK) and cyclin-dependent kinases (AGC group). The later included cyclic adenosine, guanine, and cytosine MP-dependent PKs (PKA, PKG, and PKC, respectively). The investigators also observed that none of the ePKs clustered within the tyrosine kinase group[57].
- In 2008, a comparative genomic study of apicomplexans Rab GTPases, identified and characterized peptide motifs of *Plasmodium* Rab GTPases involved in regulating membrane proteins trafficking from the apical secretory organelles (rhoptries, micronemes and microspheres) to cell cytosol, apicoplast, food vacuoles and Maurer's clefs. In contrast, motifs involved in the other direction (intracellular pathways → secretory organelles) were not identified. Because apicomplexans Rab GTPases are promising drug targets, the investigators recommended future studies focusing on identification of the peptide motifs of *Plasmodium* Rab GTPases to develop novel anti-malarial drugs[40].
- In 2009, two studies were conducted. The first study utilized RNA aptamers and intramers as treatment tools to target erythrocyte membrane protein 1 and intracellular parasite proteins of *P. falciparum*[58]. Metabolic labeling by radioisotopes demonstrated that *P. falciparum* synthesized carotenoids, an absent pathway in mammals, for parasite antioxidant protection. Accordingly, the investigators proposed carotenoid synthesis pathway a promising drug target and/or vaccine candidate[59].
- In 2010, another two studies were conducted. Spermidine synthase (SpS) enzyme, involved in *de novo* synthesis of *Plasmodium* polyamine spermidine, proved essential for survival, growth, and proliferation. Since cyclohexylamine exhibited inhibitory activity against SpS, the investigators suggested *Plasmodium* SpS a novel drug target[60].

Another metabolomic study demonstrated the essentiality of peroxisomes, specific *P. falciparum* organelles, in redox signaling and lipid homeostasis. Utilizing a comparative genomics approach, the investigators predicted peroxisomal proteins *in silico*. Since purified peroxisomes contained a highly diverse enzymatic network, the investigators observed that these proteins contributed to several crucial metabolic processes, e.g., fatty acid oxidation, ether lipids biosynthesis, and free radical detoxification. Due to their essentiality in survival and growth corresponding to the metabolic requirements of fast *P. falciparum* growth, peroxisomal proteins were suggested as potential drug targets[61].

- Rapid erythrocytic cycles require well-regulated lipid metabolic pathways that are essentially involved in survival, proliferation, and life cycle stages differentiation. Accordingly, proteomic and metabolomics studies on the role of phospholipases in *Plasmodium* spp., suggested phospholipid-hydrolyzing esterases virulence factors for their crucial role in membrane dynamics during *de novo* invasion and egress cascade, i.e., promising drug targets[62]. In this context, two additional events were observed. First, *Plasmodium* phosphatidyl-inositol-phospholipase C (PI-PLPC) was activated after RBCs invasion and induced high levels of intracellular calcium, with subsequent translocation of the molecules expressed from micronemes onto the parasitophorous vacuole membrane. Keeping in mind that increased intracellular calcium is the initial trigger for egress[63]. Second, since *Plasmodium* apicoplast is unique in its lipid composition such as cholesterol, sphingomyelin and ceramide, the investigators demonstrated that these lipids regulated apicoplast membrane permeability and activated membrane transporter proteins[64].
- Luth *et al*.[65] conducted *Plasmodium* genomic analyses and suggested dipeptidyl aminopeptidase 1 (a cysteine protease), and cyclin-dependent-like kinase 3 (a PK) as potential drug targets. Since genetic changes such as SNPs and copy number variants (CNVs) have essential roles in *P. falciparum* proteasome, the investigators recommended further analyses to identify novel drug targets[65].
- Severity of *falciparum* malaria is attributed partially to its ability to export atypical PKs (FIKKs) into infected RBCs causing major structural and functional changes. Members of FIKKs family are unique in *P. falciparum* with no orthologues in humans. Utilizing RNAi, the investigators succeeded to identify and characterize eight FIKKs as potential drug targets. Several functions were assigned including growth and survival *via* mitotic nuclear division, merozoites egress and *de novo* RBC invasion, as well as their role in infected RBC adhesive properties and cytoadherence phenomenon[66].
- Since *P. falciparum* potentiates a complex interaction with its host, a network-based integrative computational approach was utilized to conduct a GWAS study. The investigators analyzed the host-*P. falciparum* networks and the results revealed identification of two malaria disease-related genes with essential roles in enhancing parasite survival and development: C6KTD2 and C6KTB7[67]. The former was previously proposed as vaccine candidate[68]. However, a knockout study showed its essentiality in chromatin structure, histone lysine methylation, and gene expression. Thus, C6KTD2 proved essential for survival, growth and development of the erythrocytic stages, i.e., a potential drug target[69]. The second (C6KTB7) was previously established virulence factor due to its involvement in the ubiquitination pathway required for regulation of several cellular signaling crucial for *P. falciparum* virulence[70]. Notably, ubiquitination is the process of attaching ubiquitin, eukaryotic molecule to the protein of interest to be degraded.

Prevention and control

With the aim of reducing malaria transmission and subsequent eradication, applications of genomic data on protection and control programs against malaria were reviewed. The researchers focused on population genomic strategies dependent on connected metadata, e.g. clinical, epidemiological, and biological information[71]. In contrast, another review reported that although NGS technologies enabled researchers addressing a diverse range of biological and epidemiological questions regarding malaria transmission' dynamics, population genomic approach remains an inappropriately utilized resource for surveillance due to several reasons. They included 1) lack of local awareness and capacity, 2) limited access to sensitive laboratory methods, 3) shortage of associated computational tools, and 4) difficulty in interpreting genetic epidemiology data[72]. On the other hand, asymptomatic infections sustain malaria transmission since they provide a silent undetected source of infection, i.e., a major obstacle for malaria control and elimination programs. Kenyan reviewers recommended utilizing NGS technology, e.g., RNA sequencing (RNA-seq) for transcriptomic analysis of field samples collected from clearly defined asymptomatic patients[73].

One of strategies of prevention and control is acting against parasite epigenetic modifications with their crucial role in immunomodulation of host immune response. Epigenetic modifications also regulate protection and susceptibility to malaria. Among *Plasmodium* epigenetic modification were molecular mechanisms inducing phenotypic changes without genome alteration, e.g. epigenetic mechanisms that involve DNA methylation and histone modification. These epigenetic modifications showed modulation of gene expression regulating host immune response, e.g. activated T- and B-lymphocytes of the adaptive immune

response exhibited phenotypic changes[74]. A series of GWASs was reviewed with the aim of discovering genes, antigens, and molecules potentially suitable for development of an efficacious malaria vaccine. Such approaches enabled the biologists discovering new antigenic targets of antibody or T-cell responses suitable for further evaluation in clinical trials. The reviewers concluded that understanding the molecular mechanisms underlined *Plasmodium* epigenetic modifications on host T and B cell development and differentiation would certainly provide a new strategy for malaria prevention and control[75].

In fact, the potential role of epigenetic factors and their regulation in the protection of or susceptibility to malaria gained much attention in the last decade. Growing evidence suggested that epigenetics play a key role on multiple levels, including host immunoevasion and immunomodulation, tolerance, and host adaptive response. Further understanding of the mechanisms and functional significance underlying chromatin and DNA methylation changes, responsible for PTMs, will generate more strategies to develop a promising malarial vaccine[3].

Prevention of transmission

- Inhibition of zygote formation with subsequent failure of ookinete development was demonstrated by suppression of genes encoding certain components of ribonucleoprotein complex[76].
- Deletion of two *Plasmodium* genes, *p48/45* coding for 6-Cys repeat domain protein, and *hap2* led to complete sterility of the male gamete and failure to attach to the female gamete[77].
- Since xanthurenic acid (XA) is critical for ookinete development, isolated and purified organelles expressing XA enabled the investigators to identify XA metabolites essential for ookinete survival and development. An American review discussed applications of metabolomics with emergence of new strategies for prevention of malaria transmission, and proposal of a XA vaccine candidate[78].
- British scientists reviewed functional studies using reverse genetics aiming to discover several aspects of *Plasmodium* sexual development. They proposed several genes encoding certain molecules in sexual differentiation, fertility and transmission. They included mitogen activated PK-2, never in mitosis/aspergillus (NIMA) related PKs 2 and 4, and armadillo repeat motif. While the first PK-2 was proved essential for *P. falciparum* sexual replication, the second NIMA PKs were crucial for ookinete maturation. On the other hand, armadillo repeat motif is a characteristic repetitive amino acid sequence (~40 residues) found in several proteins that typically contain several tandemly repeated copies. Abnormal movement and sterility of microgametocytes was observed in the absence of armadillo repeat motif. Accordingly, the reviewers recommended further studies using CRISPR-Cas9 technology to express transgenic population of mosquitoes unable to transmit malaria[79].

Vaccine trials

- Indian reviewers claimed that *P. falciparum* proteomics and genomic sequence facilitated the discovery of novel subunit vaccines composed of purified proteins and polysaccharide antigens, and DNA vaccines, respectively. In the latter, an antigen-coding gene was inserted into suitable expression vector (plasmid) and the purified recombinant vector encoding the immunogen was injected into the host[80].
- An American proteomic study analyzed proteins expressed on the surface of *P. falciparum* infected RBCs, and identified two surface proteins (erythrocyte surface proteins 1 and 2). These were acknowledged for being encoded by a single gene copy and are conserved among the parasite isolates from different geographic areas; two criteria that make the identified proteins promising vaccine candidates as they bypassed the challenges of antigenic variations and genetic polymorphisms commonly observed in *Plasmodium* spp.[81].
- Utilizing mass spectrometry, a study analyzed the proteins on the surface of salivary gland sporozoites of *P. falciparum*. American investigators identified several expressed proteins that proved to have a crucial role in infection and RBCs invasion. Among them were circumsporozoite protein (CSP), sporozoite surface protein 3, thrombospondin-related anonymous protein (TRAP), apical membrane antigen 1 (AMA1), 6-Cys protein (P38) and heat shock protein 70 (HSP70). Since CSP and TRAP were glycosylated in *Plasmodium* sporozoites, both proteins were proposed as promising vaccine candidates for *falciparum* malaria[82].

CONCLUDING REMARKS

1. High-resolution mass spectrometry approach is a useful technique for both proteomics and proteomics related studies as metabolomics. While NGS and RNAi are used for gene sequencing and knockout, respectively, CRISPR-Cas9 is a new technology for gene editing.
2. The proteome-reverse genetic approach allows the study of the parasite systems biology. Understanding of the molecular mechanisms in host-parasite interactions was achieved by GWASs involving coding and non-coding RNA, metabarcoding studies, and SNP analyses.
3. Both *P. falciparum* metabolomics and molecular barcoding provide unique methods for diagnosis especially in asymptomatic patients. *Plasmodium* resistance studies identified molecular genetic markers in resistant strains that were valuable tools for drug resistance monitoring.
4. *Plasmodium* genome shows unique complex genomic plasticity, and drug resistance is the main obstacle in malaria elimination. This necessitates identification of novel potential drug targets and vaccine

candidates. Due to the advance in evolutionary technology by bioinformatics, few drugs, and vaccines for prevention of malaria transmission are now in clinical trials.

5. Bioinformatics tools were utilized to identify and characterize several potential drug targets including histone modifying enzymes involved in PTMs, established virulence factors, and apicoplast unique lipid composition. Besides, *P. falciparum* exported atypical PKs (FIKKs), and two malaria disease-related genes (C6KTD2 and C6KTB7) were recently identified.
6. *Plasmodium* epigenetic modifications showed several modulations of gene expression that regulate host immune response and confer innate protection. The recent technology of CRISPR-Cas9 was utilized to delete two *Plasmodium* genes (*p48/45*) developing an efficient transmission vaccine that led to complete sterility of microgametocytes. Genetically attenuated vaccines (pre-erythrocytic and erythrocytic stages) are now in clinical trials for protection against *P. vivax* and *P. falciparum*.

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