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Metabolomics and malaria biology

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Abstract

Metabolomics has ushered in a novel and multi-disciplinary realm in biological research. It has provided researchers with a platform to combine powerful biochemical, statistical, computational, and bioinformatics techniques to delve into the mysteries of biology and disease. The application of metabolomics to study malaria parasites represents a major advance in our approach towards gaining a more comprehensive perspective on parasite biology and disease etiology. This review attempts to highlight some of the important aspects of the field of metabolomics, and its ongoing and potential future applications to malaria research.

Keywords

Malaria; metabolomics

2. Introduction

Metabolomics in biology is the study of the entire repertoire of metabolites in a biological system [1]. This repertoire consists of many different classes of small molecules including sugars, amino acids and fatty acids. These small molecules perform critical functions such as energy storage, signal transduction, maintaining cellular structure, and feedback regulation of gene expression. Global as well as targeted metabolomic analyses have been widely and successfully used to study several pathogens that infect humans such as the opportunistic human fungal pathogen *Aspergillus fumigatus* [2], *Streptococcus pneumoniae* and *Staphylococcus aureus* that are common causes of pneumonia [3], hepatitis B virus [4], uropathogenic *Escherichia coli* strains [5], and *Bacillus cereus* [6] to list a few. Metabolomics of malaria is still in its infancy and only a handful of studies have been reported thus far [7-13]. Notwithstanding their recent application to malaria research, the remarkable versatility of the currently available metabolomic techniques and the extensive body of literature generated from their application in other fields, provide a strong platform to expand their application to the malaria field.

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Widespread resistance to most anti-malarial drugs and the unavailability of an effective vaccine have fueled the emergence of malaria in recent years as a major global health and economic burden [14]. Despite these hurdles, the field of malaria research has witnessed some extremely notable developments in the recent past including sequencing of the malaria genome [15], the application of proteomics to studying malaria life cycle [16], the malaria transcriptome [17], and several web resources such as MalVac (database of malaria vaccine candidates), PlasmoDB (genome database of the *Plasmodium* genus), and VarDB (database for antigenic variation gene families) [18-20].

Metabolomics adds a unique and much-desired dimension in our endeavors to better understand malaria parasite biology and discover novel therapeutic and vaccine targets. Furthermore, metabolomics represents an important tool that complements genomics and proteomics in enabling investigators to perform the most exacting and thorough analysis of biological systems [1,21]. This combinatorial systems biology approach of studying the multiple facets of a biological system would yield a detailed view of its intricate networks. In so doing, such an approach could inform many aspects of parasite biology including, but not limited to, basic biological models, drug development, biomarkers of infection, and models of human pathophysiology.

Mindful of the enormity of the field of metabolomics and the vast body of literature, this review attempts to highlight only some of its salient aspects. We provide an overview of the commonly used techniques and statistical approaches, and review the present state of malaria metabolomics research. We conclude with suggestive future applications of metabolomics in the field of malaria research, which could potentially lead us to an in-depth understanding of malaria parasites at both the basic and clinical levels.

3. Approaches to metabolomic analysis

The ultimate goal of metabolomics is to achieve a comprehensive and quantitative analysis of all the metabolites in a biological system, and to achieve the closest possible approximation of its physiology. Metabolites in a sample possess very diverse biochemical properties, and are present at varying abundance levels [22]. In addition, the characteristics of a molecule are influenced not just by its milieu but also its interactions with other molecules. A large-scale comprehensive analysis of the metabolites in a sample entails extracting and identifying thousands of compounds. Depending on the kinds of metabolites to be extracted, such analysis requires the development of tailor-made strategies for sample preparation, metabolite extraction and separation, and data analysis. Fortunately, numerous robust techniques have been developed to achieve an efficient and all-encompassing metabolomic analysis. However, it is important to note that a single extraction method cannot isolate/identify every molecule in the pool due to the diversity of the chemical structures. A prior knowledge of the types of molecules to be explored will dictate the protocols to be used, and the employment of multiple methods is essential to maximize the identification of the broadest range of molecules in a sample.

3.1 Extraction of metabolites

The different preparation methods that can be used to extract the most complete set of metabolites from a biological sample have been expertly reviewed by Dettmer *et al*. and Mashego *et al*. [23,24]. For microbes, at least two different extraction procedures (sequential and simultaneous sample processing) are employed[24]. The former involves separate and sequential quenching and metabolite extraction steps, whereas in the latter both quenching and extraction are performed in a single step [24]. The efficiency of metabolite extraction varies depending on the microbe and procedure used, and the nature of the microbe [prokaryotic (e.g. bacteria) versus eukaryotic (e.g. yeasts)] seems to dictate the best method

for extraction [24]. Extraction of compounds that are susceptible to hydrolysis (e.g. ATP and phosphorylated compounds) also presents challenges, but methods have been developed to overcome these barriers [25]. Malaria parasites undergo profound developmental and metabolic changes during the intraerythrocytic life cycle [10]. Extraction efficiency and the nature of metabolites extracted will thus depend on the extraction reagents used [11,26]. Some investigators use parasites that have been freed from erythrocytes using saponin to specifically analyze parasite metabolites, yet others have used intact infected erythrocytes [9,10].

Maintaining sample integrity to reflect the physiologic state is particularly challenging with small molecules as they can undergo rapid alteration in response to changes in environmental conditions. Sample quenching to achieve a rapid arrest of metabolic activity typically involves a rapid decrease (sometimes increase) in temperature to arrest enzymatic activity followed by extraction of metabolites with a combination of extracting solvents. The most popular quenching method involves the use of chilled methanol/water combination but several other reagent combinations have also been used [27]. Recently, a methodology for quenching and extracting metabolites was reported for the protozoan parasite *Leishmania*, which enabled the detection of ~20% of the parasite's predicted metabolome [28].

3.2 Chromatographic separation

Global metabolomic analysis begins with an extremely complex mixture of small molecules that first need to be separated. Chromatographic separation constitutes a critical initial step before the metabolites are subjected to further downstream analysis for identification. A separation step has several advantages, which include reducing isobaric interferences that can occur when compounds of the same mass appear as indistinguishable entities in downstream mass spectrometry (MS) analysis. Second, it minimizes ion suppression in which a more easily ionizable species masks the presence/detection of a less ionizable one (also known as matrix effect) [25]. Several different chromatographic techniques are currently used to separate the individual compounds in this complex mixture. These include gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) [22,29]. Also, several solvent systems can be used for resolving the compounds, which will dictate the nature and efficiency of compound separation [29].

The advantages of GC include high resolution and reproducibility. But it separates compounds based on the metabolites' inherent volatility for which the compounds need to be thermally stable [22]. Alternatively, the metabolite may be rendered volatile and thermally stable through chemical derivatization (as in the case of sugars, nucleosides and amino acids). However, this can be time-consuming [22,25]. Since derivatization requires prior knowledge of the chemistry of the metabolites in order to be able to select the best method to be used for this process, it limits the range of metabolites that can be analyzed by GC. Additionally, labile phosphorylated metabolites can easily degrade during derivatization and when exposed to the high temperature conditions involved in GC analysis. Metabolites having varying affinities for derivatizing agents could lead to potentially inaccurate quantification unless appropriate standards and normalization methods are used [22,25]. Also, formation of byproducts from derivatization, possible biochemical conversion of metabolites to other species and/or degradation of the final product(s) could lead to skewing and misinterpretation of data [25]. To get around this, a two-step derivatization method that overcomes the drawbacks associated with derivatization can provide a better and wider coverage of analyzable metabolites [22,25]. Some compounds cannot be volatilized, and therefore GC is not ideal for their analysis. Thus, a priori knowledge of the chemical properties of the compounds of interest becomes important. Despite the drawbacks, an important advantage of GC is a high separation efficiency of even isomeric compounds [25].

Furthermore, multidimensional GC and the use of two different columns, one for polar and another for non-polar compounds, can dramatically improve sensitivity [25].

In LC, the samples have to go through an extraction process prior to injection to the LC system to minimize matrix effects. But LC-based separation of compounds can be used without the need for chemical derivatization that is required for GC [22]. Liquid chromatography affords greater versatility by allowing the use of varying stationary and mobile phases during chromatographic separation enabling simultaneous qualitative/quantitative analysis of different classes of metabolites [25]. The nature of the column becomes critical for separation of metabolites by LC especially for biological fluids that contain both polar and non-polar compounds [30]. Normal phase LC columns are better at resolving polar compounds, whereas reverse phase (RP) columns work better for non-polar compounds. Hydrophilic interaction chromatography (HILIC) columns were developed to separate the more polar compounds, which offered an ideal complementary technique to the traditional RP chromatography used for the more non-polar compounds [29,30].

Ultra performance liquid chromatography (UPLC) offers the best LC separation method allowing for the pumping and injection of metabolite extracts at a tremendously high pressure (>10 000 psi), yielding improved compound separation and resolution in a shorter time than regular high performance liquid chromatography (HPLC), sharper chromatographic peaks with increased peak capacity, lower ion suppression, and increased sensitivity [29]. Ultra performance liquid chromatography also results in at least a 20% increase in the number of components detected compared to traditional HPLC, as well as superior retention time reproducibility, and signal-to-noise ratios [31].

Capillary electrophoresis offers a powerful analytical tool for qualitative and quantitative "omics" level analysis of samples. Capillary electrophoresis offers several advantages such as the ability to analyze a variety of samples including inorganic ions, organic ions, nucleic acids, proteins, and peptides [32]. Also, CE requires a smaller sample volume, offers higher resolution potential, and is highly efficient compared to other separation techniques in that it is less labor intensive and requires a relatively shorter time-period for analysis. However, its limitations include relatively low sensitivity owing to small sample injection volumes and difficulty in the identification of metabolites. These shortcomings can be overcome by coupling CE with other techniques such as MS, which improves sensitivity and allows for the identification of metabolites [32].

3.3 Metabolite profiling

Nuclear magnetic resonance (NMR) and MS are the most commonly used tools for identification of metabolites [22]. Because of the complementary nature of their analytical features, NMR and MS can be used to achieve a highly comprehensive metabolic profiling [33]. Hybrid systems that combine NMR and MS approaches are rare [34], yet this combinatorial approach offers exciting metabolomics opportunities to be explored [33,35]. Although using NMR typically does not require sample purification and only minimal sample preparation, a major limitation of NMR is that it requires large amounts of sample and lacks the sensitivity of MS [34]. Several different isotopes have been used to perform NMR-based studies. While the use of ^{1}H-NMR achieves maximum sensitivity and broadest compound coverage, using other nuclei, particularly ^{13}C, ^{31}P and ^{15}N, yields better biochemical/biological information (e.g. metabolite flux, biochemical reactions, etc.) [36].

Teng *et al*. used ^{1}H-NMR to identify more than 50 metabolites from an extract of saponin-released *P. falciparum* trophozoites [11]. In addition to identifying and quantifying amino acids, nucleotides, carboxylates and other molecules, they found that perchloric acid was the optimal extraction reagent [11]. ^{1}H-NMR metabolomics has been applied to other parasites

to study, for example, the metabolites produced and released by *Cryptococcus neoformans* in culture media [37], the lipidome of *Leishmania donovani* [38], comparing intracellular metabolites produced by long-term *in vitro* cultured *L. donovani* axenic amastigotes with those produced by promastigotes and intracellular amastigotes [39], and the *in vivo* metabolism of *Trypanosoma cruzi* [40].

Basant *et al*. used ^{1}H-NMR to analyze metabolite extracts of urine, serum and brain samples from mice infected with *P. berghei* to study metabolic alterations with disease progression [12]. Urine samples from female mice showed greater alterations in global metabolism during early stages of infection compared to samples from male mice. Global changes in serum samples were greater in male mice in the early stages, whereas changes in brain metabolism were minimal in either sex. An analysis of individual metabolites in serum and brain samples also revealed significant changes in female mice in the early stages of infection. These sexually dimorphic metabolic alterations were suggested to have a bearing on disease prognosis and treatment [12].

^{13}C-based NMR studies have been used in the malaria field to study glucose metabolism in *P. falciparum*-infected erythrocytes [8], measuring glucose flux in *P. falciparum* and *P. yoelii*-infected erythrocytes as well as the suppression of glycolytic activity in uninfected erythrocytes by malaria-conditioned medium [9,41]. ^{31}P-NMR is particularly useful in tracking phosphate metabolism in *Plasmodium* spp. [42], *Toxoplasma gondii*, and *Cryptosporidium parvum* [43], as well as in the kinetoplastids [44].

Mass spectrometry is highly sensitive and selective because it can detect moieties such as sulfates that cannot be detected by NMR. However, MS alone may fail to discriminate between certain classes of compounds depending on the ionization method used, and thus needs coupling with metabolite separation techniques such as GC and LC [22]. Olszewski *et al*. used an LC-MS/MS approach to quantitatively measure the levels of ~200 known compounds of validated identity over the 48 hour blood-stage developmental of *P. falciparum*. An important discovery was the conversion of arginine to ornithine by parasite arginase, and the potential link between parasite-induced hypoargininemia and the development of cerebral malaria [10].

3.4 Statistical tools for data analysis

Metabolomic experiments produce large volumes of data. Efficient data handling is key to achieving accurate metabolite identification and quantification, and thus generating biologically meaningful interpretations. In the recent past, a wide variety of data processing software and other tools have been developed for data handling [45]. Chemometrics tools include mathematical, statistical, graphical, and symbolic methods used to extract the maximum amount of information possible from chemical and/or spectral data, and thus constitute an integral component of the data analysis [34].

The mostly commonly used statistical tools include principal component analysis (PCA), principal component discriminant analysis (PCDA) or partial least squares (PLS), and partial least squares discriminant analysis (PLSDA) [46]. Principal component analysis is a statistical technique that determines the most optimal linear transformation for a collection of data points in such a manner that the properties of that sample are most clearly displayed along the coordinate (i.e. principal) axes. In other words, PCA allows one to plot, visualize, and cluster multiple metabolomic data sets based on linear combinations of their shared features [34]. Partial least squares discriminant analysis similarly enables one to perform efficient classification and discrimination analysis of very large data sets containing a small number of samples. A variant of PLSDA, the orthogonal-PLSDA, classifies the data into two blocks, one representing the “between-class” variation, and the other representing the

"within class" variation [47,48]. It thus separates the more meaningful data (the former) from the less meaningful (the latter) [47]. Also, statistical techniques such as cross-validation and permutation testing enable one to assess the robustness of a statistical tool used [48]. Other statistical tools such as k-means clustering, hierarchical clustering, artificial neural networks, analysis of variance, and multivariate analysis of variance are also widely used [34,48].

4. Metabolic highlights of *Plasmodium falciparum*

4.1 Malaria metabolomic databases

Metabolic pathways are typically constructed by integrating biochemical data from a diverse set of organisms. There are a number of databases describing malaria metabolic pathways including the Kyoto Encyclopedia of Genes and Genomes, Malaria Parasite Metabolic Pathways, PlasmoCyc, metaSHARK, and metaTIGER [49,50]. These databases and others such as PlasmoDB and GeneDB are regularly updated based on new findings. These databases provide powerful resources for building metabolic pathways using gene annotation information. However, investigators face several hurdles while undertaking a bioinformatics-based reconstruction of metabolic pathways. These include the lack of proper gene annotation, absence of certain reactions in a pathway if the parasite utilizes host molecules, the demands imposed on the parasite by its novel niche to acquire pathways not previously seen in nature, and the availability of experimental confirmation for only a subset of pathways. These limitations have been eloquently described elsewhere [49]. We focus on polyamines, glycerol and lipids because they are illustrative of the different approaches that have been taken by investigators to unravel important biochemical attributes of the parasite. Astute use of these approaches also carries the potential for future discovery of novel parasite biology.

4.2 Polyamines

During the past three decades, polyamine metabolism has attracted much interest as a target for anti-proliferative therapy [51]. Polyamines (putrescine, spermine and spermidine) are low molecular-weight organic cations that are found in a wide range of organisms including bacteria, plants, animals and parasites (including *Plasmodium*), and mediate several important cellular processes including cellular development and proliferation, and stress responses [52,53].

Polyamines are a major metabolite in *Plasmodium* and make up 14% of the total metabolome of isolated parasites [11]. Recent efforts have focused on chemically interfering with *Plasmodium* polyamine metabolism owing to the fact that rapid proliferation of *Plasmodium* is critically dependent on the availability of high amounts of polyamines [54]. *Plasmodium* has its own unique polyamine synthesis pathway in which arginine is converted to ornithine which then acts as the precursor for spermidine. The two rate-limiting enzymes in this pathway, ornithine decarboxylase and S-adenosylmethionine decarboxylase, differ significantly from the mammalian ones in their organization as a bi-functional protein complex [55].

The effect of polyamine depletion as an anti-malarial strategy was studied through co-inhibition of *S*-adenosylmethionine decarboxylase and ornithine decarboxylase in *P. falciparum*, and performing a comprehensive genomic, proteomic, and metabolomic analyses [7]. The latter involved an LC-MS/MS methodology to analyze ~100 metabolites including polyamines. Three perturbation-specific compensatory transcriptional responses were observed, two of which were confirmed at both the protein and metabolite levels. Specific effects on the parasite metabolome included a significant decrease in the levels of the polyamines putrescine and spermidine, and the downstream metabolite 5′-

methylthioinosine. Ornithine aminotransferase was elevated at both the transcript and protein levels suggesting the occurrence of compensatory changes in the parasite. This study demonstrates the power of a combinatorial and correlative analysis at the transcription, protein and metabolomic levels [7]. More recently, Becker *et al*. showed that cyclohexylamine (a spermidine synthase inhibitor) treatment of *P. falciparum* resulted in transcriptional arrest of genes in the polyamine biosynthesis and associated metabolic pathways as well as differential expression of the corresponding proteins. Additionally, changes were observed in the levels of polyamine metabolites [56].

4.3 Glycerol and carbon metabolism

Glucose has long been shown to be a major carbon source for the parasite, and lactate is one of the major end-products of glucose metabolism. Drugs such as chloroquine and amodiaquine accumulate in *P. falciparum* in an energy-dependent manner [57,58]. A recent study using ^{13}C-glucose-supplemented media enabled the detection of all the by-products of glucose metabolism in *P. falciparum* [8]. ^{13}C-NMR was used to confirm the generation of the expected end-products of glucose metabolism including lactate, pyruvate, and alanine [8]. Interestingly, the study also found that parasites generated glycerol and glycerol 3-phosphate from glucose, a metabolic conversion that had previously not been known to exist. These metabolites were thought to be produced due to the operation of a glycerol-3-phosphate shuttle in the parasite in response to growth under limited O_2 and elevated CO_2 [8].

Glycerol is produced during anaerobic catabolism of glucose in yeast, protozoan parasites (*Trichomonas*, *Leishmania* and *Trypanosomes*) and rumen ciliates, but not by humans [8,40]. Incidentally, all of these studies that detected glycerol had used ^{13}C-NMR spectroscopy [8]. In contrast, studies that had used HPLC or GC-MS/MS and LC-MS/MS could not detect glycerol because of its poor ionization potential [8]. Thus, a combinatorial approach is critical to achieving the most comprehensive analysis of small molecules in a biological sample.

Tracking the metabolism of ^{13}C-labelled precursors using LC-MS led to the discovery that the malaria parasite employs the TCA cycle in an unconventional manner with important biological implications [59]. Glutamate and glutamine were the major metabolites that entered the parasites tricarboxylic acid cycle to generate acetyl-CoA moieties for histone acetylation, whereas glucose-derived acetyl-CoA was used to acetylate amino sugars [59]. For an in-depth account of carbon metabolism in malaria, we refer the reader to a recent comprehensive review [60].

4.4 Lipids

Cellular lipids as a class constitute a tremendous diversity of chemical structures and qualities. For example, phospholipids constitute a major class of lipids in *P. falciparum* and are important for membrane biogenesis during parasite proliferation [61]. These parasites use numerous pathways for phospholipid synthesis. Interestingly, rodent and non-rodent malaria parasites differ in their phospholipid synthesis pathways [61]. The parasite utilizes lipids for numerous functions including membrane formation, and as metabolic co-factors, and can affect host physiology through pro- or anti-inflammatory effects [62,63]. Early metabolic labeling studies with fatty acids demonstrated that parasites can uptake host fatty acids, particularly palmitic and oleic acids [64,65] and lipoproteins [66].

Interestingly, GC analysis demonstrated that parasite elongation of ^{14}C-palmitic acid to oleic acid occurred only when the level of fatty acid supplementation of the culture media was lowered, suggesting that malaria parasites have the capacity to adapt to variations in

exogenous nutrient levels [65]. Malaria parasites also have the ability to synthesize fatty acids through the type II fatty acid synthesis (FAS II) pathway. This pathway is absent from mammalian cells, and is thus an attractive drug target [67]. The FAS II pathway is required for liver stage development in rodent malaria model but is not essential for the blood stages [68,69]. Parasites can incorporate of ^{14}C acetate into fatty acids, preferentially resulting in the production of C_{10}-C_{14} fatty acyl chains [70]. Although the FAS II pathway is inactive in the blood stages, transcriptional analysis of a subset of blood stage parasites collected from *P. falciparum*-infected patients found upregulation of FAS II genes and an overall pattern of parasite starvation [71]. However, further investigation is warranted to determine the mechanistic basis of this observation [72]. Thus, analyzing both parasite and host plasma metabolomes could address questions related to parasite adaptation in a dynamic host environment.

High performance liquid chromatography experiments of parasite extracts have identified large amounts of hydroxy derivatives of a diverse group of polyenoic fatty acids [73]. This was an unexpected finding because the parasite lacks the critical lipooxygenase enzyme involved in initiating the formation of these hydroxyl fatty acids. However, it was shown that these hydroxy fatty acids could be generated in a non-enzymatic fashion during heme catabolism. These hydroxy fatty acids were found to be inhibitory to monocyte function, potentially playing a role in parasite-mediated host immune modulation [73]. These studies suggest that parasite metabolic pathways could play a crucial role in host-parasite interactions [73].

Isoprenoids represent one of the largest groups of biologically active lipid-based metabolites. They serve as prosthetic groups for many enzymes, and precursors to molecules involved in electron transport, and the formation of glycoproteins [74]. The main difference between the isoprenoid pathways of humans and malaria parasites is that the parasites use the methylerythritol pathway whereas humans use the mevalonate pathway [75]. Couto *et al*. demonstrated using precursors of the isoprenoid pathway, ^{3}H-farnesyl pyrophosphate and ^{3}H-geranylgeranyl pyrophosphate, and ^{14}C-acetate, that *P. falciparum* has an active isoprenoid pathway [76]. Parasite octaprenyl pyrophosphate synthase activity, which is involved in isoprenoid precursor biosynthesis, was demonstrated by the formation of polyisoprenoids with eight isoprenic units [77].

Given that *P. falciparum* contains an apicoplast, a relic of the plastid, this group also investigated whether *P. falciparum* is able to produce carotenoids akin to that produced by the plastids of algae and plants. Carotenoids are derived from the isoprenoid biosynthesis pathway, and the carotenoid biosynthetic pathway is not present in mammalian cells. Using metabolic labeling with ^{3}H and ^{14}C radioisotopes, *P. falciparum* parasites were shown to possess an active carotenoid biosynthesis [78]. Carotenoids in plants mainly function as antioxidants to protect the lipophilic compartments from oxidative damage, as well as scavenge and neutralize singlet molecular oxygen and peroxyl radicals that are generated during photooxidative processes [78]. The identification of a carotenoid biosynthesis pathway in *P. falciparum* adds to the growing list of features that are similar to those in plants, and that could serve as potential targets for developing novel antimalarials [74,79,80].

5. Potential future research

Metabolomics of malaria is still in its infancy, but by drawing on concepts and themes developed in other areas, it could be promptly adapted to malaria research. The application of metabolomic analysis to malaria research is bound to provide tremendous insights into the intricacies of the malaria parasite's life cycle, transmission and pathophysiology.

5.1 Parasite and vector biology

Nearly 60% of the *P. falciparum* genome is unannotated, and a combinatorial analysis can be useful in uncovering encoded enzymatic functions. Correlative analysis of gene expression, proteomic and biochemical metabolic profiling is a powerful way to identify genes that encode enzymatic functions [81]. This can be performed either in a global whole-genome manner or focused on specific mutation(s) or loss-of-function(s), and studying the metabolomes generated from different parasite genotypes can provide insight into the underlying genetic basis of distinctiveness. For example, quantitative trait loci mapping is an attractive way to identify genetic contributors to differing levels of particular metabolites between various *P. falciparum* strains [82]. The metabolite levels serve as the equivalent of phenotypic indicators of the existence of underlying genetic contributors [82]. Witola *et al*. showed that the disruption of *P. falciparum* phosphoethanolamine methyltrasferase results in a complete loss of phosphatidylcholine biosynthesis by the serine decarboxylase-phosphoethanolamine methyltransferase pathway [83]. Global metabolomic analysis on such parasite lines could uncover the effect of loss-of-function on other metabolic pathways, and help build a network of regulatory interactions between those pathways. In *Arabidopsis*, comparing four different genotypes showed that each genotype possesses a unique metabolic profile and informed the physiology related to each set of genetic mutations [84].

Another ingenious way of investigating whether a protein functions as an enzyme is to express and purify the protein(s) of interest, and incubate it with metabolite extracts prepared from the biological system being studied. Proteins of unknown function in *E. coli* were cloned, expressed, purified, and incubated with a complex metabolite mixture [85]. Capillary electrophoresis and MS were then used to identify enzyme activity by looking for signature substrate-product combinations, as well as compounds whose levels had changed following incubation with the purified protein. This allowed assignment of enzymatic function to proteins that had previously been uncharacterized [85]. It should be noted that heterologous expression of malaria parasite proteins has been a major hurdle in performing structure-function studies [86].

Performing a targeted analysis by investigating specific organelles in the malaria parasites can be highly probative of biochemical composition and function in those compartments. Mitochondria, which retain their structural and functional integrity, have been successfully purified from yeast cells, free of contamination by other organelles [87]. In a fascinating study on the mitochondria of *P. falciparum*, it was shown that erythrocytic stage parasites maintain an active mitochondrial electron transport chain exclusively to regenerate ubiquinone, which is required as the electron acceptor for dihydroorotate dehydrogenase, an essential enzyme for pyrimidine biosynthesis [88]. Transgenic *P. falciparum* parasites expressing *Saccharomyces cerevisiae* dihydroorotate dehydrogenase, which does not require ubiquinone as an electron acceptor, were completely resistant to inhibitors of mitochondrial electron transport [88]. Expressing proteins from another organism in *P. falciparum* might trigger functions that would otherwise be hard to elicit in this parasite. Organelle purification and metabolomics have also been used to study the peroixomes, organelles that play a key role in redox signaling and lipid homeostasis [89].

Metabolomics offers a unique and powerful tool to explore the biology of the mosquito vectors and developing strategies to interrupt malaria transmission [90]. It was shown that xanthurenic acid (a derivative of tryptophan) and another anonymous factor play a critical role in exflagellation and ookinete formation in *Plasmodium* [91]. Interrupting the formation of such metabolites can help reduce malaria transmission, which appears to be an insurmountable burden at the present time. Mosquito entomological inoculation rates (and hence the rate of transmission) vary depending on the geographical location [92], and it

estimated that in some African communities as much as a 100-fold reduction in inoculation rate would be necessary to achieve reductions in parasite prevalence by 30-70% [14].

5.2 Parasite-host interaction studies

A review of the “omics” approach used in studying plant stress response provides an ideal benchmark from which to gather ideas [93]. Particularly interesting is malarial response to temperature stress. Malaria parasites are exposed to a wide range of temperatures between their invertebrate and vertebrate hosts. Subjecting malaria parasites to temperature stress *in vitro* was shown to cause marked transcriptional changes in genes involved in several cellular functions including basic metabolism, lipid/fatty acid/isoprenoid metabolism, and encoding known transporters [94]. Monitoring parasite metabolome under such stress conditions could provide insights into the association between gene expression changes and biochemical alterations.

Malaria presents an enormous global heath and economic burden. *Plasmodium falciparum* infects people in all age groups, and patients present with varying levels of disease severity. Mechanisms causing variations in infection outcomes remains poorly understood. Patients with severe disease can present with respiratory distress, coma, abnormalities in blood coagulation, and vital organ dysfunction. The molecular and biochemical bases of disease in each of these clinical manifestations may vary, and analysis of the small molecule repertoire in such patients may shed light on disease etiology. For example, our group recently used a microarray-based approach to study parasites collected directly from patients and identified novel transcriptional states of *P. falciparum* in the human host that had previously not been known to exist [71]. One could hypothesize that these novel parasite transcriptional states could produce characteristic metabolic outputs, which in a genetic-metabolic feedback loop could in turn trigger particular transcriptional profiles.

Human populations carry genetic adaptations that have evolved in response to diverse climatic conditions, exposure to infectious diseases, and diets [95]. These factors are thus critical considerations in developing disease models using a combinatorial approach involving metabolomics, genomics, and proteomics. Metabolomics, for example, could be used to reveal the impact of dietary nutrients on the biochemistry of humans and their susceptibility/resistance to diseases [96,97]. Moreover, current metabolomics techniques are highly sensitive requiring very small amounts of human plasma or tissue samples, thus making these kinds of analyses feasible.

Metabolomics has been implemented as a diagnostic tool in a number of human diseases and could also be applied to malaria [98]. For example, ^{1}H-NMR spectra of human serum samples allowed >90% of subjects with significant coronary vessel blockages to be distinguished from subjects with angiographically-normal coronary arteries with a specificity of >90% [99]. In addition, metabolomics could potentially be used to detect organ-specific involvement in disease that could have prognostic or treatment implications in malaria. For example, cerebral malaria can be difficult to discern clinically in comatose malaria-infected children who have died of alternative causes [100]. Similarly, placental immunopathology, which causes anemia, low birth weight, and fetal loss due to infection at submicroscopic levels, can underestimate the incidence of placental malaria diagnosed using microscopic detection [101]. Metabolomics could potentially identify markers of such clinical conditions and improve clinical outcomes for these patients. Animal models for malaria afford us the opportunity to circumvent some of the problems associated with performing experiments on human subjects such as availability of tissue/organ samples and knockout animals [102]. Combining such metabolomic analysis with genomic and proteomic analyses can provide a comprehensive and correlative understanding of parasite biology.

Also, humans can be co-infected with multiple species of *Plasmodium* as well as other pathogens. In an interesting study in semi-immune children in Papua New Guinea infected with multiple species of *Plasmodium*, it was found that the total parasite density was maintained at a constant level [103]. This study also revealed that peaks of infection with each species occurred in a mutually exclusive manner. This was hypothesized to be due be a cross-species regulatory effect. It was proposed that the sequential episodes of infection were produced as a consequence of interactions between the density-dependent regulation of parasite levels and species-specific immune responses. However, the factor(s) though to be involved in this phenomenon has remained elusive till date [103,104]. Metabolomics and proteomics, and the use of animal models, could prove useful in identifying the factor(s) that mediates this regulation, and assess whether or not immune regulatory mechanisms operate to bring about this effect [104-107].

6. Concluding remarks

Metabolomics has been a revelation in other fields. Several different approaches have been adopted, and a wealth of basic and clinically relevant information has been collected on other pathogens and clinical conditions. The successful application of metabolomics in other fields should provide us the impetus to adapt it to malaria research. This will enable us to unveil novel aspects of parasite and vector biology. It will also provide insights into the biochemistry of the host, and nature of host-parasite interactions during infection, and makes it feasible for us to discover species-specific biomarkers that might correlate with various clinical manifestations of malaria. Ultimately, it will foster our efforts towards designing improved antimalarials and better vector control measures, and discovering more effective vaccine candidates.

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