Parasitology Research
https://doi.org/10.1007/s00436-022-07673-7

REVIEW

Recent metabolomic developments for antimalarial drug discovery

Lúcia Mamede[1] · Fanta Fall[2] · Matthieu Schoumacher[3] · Allison Ledoux[1] · Pascal De Tullio[3] · Joëlle Quetin-Leclercq[2] · Michel Frédérich[1]

Received: 13 June 2022 / Accepted: 14 September 2022
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Abstract
Malaria is a parasitic disease that remains a global health issue, responsible for a significant death and morbidity toll. Various factors have impacted the use and delayed the development of antimalarial therapies, such as the associated financial cost and parasitic resistance. In order to discover new drugs and validate parasitic targets, a powerful omics tool, metabolomics, emerged as a reliable approach. However, as a fairly recent method in malaria, new findings are timely and original practices emerge frequently. This review aims to discuss recent research towards the development of new metabolomic methods in the context of uncovering antiplasmodial mechanisms of action in vitro and to point out innovative metabolic pathways that can revitalize the antimalarial pipeline.

Keywords Metabolomics · Malaria · *Plasmodium* sp. · Mass spectrometry · Nuclear magnetic resonance · Mechanism of action

Malaria: understanding the *Plasmodium* spp.

Malaria is a vector-borne parasitic disease that remains a global health issue (WHO 2021). Clinically speaking, malaria is caused by an obligate intracellular protozoan with a complex life cycle that alternates between an invertebrate and a vertebrate host — see Fig. 1 (Russell and Cooke 2017). The *Plasmodium* spp. are host specific, and five recognized species infect mankind: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale*, and *P. knowlesi*, this last one being a zoonotic parasite that has more recently crossed the species barrier (Russell and Cooke 2017). The latest data indicate that there were 241 million estimated malaria cases in 2020, of which 95% originated in sub-Sharan Africa (WHO 2021). The death toll was 627,000 deaths in the same year, with 77% representing children, an important decline from 87% in 2000 (WHO 2021). Indeed, global malaria incidence and mortality diminished since 2000, but at a relative slower rate since 2015, which jeopardizes the WHO' malaria eradication plan (WHO 2020).

Presently, malaria control methods include insecticide-treated mosquito nets, prophylactic drugs, and the WHO recommendation of the RTS,S/AS01 malaria vaccine since October 2021 (WHO 2021). Antimalarial drugs, thus, play a pivotal role in disease management and in the eradication plan. All antimalarial drugs have been impacted by the emergence of resistance, and, to tackle this issue, treatment evolved in the sense of artemisinin-based combination therapies (Khan et al. 2019; Tse et al. 2019; WHO 2020). Although this strategy has been successful in delaying the spread of resistance and diminishing treatment failure, the necessity for antimalarial drugs with innovative mechanisms of action (MoA) is irrefutable. Antimalarial drug discovery is the key for malaria eradication, and tools to aid and optimize it are needed.

Malaria pathological mechanisms presuppose a tight connection between the host cell and the parasite that can be used against the latest. In fact, the presence of the parasite inside the red blood cell (RBC) serves the function of

Section Editor: Dietmar Steverding

✉ Michel Frédérich
M.Frederich@uliege.be

[1] Laboratory of Pharmacognosy, Center of Interdisciplinary Research On Medicines (CIRM), University of Liège, CHU B36 Av Hopital 1, B36 4000 Liege, Belgium

[2] Pharmacognosy Research Group, Louvain Drug Research Institute (LDRI), UCLouvain, Avenue E. Mounier B1 72.03, B-1200 Brussels, Belgium

[3] Laboratory of Pharmaceutical Chemistry, Center of Interdisciplinary Research On Medicines (CIRM), University of Liège, Liege, Belgium

Published online: 04 October 2022

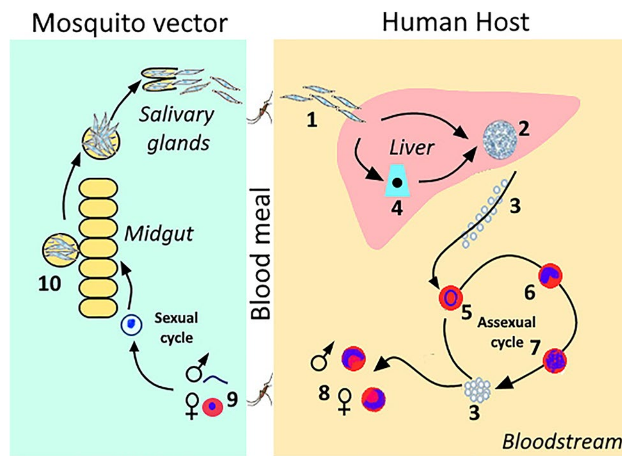

Fig. 1 Human-infecting *Plasmodium* spp. lifecycle. Upon a bite from the infected *Anopheles* sp. mosquito, the invertebrate host, sporozoites (1) migrate to the liver of the vertebrate host where a schizont (2) will develop in the hepatocytes, releasing merozoites (3) into the bloodstream. Alternatively, in *P. vivax,* the hypnozoite (4) form might also develop in the liver, staying in stasis until eventual re-activation. Merozoites invade reticulocytes or erythrocytes, depending on the species, and develop into early trophozoites, also known as the ring stage (5). Over the course of one asexual cycle, the ring stage evolves to a more characteristic trophozoite (6) until it begins schizogony. The schizont (7) releases its merozoites into the bloodstream, recommencing the cycle. Alternatively, merozoites, upon invasion, may develop into male and female gametocytes (8) (stages I–V) that, when taken in a bloodmeal by the invertebrate host, develop into gametes (9) and undergo the sexual part of the cycle in its midgut. Oocysts (10) release sporozoites that migrate to the salivary glands where they readily infect a new vertebrate host upon a new feeding (Venugopal et al. 2020)

protection, but implies an adaptation cost in order for it to also be a nutritional source (Olszewski et al. 2009). The *Plasmodium* is auxotrophic for many essential substances: purines, such as hypoxanthine; vitamins, such as B_5 and B_7; and isoleucine (Müller and Kappes 2007; Olszewski et al. 2009; Sengupta et al. 2016; Krishnan et al. 2020). This implies that the parasite is partially reliant on external nutritional sources that are relevant to characterize it and, most importantly, that their metabolic pathways can become innovative antimalarials targets.

In order to characterize this new venue for antimalarial development, metabolomics became a stepping stone. This omics discipline that studies the metabolome in depth, including the lipidome (lipidomics) and the metabolic flux (fluxomics), is an invaluable tool in elucidating many aspects of the malaria disease (Ghosh et al. 2018; Sexton et al. 2019; Yu et al. 2021). The metabolome comprises metabolites and small molecules (< 1500 Da) that reflect accurately and rapidly the activity of enzymes, proteins, and pathways, leading to a faithful snapshot of the parasite's status (Sexton et al. 2019; Yu et al. 2021). Some examples include amino acids, vitamins, cofactors, nucleotides, and fatty acids, among others; all compounds that provide energy, signaling, or building blocks are essential for parasitic survival.

This snapshot can reveal the dynamics of a complex organism like the *Plasmodium* sp. when exposed to a drug, thus establishing a metabolic profile under those particular conditions and portraying the drugs' effects (Jang et al. 2018; Cobbold and McConville 2019; Allen and Young 2020; Bao and Liu 2020). This type of study has become, in the last decade, a significant tool in antimalarial drug discovery (Bao and Liu 2020; Tewari et al. 2020). In fact, antimalarials are recognized to act by disturbing metabolic pathways that are essential to the *Plasmodium*, but remain segregated from the host, such as folate synthesis or hemoglobin proteolysis (Kafsack and Llinás 2010). Even if the MoA is not known, the metabolome is capable of capturing the senescence cascade by reflecting how other systems are affected, e.g., the antioxidant system. Knowledge on essentiality of pathways or variating metabolites is crucial to find new targets and for rational drug development (Plata et al. 2010; Sexton et al. 2019; Cobbold and McConville 2019; Krishnan et al. 2020). Likewise, the metabolome also echoes resistance mechanisms that can then be prevented. Therefore, metabolic profiling, or fingerprinting, can be repurposed for the discovery and description of drug MoA, thus aiding in the expansion of the antimalarial arsenal (Cowell and Winzeler 2019; Tse et al. 2019).

In the recent decade, many important studies regarding the metabolic overview of the *Plasmodium* emerged, particularly in the field of target validation and drug discovery (Cowell and Winzeler 2019; Birrell et al. 2020; Moreno-Pérez and Patarroyo 2020; Yu et al. 2021). These advancements have been reviewed before with variant focuses (Besteiro et al. 2010; Cowell and Winzeler 2019; Khan et al. 2019; Sexton et al. 2019; Allen and Young 2020; Moreno-Pérez and Patarroyo 2020; Yu et al. 2021). This review aims to discuss recent and groundbreaking research on the characterization of antiplasmodial targets and MoA, with a focus on metabolomic in vitro studies. To do so, we shed light on recent metabolomic analytical method developments in the field of malaria and point to pertinent new directions in antimalarial drug discovery.

Metabolomics applied to the *Plasmodium* spp. parasite

Innovation in metabolomics methodologies

Metabolomics is a broad discipline that has been used extensively in medical field to elucidate biomarkers and mechanisms of disease or aid in the rational development of drugs (Jang et al. 2018; Bao and Liu 2020). Despite its huge potential, metabolomics is not without challenges. The

metabolome is a very complex assortment of molecules, so it is difficult to detect, characterize, or quantify them all simultaneously, as this omics proposes (Besteiro et al. 2010; Bao and Liu 2020). These and other hurdles exist in metabolomic studies on the *P. falciparum* parasite as discussed ahead.

A metabolomic experiment is defined by a sequence of necessary steps, also called the workflow — see Fig. 2 (Price et al. 2018). Key steps in malaria in vitro studies include the sampling and isolation of the parasitic cell from the environment, cessation of all metabolism (quenching), and successful extraction according to the coveted coverage (Jang et al. 2018; Price et al. 2018). These points are of extreme importance, since they can alter the results and, thus, their interpretation. A discussion on each of these decision steps is given ahead.

Study design

Metabolomic studies are key to elucidating drug's MoA due to their unbiased nature, global overview, and sensitivity (Yu et al. 2021). Most study designs of this type in antiplasmodial drug discovery do so through treatment experiments in vitro, in which the parasite is exposed to a compound of interest and the modified metabolome is compared to a control. This allows the identification of changes caused by the drug, which are then studied through statistical models and metabolite identification in search for a biological meaning (Sadeghi Tafreshi et al. 2019; Cobbold and McConville 2019; Tewari et al. 2020). This is the basis of metabolic fingerprinting, but the extracellular medium can also be collected and analyzed, thus providing the so-called footprint. Ideally, both the fingerprint and footprint should be used for the most complete dataset possible (Price et al. 2018).

The key aspect of the design is that the collected sample reflects the most directly possible the desired metabolome (Jang et al. 2018; Price et al. 2018). Metabolomic experiments can also reveal other aspects of parasite biology, such as merozoite invasion and egression, gametocytogenesis, schizogony, and mosquito transmission, among others. For each case, the appropriate sampling choice must be considered, likewise the number of samples or parasites *per* sample and their species, stage, or synchronicity (Price et al. 2018).

The workflows applied to in vitro *P. falciparum* studies regarding the evaluation of drug's MoA published in the literature since 2015 are resumed in Table 1. These were simplified by the key steps, from the parasitic stage to data processing and analysis tools, and will be detailed more in-depth in the following sections.

Sample choice and sampling

Metabolomics can reveal fundamental aspects of the metabolism, irrespective of the parasitic stage or species, provided they are not quiescent. The in vitro life stages, be it the intraerythrocytic asexual or sexual, characterize the human infection and transmission, respectively. These timepoints of interest for drug development are therefore easily available, and information on activity and transmission-blocking capabilities can be readily attained (Ross and Fidock 2019).

Across the intraerythrocytic asexual life stages, most studies focus on the trophozoite and schizont phases, as they are easily magnetically concentrated and are considered metabolically active (Creek et al. 2016; Allman et al. 2016; Dumont et al. 2019; Birrell et al. 2020). This selection occurs due to the presence of paramagnetic hemozoin, which is retained when in presence of a magnetic field, thus allowing non-invasive synchronization and concentration of late trophozoite and schizont stages (Carey et al. 2018). A metabolomic study by Beri et al. aimed to discover metabolites as potential malaria biomarkers (Beri et al. 2019). It determined that, of the intraerythrocytic asexual life stages, the ring stage was the least metabolically active. Of the 141 metabolites identified, only 10 were common between all stages, reflecting the adaptability and swift changing needs of the parasite over the course of one lifecycle. Additionally, 34% were assigned to lipid metabolism, that proved essential throughout the lifecycle, and 24% were attributed to amino acid biosynthesis, which was upregulated at the trophozoite (24 h) and schizont (40 h) stage. Interestingly, the assay was stopped at the 40 h mark in order to prevent contamination with the RBC metabolome resulting from lysis and merozoite egression (before reaching 48 h), which most studies in the literature do not account for (Beri et al. 2019).

The ring form is harder to study than other stages, as it is more difficult to isolate in adequate quantities (Carey et al. 2018; Beri et al. 2019). Still, by isolating schizonts, rings can be studied as well after a few hours (Carey et al. 2018; Murithi et al. 2020; Zimbres et al. 2020). Whenever possible, metabolomic fingerprinting studies try to distinguish the candidate drug profile across the asexual lifecycle, starting at the ring stage. Dihydroartemisinin is frequently used as a standard to confirm or compare profiles, since it is recognized that its peak activity happens during this stage (Murithi et al. 2020). Consequently, and due to the emergence of artemisinin resistance that leads to an unusual prolongation of this form from around 8 h to up to 14 h, this stage is one of the most interesting (Khoury et al. 2020). A drug metabolomic fingerprint screening performed by Murithi et al. revealed the different profiles and peak activity of 36 compounds, including standard antimalarials (Murithi et al. 2020). Remarkably, by comparing the fingerprints between chloroquine and other compounds that were theorized to affect hemoglobin catabolism, unique peak activity was found in early ring stages, indicating that hemoglobin digestion might begin as early as under 8 h, before the digestive vacuole (DV) is formed. Multiple drugs with differing

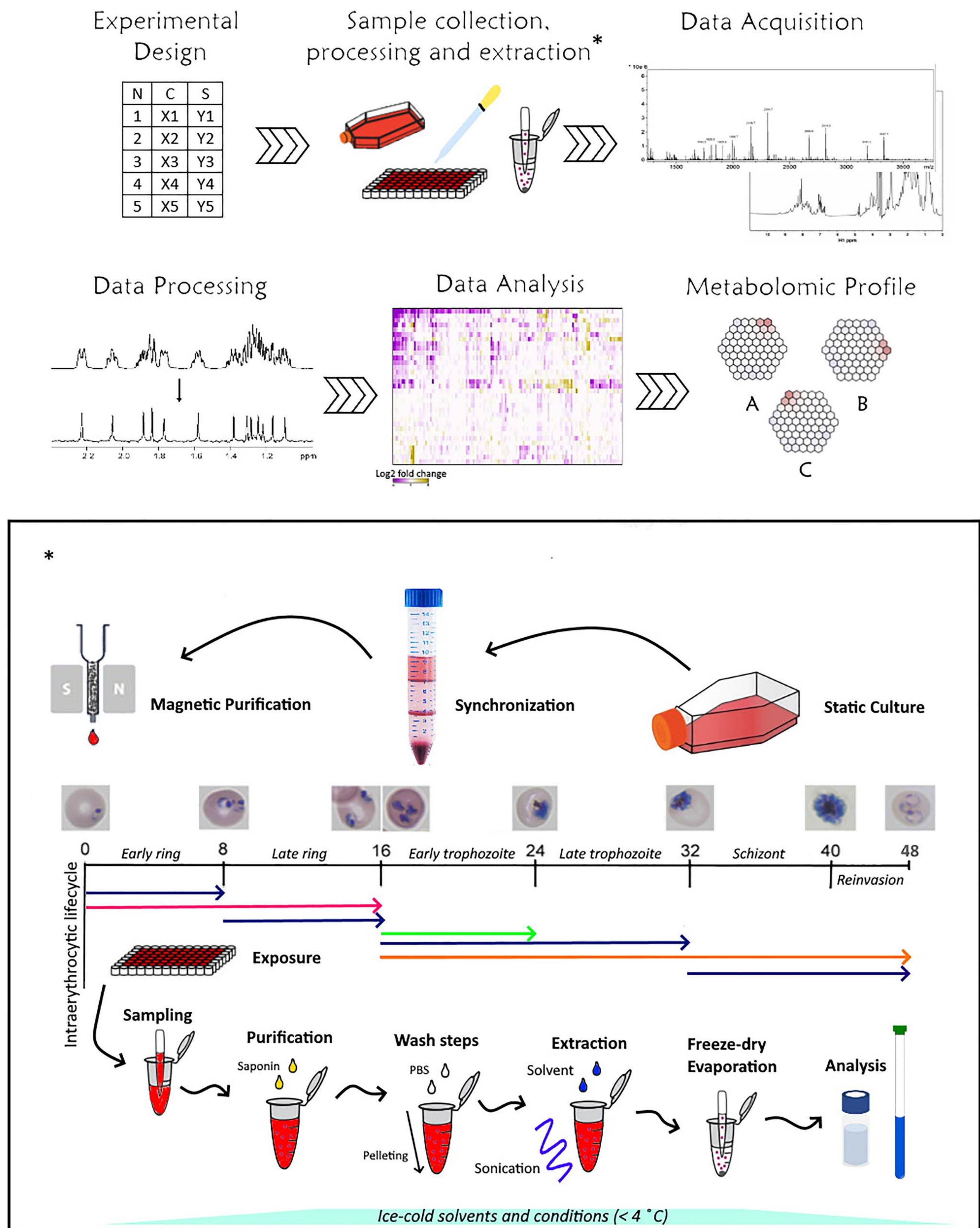

Fig. 2 Workflow of an in vitro *P. falciparum* metabolomic test. Following the preset of the experimental design steps, an in vitro assay can yield individual and representative samples that, through processing and extraction (depiction of critical steps, including synchronization techniques, magnetic purification, study design (e.g., of an intraerythrocytic time-resolved exposure scheme), purification, washing, extraction, and storage), are analyzed through one or more robust metabolomics techniques

Table 1 Methods used in *Plasmodium* spp. metabolic studies published since 2015

Parasitic stage	Number of cells/sample	Purification	Quenching	Metabolite extraction method	Storage	Data acquisition method	Data analysis	Reference
Intraerythrocytic stages	1×10^8	-	Ice-cold PBS	Acetonitrile:water (4:1,v/v)	-	LC–MS (SeQuant ZIC-pHILIC, Q-TOF)	MAVEN, *R*, MetaboAnalyst 3.0, BioCyc, XCMS	Cobbold et al. (2016a, 2021); Dickerman et al. (2016); Hapuarachchi et al. (2017); Kennedy et al. (2019); Cobbold and McConville (2019)
Intraerythrocytic stages	-	-	Dry ice-ethanol bath	Methanol	−80 °C	RP UPLC-MS/MS and HILIC UPLC–MS/MS (Q-Exactive, Orbitrap)	MATLAB, MetaboAnalyst, HMDB, SMPDB, KEGG	Tewari et al. (2020)
Intraerythrocytic stages — trophozoite	5×10^7	-	-	Methanol	−20 °C/−80 °C	LC–MS (SeQuant ZIC-pHILIC, Q Exactive)	IDEOM, XCMS, TraceFinder, MetaboAnalyst	Creek et al. (2016); Siddiqui et al. (2017); Birrell et al. (2020)
Late-stage gametocytes	-	-	-	Direct media was tested	-	^{1}HNMR	ProMetab, MATLAB, HMDB, KEGG	Sadeghi Tafreshi et al. (2019)
Intraerythrocytic stages, Mid to late stage gametocytes (III-IV)	1.0×10^5 to 1×10^8	-	-	90% methanol	−80 °C	UHPLC-MS (Phenomenex Hydro-RP C18, Orbitrap)	MAVEN, *R*	Allman et al. (2016); Antonova-Koch et al. (2018); Murithi et al. (2020); Vanaerschot et al. (2020); Mok et al. (2021)
Intraerythrocytic stages	5×10^7	0.1% saponin	Liquid nitrogen	Chloroform and methanol/water (3:1) (GC–MS) 80% acetonitrile (LC–MS)	−80 °C	GC–MS (BD5 capillary column, EI-quadrupole MS) LC–MS (SeQuant ZIC-pHILIC column, Q-TOF)	ChemStation, MAVEN, METLIN	Dumont et al. (2019)
Intraerythrocytic stages — trophozoite	-	-	Ice-cold PBS	Methanol	−80 °C	GC–MS (DB5 capillary column)	IDEOM, XCMS, mzMatch, *R*	Creek et al. (2016)
Intraerythrocytic stages — early rings	-	0.15% saponin	Liquid nitrogen	Methanol	−80 °C	UPLC-MS/MS (HILIC, Q Exactive)	*R*	Carey et al. (2018)

Table 1 (continued)

Parasitic stage	Number of cells/sample	Purification	Quenching	Metabolite extraction method	Storage	Data acquisition method	Data analysis	Reference
Intraerythrocytic stages	1×10^5	0.07% saponin	-	Series of organic and aqueous extractions 10 v/v 0.1% formic acid in methanol	− 80 °C	GC–MS (Trace DSQ fast scanning single-quadrupole) HPLC–MS/MS (Zorbax Eclipse Plus C18, QQQ)	GraphPad Prism 5.0, *R*	Beri et al. (2019)
Intraerythrocytic, gametocytes and ookinetes	1×10^6	-	Dry ice-ethanol bath	Chloroform/methanol/water (1:3:1, v/v), sonication	− 80 °C	GC–MS (VF5-MS column)	Agilent ChemStation	Srivastava et al. (2016)
Intraerythrocytic stages	8×10^7	-	Dry ice-ethanol bath	Methanol, methanol/water (4:1), sonication	− 80 °C	uHPLC-MS (Hypersil-GOLD C18, QQQ)	-	Olszewski and Llinás (2013); Swift et al. (2020)
Intraerythrocytic stages	1×10^8	-	-	Chloroform/methanol/water (1:3:1 v/v)	-	GC–MS (DB5 capillary column)	Mass Hunter, IDEOM, MAVEN	Cobbold et al. (2016a)
Intraerythrocytic stages — trophozoite and late-stage schizont	1.2×10^{10}	-	-	Acetonitrile:water (4:1 v/v)	-	LC–ESI–MS/MS (Acquity UPLC BEH C18, Reaction Monitoring (SRM) Method, Quattro Premier XE MS)	-	Moles et al. (2017)
Intraerythrocytic stages	-	-	-	Acetonitrile (2:1, v/v)	-	LTQ-FTMS (C18)	Metlin Mass Spectrometry Database, apLCMS, KEGG	Park et al. (2015)
Intraerythrocytic stages – early ring	-	-	-	90% methanol	− 80 °C	Reversed-Phase Ion-Pairing LC–MS, Exactive Mass Spectrometer	MAVEN, *R*	Lee et al. (2018)
Intraerythrocytic stages	-	0.02% saponin	-	Sonication, 1.8 mM perchloric acid and pH adjusted with 5.4 M KOH	− 20 °C	^{1}HNMR	Mestrec software, MATLAB, HMDB, Metaboanalyst	Parvazi et al. (2016); Elmi et al. (2020)
Intraerythrocytic stages	-	-	-	Methanol:acetonitrile:water 5:3:2	− 80 °C	uHPLC-MS (Hypersil-GOLD C18, Orbitrap Elite)	GraphPad Prism v 7.04, *R*, HMDB, Metlin, and KEGG	Na et al. (2021)

Table 1 (continued)

Parasitic stage	Number of cells/ sample	Purification	Quenching	Metabolite extraction method	Storage	Data acquisition method	Data analysis	Reference
Intraerythrocytic stages	1×10^9	0.1% saponin	-	Chloroform/methanol/acetonitrile (2:1:1, v/v/v), 2 vols water	−80 °C	LC–MS/MS (Ion-pair reverse-phase chromatography, 4000 QTRAP)	-	Imlay et al. (2015); Guggisberg et al. (2018)
Intraerythrocytic stages and gametocytes	-	0.1% saponin	-	Isopropanol:hexane (1:2) and KCl (2 M):MeOH (4:1)	-	LC–MS (Zorbax Rx-Sil, QQQ)	MATLAB	Gulati et al. (2015)
Intraerythrocytic stage — late-stage schizonts	-	Hypotonic buffer (5 mM KH_2PO_4, pH 7.4)	-	Methanol	-	HPLC–MS (ReprosilPur Basic C18, LTQ-Orbitrap Discovery MS) HPLC–MS/MS (HSS T3, TSQ triple quadrupole MS)	XCALIBUR, XCMS, HMDB and METLIN	Rijpma et al. (2016)
Intraerythrocytic stages	5×10^7	-	-	N-ethylmaleimide in 80% methanol with 10 mM ammonium formate, and acetonitrile	−80 °C	HPLC–MS (HILIC-Z column, Q-Exactive Orbitrap)	QuanBrowser 4.2, GraphPad Prism 8.0.2	Siddiqui et al. (2022)
Intraerythrocytic stages	3.5×10^8 iRBC	0.15% saponin	-	Ethanol/0.01 M phosphate buffer (85:15, v/v), sonication	−80 °C	UPLC-MS/MS (ACQUITY, Xevo TQ-S)	MetIDQ, *R*	Brown et al. (2020)
Intraerythrocytic stages — extracellular vesicles	-	Ultrafiltration and size-exclusion chromatography	-	Chloroform:methanol (2:1)	4 °C	MALDI-TOF	-	Borgheti-Cardoso et al. (2020)

clustering profiles seemed to have peak activity at the ring-stage, opening the landscape to the possibility of multiple druggable targets at this stage. Similarly, another study that investigated ozonides' MoA revealed that hemoglobin-derived peptides were impacted as early as 6–12 h, further attesting the hemoglobin digestion theory (Giannangelo et al. 2020). More studies analyzing the hemoglobin catabolism and proteosome in this early stage would undoubtably advance the understanding of the beginning of the parasitic lifecycle and how it could be targeted in alternative ways to dihydroartemisinin.

Other lifecycle stages should also be considered noteworthy, since they represent timepoints in which interference can prevent transmission or resistance spread (Reader et al. 2015; Allman et al. 2016; Antonova-Koch et al. 2018; Delves et al. 2018; Sadeghi Tafreshi et al. 2019; Schalkwijk et al. 2019). This is the example of gametocytes. Only 1–2% of parasites differentiate in these sexual intraerythrocytic forms; however, they are solely responsible for perpetuating the sexual cycle to the invertebrate host (Delves et al. 2018; Ross and Fidock 2019). Gametocytogenesis is universal in the human-infecting *Plasmodia*, but it changes depending on the species, namely in the number of stages and the cycle duration, for example, five stages (gametocytes I–V) in approximately 10–12 days, in the case of *P. falciparum* (Lamour et al. 2014; Reader et al. 2015; Delves et al. 2018). Despite not causing symptoms, a drug with effective gametocidal activity would prove invaluable in blocking transmission. Unfortunately, gametocyte metabolomic studies are not easy, since it is difficult to generate enough biological material in vitro. Additionally, the late stage, responsible for transmission, is quiescent, awakening only in the conditions of the mosquito's midgut, thus limiting its metabolic studies and hindering drug targetability (Reader et al. 2015; Delves et al. 2018; Jennison et al. 2019). Presently, methods that account for up to 4% gametocytemia in in vitro culture exist (Reader et al. 2015). Notably, accumulation of homocysteine and redox metabolites in culture was associated with differentiation into sexual stages (Beri et al. 2017). Despite being intraerythrocytic parasitic forms, the metabolism between sexual and asexual stages is sufficiently different to allow future development of targeted gametocidal drugs. Specifically, glycolysis and mitochondrial processes were found to be activated distinctly in asexual and sexual life stages, respectively (Srivastava et al. 2016; Valenciano et al. 2019). Exclusively during gametocytogenesis, the mitochondria develop tubular cristae and expand in size (Valenciano et al. 2019). It is theorized that these modifications are correlated to an increased function of the tricarboxylic acid (TCA) cycle during gametocyte maturation in *P. falciparum* and *P. berghei*, despite these two species' different metabolic needs (Srivastava et al. 2016). Additional studies are needed to ascertain and screen compounds MoA against this stage, so that a transmission blocking treatment can be developed.

The mosquito parasite stages represent additional complications, since they require supplementary laboratory apparatus and produce small biological samples, especially in comparison to in vitro intraerythrocytic cultures (Antonova-Koch et al. 2018; Delves et al. 2018). One naturally infected mosquito carries between 2 and 5 oocysts, while feeding assays can reach a saturation of around 400 oocysts, in comparison to tens of millions of parasites in the human intraerythrocytic stages (Delves et al. 2018). An ambitious study by Antonova-Koch et al. screened half a million compounds for their capability of inhibiting hepatic invasion, hence possessing chemoprophylactic properties, by dissecting more than one million infected mosquitoes over a 2-year period (Antonova-Koch et al. 2018). This demonstrates the difficulties in generating enough mosquito infective forms to study these and downstream stages. This study found, through phenotypic and metabolic profiling approaches, 58 potential antimalarial drugs, indicated as mitochondrial inhibitors, that would benefit from being explored further (Antonova-Koch et al. 2018). Additionally, parasitic stages in the mosquito host are not stationary and differ between female and male. In a metabolomic and genomic study, upon ingestion by mosquitos, *P. berghei* female and male gametocytes were shown to develop into gametes through different processes (Srivastava et al. 2016). Females conserve the mitochondria, while it degenerates in the male counterpart. The latter is thought to be more vulnerable to drugs because of its higher necessity of glucose for both mobility and the fast-multiple rounds of nuclear replication (Srivastava et al. 2016). Alternatively, female gametes can use glutamine and the TCA cycle to fuel ATP synthesis, making them less dependent on energy storage and more prone to interchanging the carbon source pathway. Other changes related to glucose availability, like the midgut pH, have also been shown to metabolically induce *P. berghei* sexual development (Wang et al. 2021). Furthermore, ookinetes remain metabolically active and dependent on both glucose and the TCA cycle. CoA synthesis seems to also play an important role, both in carbon metabolism and lipid synthesis, thus affecting ookinete to oocyst conversion and oocyst sporogony. Hence, compounds that could disrupt these pathways could prove important transmission blocking properties (Srivastava et al. 2016; Delves et al. 2018).

Lastly, hepatic schizonts and hypnozoites are also important, permitting intervention before the disease manifests and the depletion of *P. vivax* and *P. ovale* reservoirs (Reader et al. 2015; Pewkliang et al. 2018; Delves et al. 2018). However, hypnozoites are unsusceptible to most antimalarials as they remain quiescent, which also hampers their metabolic evaluation. Additionally, it is difficult to culture the hepatic stages, as hepatocytes are harder to maintain and have smaller life

spawns than RBC. Hence, robust in vitro hepatic stage models are an area of interest for malaria research, which would facilitate metabolomic testing. Most models still have issues relative to deficient surface receptors, abnormal cell regulation, and loss of hepatic function, responsible for poor sporozoite invasion rates and difficulties in maintaining and in-depth studying this stage (Pewkliang et al. 2018; Roth et al. 2018). Recent developments of culture systems for primary human hepatocytes and immortalized hepatocyte-like cell line models represent important advancements that will enable studying these forms in the future (Flannery et al. 2018; Pewkliang et al. 2018; Roth et al. 2018).

Finally, a few key obstacles need to be considered when choosing the sample and sampling for metabolomic studies. As metabolites or pathways are sometimes overlapping between host and parasite, care is important when treating data to account for the metabolome that does not originate from the parasite. Specifically, in the case of in vitro assays, cultures usually have parasitemia up to 10%, meaning there are significantly more RBC than parasites (Olszewski and Llinás 2013; Carey et al. 2018). Some experimental steps can be performed to selectively extract the parasitic metabolome, for example, through saponin treatment. Saponin at a given concentration was demonstrated to lyse RBC cellular membranes, while maintaining the parasitic membrane intact (Crary and Haldar 1992). Additionally, special culturing protocols have allowed to, through this process, have parasites directly in culture without the RBC (Cobbold et al. 2016b). Even if for a limited time, this so-called functional isolation enabled different approaches on the study of uptake of certain metabolites and drugs. The saponin isolation method is used recurrently in the methods described in Table 1. Care is still important even when using this technique, considering that RBC remains may stay attached to the parasite and influence analysis (Carey et al. 2018). Another method to induce lysis is incubation with streptolysin, yet it is less used because it is recognized that it does not disintegrate the RBC's membranes, leading instead to pores and increased sample contamination (Moll et al. 2008; Besteiro et al. 2010). Other lysis options exist, based on solutions with NH_4Cl, $KHCO_3$ and EDTA; however, incubation times of 15 min prevent them from being used in metabolomic workflows, despite their advantage of allowing membrane purification and study, which is important for lipidomics (Moll et al. 2008). The lysis step, when performed, should last from a few seconds to a couple of minutes and usually bellow 4 °C.

Additionally, studies typically do not distinguish or note the age of RBC used in culture, thus not accounting for a factor that, as mentioned above, can influence parasitic growth and antiplasmodial activity (Olszewski et al. 2011). In culture maintenance, some protocols specify that the conservation of RBC should not surpass 1 month, ideally 2 weeks (Trager and Jensen 1976; Radfar et al. 2009; Tewari et al. 2021a). This assures that the parasites have fresh cells to infect and ascertains the quality of these cells. Since *P. falciparum*, the most recurrent species in culture, can infect RBC of any age, age or type of cells is not usually a problem and, hence, goes unnoticed. Even if type or age is not noted, RBC of the same donor should be used to account for the least variance possible. This was demonstrated to be an important factor in a recent metabolomic study by Tewari et al. (2021a). This team verified if there were significant batch differences or metabolic biases that could inadvertently come from the RBC in vitro. As such, this assay maintained uninfected RBC in the same conditions as originally intended for infected red blood cell (iRBC) and tested the metabolome through high-resolution metabolomics. Despite inter-study consistency, individual donors were responsible for metabolic variations, pointing to the necessity of more batch controls in metabolic studies (Tewari et al. 2021a). Parallelly, blood storage was proven to be an important factor, as peptide modifications, due to degradation of methemoglobin, alongside with variations in glycolysis and in synthesis of glutathione were detected (Tewari et al. 2021a). These variations are often overlooked in metabolomic profiling in *Plasmodium* studies, but could prove to adulterate results and should be carefully analyzed to eliminate biases (Tewari et al. 2021a).

When performing metabolomic studies, care should be given to possible contaminations from the RBC's metabolome or the extracellular medium. This can be done by incorporating washing steps, by sampling RBC standard metabolomes for comparison, and through fluxomics. Via enrichment techniques, it is possible to follow some metabolites and confirm branching or extension of pathways. Indeed, studies using metabolically labeled ^{13}C-glucose have successfully distinguished pathway activation between parasite and RBC. Notably, in a study by MacRae et al., TCA cycle intermediates were found in ring stage-iRBC at five times the concentration of uninfected RBCs, with significant labeling of these compounds in the infected sample (MacRae et al. 2013). The difference in labeling between uninfected and iRBC successfully discerned the extensiveness and rate of metabolic fluxes of the TCA cycle (MacRae et al. 2013). More recently, Cobbold et al. performed an untargeted LC–MS metabolomic assay with unsupervised isotopologue grouping to describe the metabolic capacity of the *P. falciparum* trophozoite–infected RBC and RBC (Cobbold et al. 2021). The basis for the study was that significant protein and metabolic-encoding *P. falciparum* genes are still unannotated, thus failing to shed light on enzyme promiscuity and ambiguous metabolites (Cobbold et al. 2021). By characterizing the metabolites and successfully distinguishing them between infected and non-infected RBCs, this research hoped to identify unexpected parasitic metabolic pathways,

complement gene annotation, and successfully remove RBC biases. This study was able to describe over 70% of parasite gene-encoded metabolic reactions, detecting 911 and 577 metabolites for the iRBC and RBC, respectively (Cobbold et al. 2021). It was found that the largest group of undescribed metabolites was related to parasitic damage-repair systems, which still need further investigation (Cobbold et al. 2021).

In order to explore the impact of RBC metabolome and media contamination, Carey et al. sought to define parameters that could optimize critical and specific *Plasmodium* factors (Carey et al. 2018). They demonstrated in an untargeted metabolomic study of intraerythrocytic *P. falciparum* that, despite varying multiple experimental conditions, both the RBC and the culture medium contaminated the samples, influencing the final results. Particularly, over 68% of parasites remained attached to "ghost" RBC, namely cell membranes, that influenced the metabolite counts, predominantly the lipidic component (Carey et al. 2018). Similarly, normalization was found to be the second most influential parameter and the method choice skewed greatly the results interpretability. Normalization by DNA content or parasite number still failed to eliminate the contamination effect from the datasets. Additionally, this study showed how the manual elimination of media and RBC contaminants, such as HEPES or phenol red, from the datasets is erroneous and should, instead, be considered for analysis, both for quality control and to evaluate the extension and type of contamination (Carey et al. 2018).

When possible, other enzymatic, membrane potential or omics methodologies should be employed alongside metabolomics to confirm or obtain further insight into parasitic-specific mechanisms. The presence of HEPES or other culture media exclusive components, as well as RBC-specific metabolites, should be incorporated as markers to give information on media contamination of samples or overall quality of the washing steps of the workflow. This would prevent result bias and improve overall assay quality.

Quenching

Metabolism shutdown is a crucial step in a metabolomics workflow. This key point, known as quenching, ensures enzymatic arrest as quickly as possible and, thus, that the sample reliably correlates to the experimental conditions (Creek et al. 2016; Price et al. 2018; Cobbold and McConville 2019). However, despite its essentiality, this step is sometimes overlooked in workflows. In fact, quenching is not easy to implement and its methodology needs to be adapted to the study's design.

Quenching methodologies are usually based on variating sample temperature and/or adding solvents, although there is no universal method (Jang et al. 2018; Price et al. 2018). For cultures, cells are frequently placed in contact with cold organic solvents (4 °C, usually lower), either after media aspiration, for adherent cells, or filtration, for non-adherent. The objective is to slow the enzymatic activity by diminishing the temperature and to assure enzyme denaturation, which happens permanently with organic solvents (Jang et al. 2018). Despite being non-adherent cells, in vitro *Plasmodium* metabolomic assays do not commonly include filtration steps in their workflow. However, fast-filtering could present advantages in the speed of segregating the cells from the media in comparison with current centrifugation cycles present in some workflows (Jang et al. 2018; Price et al. 2018).

Any steps that may alter the metabolism or induce stress are usually advised against. These include cell pelleting and wash steps prior to quenching. However, in the workflows presented in Table 1, it is noticeable that most studies perform cell pelleting. It is a frequent antiplasmodial metabolomic workflow to incubate the sample with saponin below 4 °C, followed by pelleting and washing with cold PBS before extraction. Despite uniformity in the quenching methods in the literature and in Table 1, metabolomics in the malaria field still needs research and viable alternatives in order to optimize this step of the workflow (Srivastava et al. 2016; Creek et al. 2016; Cobbold and McConville 2019).

Metabolite extraction

After quenching, and in this case separation between RBC and parasite, the next step is to obtain the latter's metabolites. The extraction is a key mandatory step in sample processing, allowing the isolation of the metabolome. Organic solvents play multiple roles at this point of the workflow: breaking down membranes, retaining the metabolome, and enzymatic denaturation (Jang et al. 2018). An additional role of the extraction step with organic solvents is sample deproteinization, which cleans the sample of interferents for posterior analysis (Wishart 2010; Olszewski and Llinás 2013). This is important, since *Plasmodium* in vitro cultures contain plasma, and hence proteins and lipoproteins, not to mention the presence of hemoglobin and other parasitic proteins. The solvent choice is crucial, because it influences the range of attained metabolites, based on their physicochemical characteristics (Cobbold and McConville 2019; Bao and Liu 2020). Additionally, the extension of metabolite extraction depends on the time, type, and possible mixture of solvents chosen. Hence, these factors need to be chosen carefully.

As shown in Table 1, the most common extraction solvents in *P. falciparum* in vitro metabolomic studies are acetonitrile, methanol, chloroform, and water, in various gradients, with few exceptions, such as hexane (Cobbold et al. 2016a; Dickerman et al. 2016; Carey et al. 2018). Hence,

by variating the gradients and mixtures, the extraction of the most differentiated molecules possible is assured, which increments the likelihood of attaining significant biological data (Bao and Liu 2020). The time of contact also variates from a few minutes to 30 min, which reveals the compromise between long experimental times and the extensiveness of metabolic extraction. Multiple cycles of extraction with a fresh volume of solvent are also frequent to try to obtain a most complete metabolome as possible. Finally, solvents are usually removed through evaporation under nitrogen stream or freeze-drying (Olszewski and Llinás 2013; Price et al. 2018).

Data acquisition

After a sample has been collected and processed appropriately through the methodologies presented previously, it needs to be analyzed. As such, the objective of data acquisition is to detect accurately, and potentially quantify, as many metabolites as possible (Jang et al. 2018).

Robust and powerful analytical technologies are needed to perform metabolomic studies (Sana et al. 2013). Platforms based on chromatography, such as liquid chromatography (LC) and gas chromatography (GC), coupled with mass spectrometry (MS), or nuclear magnetic resonance (NMR) made metabolomics possible because of their sensitivity, selectivity, and reproducibility (Wishart 2010; Emwas 2015). These analytical methodologies are versatile in characterizing samples by their chemical composition, be it the affinity for the stationary phase of a column, the ionization profile, or chemical shift, among other factors. Their features allow them to be used in diverse circumstances, making them universal in metabolomic studies, including in malaria studies (Bao and Liu 2020). However, different platforms have unique advantages and disadvantages and may require care during the sample treatment stages. These analytical techniques have been reviewed extensively elsewhere (Saini et al. 2021; Harrieder et al. 2022; Moco 2022).

In the *Plasmodium* in vitro context, NMR is not utilized as frequently as MS. This can be because of the lack of access to different platforms or the relatively lower sensitivity of this technique. This characteristic does imply that NMR requires more parasites per sample than MS, above 10^8 parasites. Interestingly, some studies have pointed out the benefits of using NMR instead of MS. For example, glycerol was left undetected in the *Plasmodium* until an NMR assay successfully detected it (Lian et al. 2009). Glycerol is a glucose catabolism end product that had been identified in other human parasitic protozoa, but not in the *P. falciparum.* This because glycerol has no chromophore and ionizes poorly through ESI; thus, UV detection or MS failed to detect it (Lian et al. 2009). Extensive derivatization might have been relevant to make this metabolite detectable, but in global metabolomics, there is no a priori knowledge of the necessity to implement such techniques, as the content of the metabolome is unknown or putative. ^{13}C NMR successfully detected and identified glycerol in *P. falciparum* samples, suggesting that the parasite maintains carbon and redox equilibrium through the ramification of the use of glucose (Lian et al. 2009). This process is significantly different in RBC, which uses glucose exclusively for glycolysis. This discovery paved the way for more studies that focused on the carbon metabolism of this parasite.

Data analysis and interpretation

Due to the metabolome's complexity, the interpretation of the obtained chromatograms and spectra is challenging. This is inherent to omics data because one sample originates dozens to thousands of features that are not readily interpretable. These data needs to be simplified and analyzed per sample, frequently without any preconception of the metabolites to be found (Price et al. 2018). Malaria-derived data is treated similarly to other metabolomics sets, using the same processing software and statistical models.

Only the databases represent a significant difference. Databases are used to facilitate metabolite annotation through the concentration of information from multiple studies that are interchangeable between laboratories (Emwas et al. 2019; Bao and Liu 2020; Phelan 2020). Their construction is specific and time and labor-consuming, but they allow a fast exploration of metabolic data. Some databases have the option for *P. falciparum* metabolites or are more general, containing biological metabolites from many organisms (Pang et al. 2021). Plasmodia-oriented databases are the PlasmoDB (https://plasmodb.org/) or Malaria Parasite Metabolic Pathways (https://mpmp.huji.ac.il/) (Sana et al. 2013; Cowell and Winzeler 2019; Valenciano et al. 2019; Mok et al. 2021). These do contain not only exclusively metabolic reactions, but also the genome and other parasite-related data, and their principal objective is to link genes to other structures in order to annotate their functional meaning. Examples of freely accessible databases with information on both MS and NMR analysis are the Human Metabolome Database (HMDB — https://hmdb.ca/) and the Kyoto University Encyclopedia of Genes and Genomes (KEGG — https://www.genome.jp) (Emwas et al. 2019; Bao and Liu 2020; Phelan 2020). Another example that works exclusively with NMR spectra is Chenomx NMRSuite (Price et al. 2018; Emwas et al. 2019). The matrix tables resulting from data analysis can be uploaded to platforms that annotate and map metabolic pathways such as Metaboanalyst or Workflow4Metabolomics, which have a *P. falciparum* option (Jang et al. 2018; Pang et al. 2021). This allows

not only to putatively identify unknown metabolites but also to interpret their biological function (Bao and Liu 2020). Generally, multiple databases are used in plasmodial metabolomic studies.

The final step of metabolomic data treatment is statistical analysis, although other data mining and analysis methods can be performed (Phelan 2020). This analysis can be performed before annotation, in a chemometric approach, or after, in quantitative metabolomics. In *Plasmodium* in culture assays, it is more frequent to encounter chemometrics due to its unbiased nature and potential for automatization (Wishart 2010).

Finally, it is worth mentioning that metabolomics data can also be used to confirm computational models' predictions or provide experimental data to build said models. These in silico assays can mirror the metabolic network of the *Plasmodium* through algorithms and thus predict outcomes, essentiality and exclusivity by virtually evaluating the impact of several factors (Oyelade et al. 2018; Tewari et al. 2019, 2021b). Despite limitations, these models are complementary to experimental studies and are sure to simplify malaria drug discovery in the future (Tewari et al. 2017, 2022).

Troubleshooting

As with any troubleshooting, after identifying the weaknesses and shortcomings of the workflow processes, it is possible and worthwhile to find workarounds and solutions. Various indicators towards optimization were pointed out so far, depending on the workflow step. In terms of study design, time or concentration-resolved experiments and fluxomics can be implemented to increase the screen's dimensionality and distinguish unspecific metabolic variations and help to establish causality. In the experimental procedures, in order to reduce or attenuate the host cell or the media impacts, some steps can be foreseen. One is parasite purification, by isolation or enrichment, by increasing the parasite:RBC ratio. Another is tracing analytes not metabolized or synthesized by the parasite, such as HEPES or phenol red, that are externally added through the culture media. Finally, samples collected from said media, RBC metabolome or the different washing steps can provide insight on contamination. Microscopy can also be helpful in confirming the integrity of parasitic membranes and the presence or absence of RBC membrane residues still attached, before continuing the metabolomic assay. Isotope marking can also be used to improve data quality, as marked species can be used to confidently remove background or degeneracy features, which can represent over 90% of all detected features in LC–MS (Cobbold et al. 2021). Non-singular acquisition methodologies should be universally implemented in order to attain and explore as further as possible individual metabolites. This would enable identification and interpretability with high degrees of confidence and thus the building of plasmodial databases that could be exchanged between laboratories. And lastly, the data processing and normalization procedures should be validated and standardized, in order to improve analytical reproducibility across plasmodial metabolomic studies (Carey et al. 2018; Cobbold and McConville 2019; Birrell et al. 2020; Phelan 2020).

Metabolomics in the discovery of mechanisms of action of antimalarials

The aforementioned workflow warranted the discovery and validation of metabolic targets of antimalarials and other potential compounds in the research pipeline (Cobbold et al. 2016a; Creek et al. 2016; Allman et al. 2016; Birrell et al. 2020; Murithi et al. 2020). Generally, compounds can have one specific target or be pleiotropic, consequently posing difficulties in defining the MoA through traditional means (Cobbold et al. 2016a; Cobbold and McConville 2019; Yoo et al. 2020). A metabolomic profile is able to disclose a compound's distinguishable fingerprint and evidencing downstream events that better correlate to parasite death. This means that the MoA would not relate to a single target, but to a series of interactions that relay to how the parasite reacted to the drug, hence also shedding light on possible resistance mechanisms. When essential parasitic pathways are targeted, their exclusiveness can increase the molecule's antiparasitic efficacy and downplay side effects. Through metabolomics, traditional antimalarials and promising compounds can be studied, both to optimize current therapies and to guide drug development to a treatment that will lead to the eradication of malaria.

With this purpose, multiple established and potential antimalarials were metabolomically studied throughout the last decade (Cobbold et al. 2016a; Creek et al. 2016; Allman et al. 2016). Table 2 summarizes the data collected in this context with a focus on the discoveries since 2015. Overall, seven metabolomic profiles were identified according to the pathway affected, as simplified in Fig. 3: hemoglobin metabolism and protein degradation, pyrimidine biosynthesis and the mitochondria electron transport chain (mitETC), isoprenoid biosynthesis, fatty acid metabolism, folate biosynthesis, homeostasis, and unknown (Cobbold et al. 2016a; Creek et al. 2016; Allman et al. 2016). These profiles are described in the next paragraphs.

Hemoglobin metabolism and protein degradation

Protein catabolism is recognized as one of the most important plasmodial metabolic pathways to target by antimalarial therapies. This stems from the fact that this genus has rudimentary amino acid biosynthesis and has adapted to obtain

them from other sources (Uppal et al. 2017). The *Plasmodium* scavenges amino acids in order to fulfill its protein requirements, both in the invertebrate and vertebrate hosts. In the intraerythrocytic cycle, the most common sources are the RBC hemoglobin and the importation of free amino acids from the host (Birrell et al. 2020; Murithi et al. 2020). These metabolites are in turn used by the parasite for various purposes, like signaling, replication, and survival.

This pathway can be disturbed at multiple points: during hemoglobin uptake, catabolism, or detoxification; amino acid transport; and DV homeostasis or its enzymes (Murithi et al. 2020). When antimalarials interfere with these processes, their metabolomic profile shows variations of hemoglobin-derived peptides, such as prolyl-glutamate, prolyl-aspartate, prolyl-glutamyl-glutamate and aspartyl-leucyl-histidine, and amino acids (Cobbold et al. 2016a). One example is leucine, whose efflux out of the iRBC is well described and is considered an indirect measurement of hemoglobin uptake and catabolism. This is mostly because uninfected RBC amino acid efflux is negligible and hemoglobin amino acid composition directly correlates to amino acid relative efflux rates (Ghavami et al. 2016). Further omics are able to detect modifications regarding hemoglobin, heme, hemozoin, and intermediary peptides (Cobbold et al. 2016a; Creek et al. 2016; Allman et al. 2016; Birrell et al. 2020; Murithi et al. 2020).

Because these essential processes are non-concomitant with the host metabolism, they represent an effective target against the malaria parasite. Indeed, many antimalarial therapies are based on drugs whose main MoA is the interference with this metabolic pathway, as displayed in Table 2 (Reader et al. 2015; Creek et al. 2016; Allman et al. 2016; Murithi et al. 2020). One of the most known is the 4-aminoquinoline class, such as chloroquine, that is a recognized inhibitor of the heme biomineralization pathway. Chloroquine is a diprotic weak base that is accumulated in the DV, whose pH differential with the parasitic cytoplasm assures drug retention (Khan et al. 2019). Resistance to chloroquine is well described and occurs through the *P. falciparum* chloroquine resistance transporter (PfCRT) (Lee et al. 2018). PfCRT is a membrane pump responsible for multiple activities, such as the regulation of hemoglobin metabolism and ionic balance of the DV (Khan et al. 2019; Ross and Fidock 2019). In resistant parasites, it is also responsible for drug efflux, effectively reducing its amount inside the DV, preventing its activity (Ghavami et al. 2016; Lee et al. 2018). Many metabolomic assays explored the profile of chloroquine, either as a reference or as a focus. For example, a ^{1}HNMR spectroscopy assay by Elmi et al. evaluated the antiparasitic effect of a nanocomposite, a dendrimer chloroquine-curcumin conjugate, on the metabolism of *P. falciparum* asexual ring stage (Elmi et al. 2020). This study found that the nanocomposite improved chloroquine and curcumin's individual activity, possibly due to synergy, and that metabolites from the glyoxylate and dicarboxylate metabolism were the most affected in parasites treated with the nanoparticle (Elmi et al. 2020). This was proven to be significantly different from the metabolism changes induced by chloroquine alone, or the control group, evidencing venues of novel targets (Elmi et al. 2020). Another example is the study by Na et al. that sought to characterize the mechanisms underlaying chloroquine resistance at a metabolic level by comparing the signatures of chloroquine-resistant and sensitive strains after chloroquine treatment (Na et al. 2021). Phenotypes between strains pointed to multiple factors of chloroquine resistance. Reduced levels of glutathione and its precursors pointed to oxidative stress in resistant strains (Na et al. 2021). Additionally, depletion of fructose-1,6-bisphosphate and alanine could indicate induction of glycolysis to compensate the increased consumption of inosine monophosphate, necessary for replication, in resistant strains (Na et al. 2021). In addition to changes in phenylalanine metabolism, these variations from the chloroquine-sensitive strain profile could be indicative of metabolic changes that assure fitness under resistance conditions.

Another drug class example is the aryl amino-alcohol group of antimalarials, which include lumefantrine and mefloquine. These two drugs do not act through the same mechanism. While lumefantrine is thought to interfere with hemoglobin uptake, mefloquine is thought to be pleiotropic and its main MoA is still a topic of discussion (Ghavami et al. 2016; Wong et al. 2017; Birrell et al. 2020; Murithi et al. 2020). Initially, while trying to elicit resistance to mefloquine through selective pressure in vitro, it became evident that its primary resistance factor is the *P. falciparum* multidrug resistance 1 (PfMDR1) that encodes the Pgh-1 protein, a DV membrane transporter that is originally responsible for solute influx (Wong et al. 2017; Birrell et al. 2020; Murithi et al. 2020). Subsequently, studies have suggested mefloquine's MoA is similar to chloroquine or lumefantrine, albeit with lesser activity. Metabolomic studies profiled these antimalarials and showed that lumefantrine clustered with GNF-Pf-5660, a known hemoglobin uptake inhibitor that does not affect hemozoin detoxification, while separating greatly from mefloquine's profile (Murithi et al. 2020). The former dissociated also from chloroquine and other hemoglobin catabolism inhibitors, indicating that its overall MoA is in fact different (Murithi et al. 2020). Metabolomic profiles also evidence stage peak activity: Lumefantrine and chloroquine are active in both ring and trophozoite stages equally, whereas mefloquine peaks in trophozoite only, further distinguishing their targets (Murithi et al. 2020). It is believed that mefloquine's cytosolic target may be the Pf80S ribosome, where the direct binding inhibits protein synthesis (Wong et al. 2017). Despite different MoA, both lumefantrine and mefloquine are affected by the

Table 2 Profiles of antiplasmodial compounds found or confirmed through metabolomics

Compound	Stage	Metabolic pathway = profile	Putative target/mode of action	References
3361	Ring	Glycolysis	Plasma membrane hexose transporter	Cobbold et al. (2016a); Creek et al. (2016)
2-deoxyglucose	Trophozoite	Homeostasis	Glycolysis	Allman et al. (2016)
Artefenomel (ozonides)	Trophozoite	Redox homeostasis	Inductor of oxidative stress	Siddiqui et al. (2022)
Atovaquone	Late trophozoite Gametocyte[1]	Pyrimidine synthesis and mitochondrial potential	Mitochondrial electron transport chain, *Bc1* complex	Reader et al. (2015); Cobbold et al. (2016a); Creek et al. (2016); Allman et al. (2016); Birrell et al. (2020); Murithi et al. (2020)
C7 (MMV665915), E4 (MMV666600) and P2_A3 (MMV000634)	Trophozoite	Lipid metabolism	Alpha-linolenic acid (ALA) pathway	Creek et al. (2016)
Chloroquine	Ring, trophozoite, early gametocyte	Hemoglobin metabolism	Hemoglobin catabolism Heme detoxification	Reader et al. (2015); Creek et al. (2016); Allman et al. (2016); Murithi et al. (2020)
Cycloguanil	Ring	Folate biosynthesis	Dihydrofolate reductase-thymidylate synthase (DHFR-TS)	Cobbold et al. (2016a)
Dihydroartemisinin[2]	Ring, Trophozoite	Hemoglobin metabolism Redox homeostasis Pyrimidine synthesis	Multiple hemoglobin-digesting proteases Inductor of oxidative stress	Creek et al. (2016); Birrell et al. (2020); Murithi et al. (2020)
DSM265	Late trophozoite	Pyrimidine synthesis and mitochondrial potential	Dihydroorotate dehydrogenase (DHODH)	Allman et al. (2016); Murithi et al. (2020)
Ferroquine	Ring	Hemoglobin metabolism Redox homeostasis	Heme detoxification Fenton reaction	Murithi et al. (2020)
Fosmidomycin	Trophozoite	Isoprenoid biosynthesis	2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway	Cobbold et al. (2016a); Gisselberg et al. (2018); Dumont et al. (2019); Murithi et al. (2020)
Indolmycin	Trophozoite	Isoprenoid biosynthesis	Apicoplast tryptophanyl-tRNA synthetase (TrpRSapi)	Kennedy et al. (2019)
JPC-3210 (MMV 892,646)	Trophozoite	Hemoglobin metabolism	β-hematin polymerization	Birrell et al. (2020)
KAE609 (cipargamin), (+)-SJ733, and KAF246	Trophozoite	Homeostasis	*Pf*ATP4	White et al. (2014); Jiménez-Díaz et al. (2014); Allman et al. (2016)
KAI407	Schizonts	Hemoglobin metabolism	Phosphatidylinositol 4-kinase (PI4K)	Murithi et al. (2020)
Lumefantrine	Ring, trophozoite	Hemoglobin metabolism	Hemoglobin uptake	Murithi et al. (2020)
Mefloquine	Trophozoite	Hemoglobin metabolism	Heme detoxification	Birrell et al. (2020); Murithi et al. (2020)
Methylene blue	Ring, trophozoite, gametocytes	Hemoglobin metabolism Redox homeostasis	Heme detoxification Glutathione reductase Inductive of oxidative stress	Cobbold et al. (2016a); Creek et al. (2016); Sadeghi Tafreshi et al. (2019); Murithi et al. (2020)

Table 2 (continued)

Compound	Stage	Metabolic pathway = profile	Putative target/mode of action	References
MMV007571 and MMV020439	Trophozoite	New permeability pathways Pyrimidine synthesis and mitochondrial potential	Mitochondrial electron transport chain, *Bc1* complex DHODH	Dickerman et al. (2016); Rawat and Verma (2020)
MMV007839 and MMV000972	Trophozoite	Homeostasis	Formate nitrite H^+-transporter (PfFNT)	Hapuarachchi et al. (2017)
MMV08138	Trophozoite	Isoprenoid biosynthesis	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (MEP/IspD)	Wu et al. (2015)
MMV667487 and P218	Trophozoite	Folate Biosynthesis	DHFR-TS	Allman et al. (2016)
Naphthoquinone	Ring	Hemoglobin metabolism	Heme detoxification	Murithi et al. (2020)
Piperaquine	Ring, Trophozoite	Hemoglobin metabolism	Heme detoxification	Creek et al. (2016); Murithi et al. (2020)
Primaquine[2,3]	Trophozoite, gametocyte, hypnozoite	Homeostasis	Inductive of oxidative stress	Reader et al. (2015) (Review Marcsisin 2016)
Proguanil[3]	Ring	Protein degradation	Arginase enzyme or transporters	Cobbold et al. (2016a)
Pyrimethamine	Trophozoite	Folate biosynthesis	DHFR-TS	Rijpma et al. (2016); Allman et al. (2016)
Sal A	Trophozoite, schizont	Lipid metabolism	Serine hydrolases	Yoo et al. (2020)
Tafenoquine[2,3]	Trophozoite, gametocyte	Homeostasis	Inductive of oxidative stress	Reader et al. (2015); Jennison et al. (2019); Birrell et al. (2020)
Torin 2	Ring, gametocyte, hypnozoite	Hemoglobin metabolism	Hemoglobin catabolism	Cobbold et al. (2016a)

[1]Atovaquone has limited gametocidal activity. Metabolic fingerprint resulted from 1 μM exposure for 2.5 h to stage III–IV gametocytes

[2]Exact mechanism of action is unknown

[3]Is a pro-drug

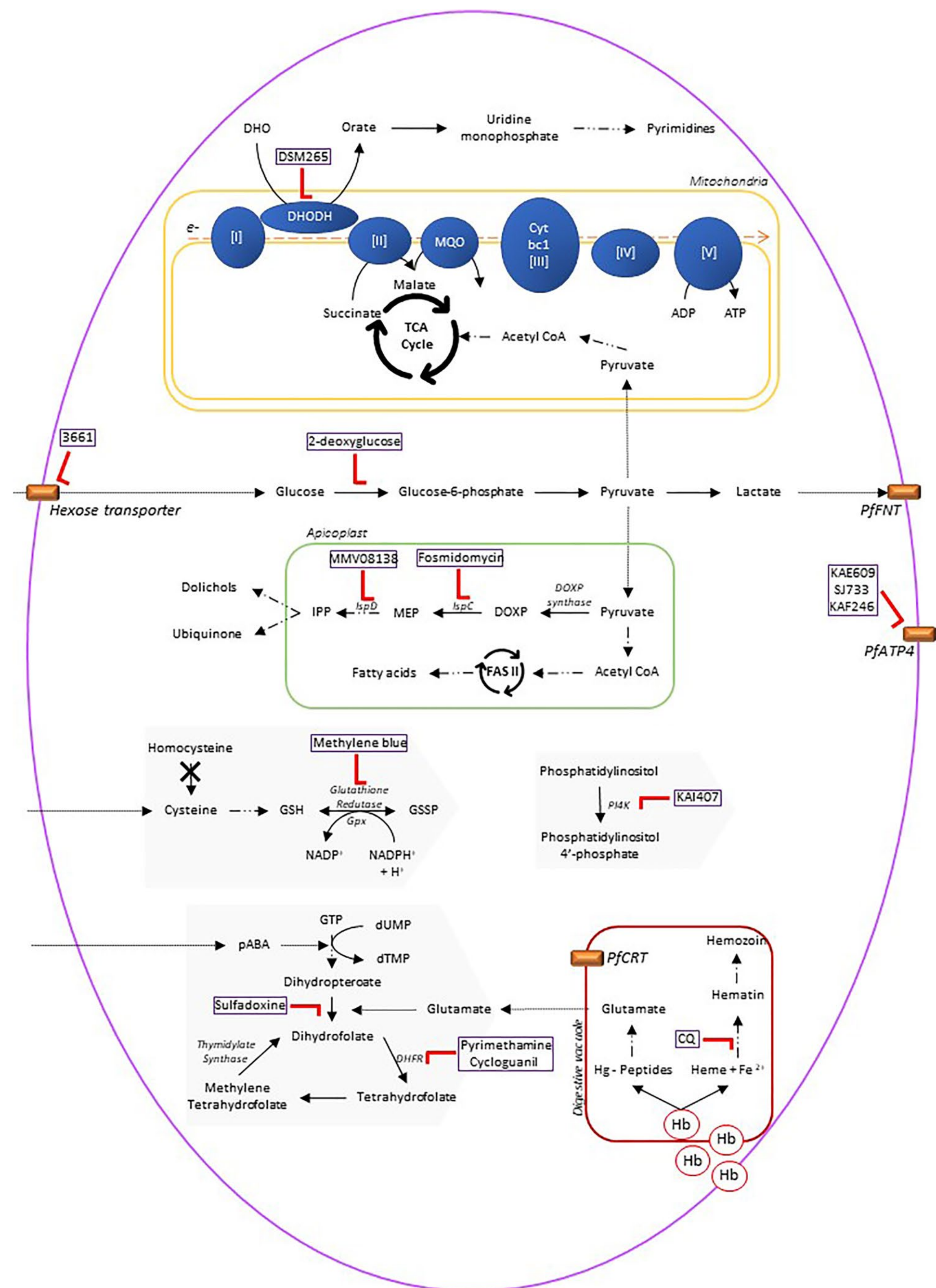

Fig. 3 Schematic view of primary and innovative metabolic targets in the asexual life stages of the human-infecting *Plasmodium* spp. Abbreviatures: CQ, chloroquine; Cyt bc1, cytochrome bc1; DHFR, dihydrofolate reductase; DHO, dihydroorotate; DHODH, dihydroorotate dehydrogenase; DOXP, 1-deoxy-D-xylulose 5-phosphate; FAS II, fatty acid synthesis; Gpx, glutathione peroxidase; GSH, reduced glutathione; GSSP, oxidized Glutathione; Hb, hemoglobin; Hg-Peptides, hemoglobin-derived peptides; MQO, *L*-malate:quinone oxidoreductase; *Pf*CRT, *P. falciparum* chloroquine resistance transporter; *Pf*FNT, *P. falciparum* formate nitrite transporter; PI4K, phosphatidylinositol 4′-kinases

Pfmdr1 (Nzila and Mwai 2009; Wurtz et al. 2014). This, however, suggests a level of adaptation that confers indiscriminate protection to multiple drugs, not specific resistance, as amodiaquine and artemisinin derivatives efficacy is also affected by this gene (Murithi et al. 2020). Curiously, chloroquine resistance is not associated with this transporter, which confirms the distinguished metabolomic profile found in multiple studies between these drugs (Nzila and Mwai 2009; Wurtz et al. 2014; Ghavami et al. 2016; Birrell et al. 2020; Murithi et al. 2020). Dihydroartemisinin, the active metabolite of artemisinin derivatives, is also thought to affect hemoglobin metabolism as one of its pleiotropic ways (Birrell et al. 2020; Murithi et al. 2020). This occurs upon activation with heme-bound iron that induces the formation of cytotoxic radicals (Khan et al. 2019; Krishnan et al. 2020). In untargeted metabolomic studies, however, it has become evident that this is not its main MoA, as its profile diverges greatly from those of chloroquine, lumefantrine, or mefloquine (Murithi et al. 2020).

In spite of the existing widespread resistance to this group of antimalarials, hemoglobin digestion and amino acid and protein biosynthesis are still interesting targets

for new drugs, the reason being multiple opportunities are present within this pathway while being exempt of PfCRT or *Pfmdr1* interference. In this context, one MoA that has gained attention is the phosphatidylinositol phosphate pathway. It includes three ubiquitous kinases that phosphorylate lipids at different positions with the purpose of regulating cellular processes, such as survival, proliferation, signaling, and membrane trafficking (Hassett and Roepe 2018). In the case of the *Plasmodium*, despite initial uncertainty regarding their presence, two have been validated as drug targets through metabolomic studies: phosphatidylinositol 3′-kinases (PI3Ks) and phosphatidylinositol 4′-kinases (PI4Ks) (Hassett and Roepe 2018). PI4K was validated as the target for imidazopyrazines, such as KAI407, while PI3K was described as the target of Torin 2 (McNamara et al. 2013; Murithi et al. 2020). KAI407 was shown to act selectively during the late-trophozoite to schizont stages, while exposure from early-rings up to 24 h showed no loss of parasitic viability (Murithi et al. 2020). In fact, exposure between 34 and 44 h blocked parasitic reinvasion and left an accumulation of multinucleated schizonts (McNamara et al. 2013). Although this kinase is present throughout the entire intraerythrocytic cycle, the consequences of the depletion of phosphatidylinositol-4-phosphate pools may become only apparent with merozoite formation, justifying why KAI407 peak activity occurs at this time (Murithi et al. 2020). Interestingly, in the metabolomic study by Murithi et al., KAI407 fingerprint clustered alongside lumefantrine and other hemoglobin catabolism interfering drugs (Murithi et al. 2020). This evidences the interference at the hemoglobin-derived amino acid level that is perceived in a phenotype of disorganized membrane segmentation and leads to defective merozoites (McNamara et al. 2013). Torin 2 was developed as an inhibitor of the mammalian target of rapamycin (mTOR), a protein kinase of the PI3K family (Cobbold et al. 2016a). This compound is reported to be active throughout all human parasitic stages, albeit without certainty of its MoA, since the mTOR was never described in the *Plasmodium*. Studies have demonstrated that Torin 2 interacts with various parasitic proteins, one of which is a putative nutrient transporter present in the asexual stage in the DV and parasite plasma membrane (Cobbold et al. 2016a). This interaction is pertinent because Torin 2 metabolomic fingerprint showed a rapid decrease of hemoglobin-derived dipeptides and tripeptides as soon as within 1 h of treatment, maintaining this tendency at every time point over the 6-h study (Cobbold et al. 2016a). Torin 2 may be pleiotropic, but considering the low fold-change of other metabolites, it is likely that it targets hemoglobin catabolism through this putative transporter. Lastly, JPC-3210 is a 2-aminomethylphenol synthesized from the nonquinoline chlorophenylphenol WR 194,965 (Chavchich et al. 2016). Despite optimization, its MoA was uncertain and a multi-omics study was conducted to determine it. Untargeted metabolomics portrayed significant depletion of hemoglobin-derived peptides within 1 h of exposure, clustering the profiles of JPC-3210, mefloquine and dihydroartemisinin together (Birrell et al. 2020). Both long- and short-chained peptides were found, suggesting early digestion interference, like hemoglobin uptake or DV changes, rather than heme detoxification, as evidenced by the differential metabolomic profile in regard to chloroquine and other 4-aminoquinolones. Additionally, JPC-3210 proved active against mefloquine-resistant *P. falciparum* strains, suggesting that its activity is independent from PfMDR1-associaded transporter and cross-resistance is unlikely. This compound's fast activity and promising pharmacokinetics have made it a lead for future preclinical studies. Globally, new antimalarials that target this pathway remain relevant, if proven to bypass resistance mechanisms, be fast-acting, and target innovative mechanisms. The PI3K (Hassett et al. 2017) or the PI4K (Fienberg et al. 2020; Sternberg and Roepe 2020) are good examples that are currently being purified and studied in order to develop assays to easily evaluate antienzymatic activity, with some inhibitors having reached clinical development (further reviewed by Arendse et al. (2021)) (McCarthy et al. 2020).

Pyrimidine biosynthesis and the mitETC

Similarly to the DV, another structure that is absent in the RBC but is crucial to the malaria parasite is the mitochondria. Despite its presence in other human cells, the parasitic mitochondria possess exclusive and essential enzymes and structures that ascertain a tangible difference and, thus, a target opportunity for antimalarial drugs (Sakata-Kato and Wirth 2016; Beri et al. 2019). Indeed, the RBC depends exclusively on glycolysis for ATP production, whereas the *Plasmodium* utilizes both the glycolysis and the TCA cycle, to different extents depending on the life stage. Irrespectively of the energetic pathway, the mitochondria has other functions: the mitETC is intimately involved in pyrimidine biosynthesis (Srivastava et al. 2016). It is in the mitochondria inner membrane that five dehydrogenases are present and their re-oxidation is maintained by the ETC, assuring their availability for RNA/DNA synthesis. Thus, the mitochondrial membrane potential is maintained through the TCA cycle in order to produce essential pyrimidines and ATP (Murithi et al. 2020).

The redox balance and membrane potential are partially sustained by two metabolites: pyruvate and glutamate. Pyruvate is diverted from glycolysis and enters the TCA cycle by, for example, converting to acetyl CoA (see Fig. 3) (Cobbold and McConville 2014). Glutamate enters the TCA cycle as 2-oxoglutarate, and it is synthesized from glutamine,

obtained from hemoglobin digestion. Alternatively, glutamate can be further transformed to γ-aminobutyric acid (GABA) (Srivastava et al. 2016). GABA represents a significant metabolic difference between *Plasmodium* and RBC, as it is only present in iRBC. This is due to the incomplete GABA shunt in the *Plasmodium* that, contrarily to Apicomplexan *Toxoplasma gondii*, lacks the enzymes to reroute GABA back to the TCA cycle, leading to its accumulation (MacRae et al. 2013; Srivastava et al. 2016; Beri et al. 2019). Its high levels in malaria patients could make it a viable marker of infection, and its effects as an inhibitory neurotransmitter are thought to be involved in cerebral malaria pathology (Beri et al. 2019). In metabolomic studies, GABA and other amino acids can be used to evaluate disturbances in the mitETC and establish drug profiles (Creek et al. 2016).

The TCA cycle interacts with the mitETC through two of the five membrane dehydrogenases, succinate:quinone reductase (SQR or complex II), and *L*-malate:quinone oxidoreductase (MQO) — see Fig. 3 (Hartuti et al. 2018). Of this chain, complex III, MQO and DHODH are known to be essential for intraerythrocytic parasite survival (Hartuti et al. 2018). Interestingly, mitochondrial ATP production is non-essential at this parasitic stage, accentuating the involvement of the mitETC in other key biological processes (Srivastava et al. 2016).

Atovaquone is the hydroxynaphthoquinone component in Malarone® and is a known inhibitor of the complex III used in combination with proguanil (Hartuti et al. 2018; Birrell et al. 2020). Though resistance is well described through single mutations in the *cytB* gene, it is still used in association (Birrell et al. 2020; Murithi et al. 2020).

As the mitETC is considered a valuable antimalarial target, other molecules are currently being developed, such as DSM265. DSM265 is a triazolopyrimidine-based inhibitor of DHODH, the enzyme of the forth step in the pyrimidine de novo biosynthesis, hence blocking DNA and RNA synthesis (Llanos-Cuentas et al. 2018). Clinical data confirmed patient safety and parasitic clearance with a single-dose regiment, but revealed a subset of *P. falciparum* infection recurrence due to a single-point mutation in the *Pfdhodh* gene (Llanos-Cuentas et al. 2018). In vitro assays confirmed this mutation protected against DSM265 treatment, further ascertaining, as with atovaquone, the necessity to use combination therapies (Llanos-Cuentas et al. 2018; Ross and Fidock 2019). In addition to this factor, metabolomic studies have shown that atovaquone and DSM265 are active in intraerythrocytic asexual stages only against late trophozoites, further limiting its use in monotherapy (Llanos-Cuentas et al. 2018; Murithi et al. 2020). This life-stage specificity is predictable with the DNA synthesis peak before proceeding to replication (Srivastava et al. 2016; Murithi et al. 2020).

The metabolomic profile of antimalarials that target the mitETC is characterized by higher levels of pyrimidine precursors, dihydroorotate, and N-carbamoyl-L-aspartate most notably, among other precursors of the TCA cycle (Dickerman et al. 2016; Allman et al. 2016; Sakata-Kato and Wirth 2016; Hartuti et al. 2018; Birrell et al. 2020). This is true for both complex III and DHODH inhibitors, as cytochrome bc1 complex inhibition indirectly inhibits the DHODH because of the interference with ubiquinol oxidation (Creek et al. 2016; Birrell et al. 2020). Hence, in this case, atovaquone and DSM265 inhibit two different targets of the same chain, but present the same metabolomic profile (Murithi et al. 2020).

Interestingly, it is recognized that the mitochondria are active differently across different parasitic stages. These differences are aligned with their requirements, as the asexual forms depend mostly on glycolysis for energy, whereas gametocytes, having limited access to glucose, namely in hypoglycemic malaria patients and in the mosquito hemolymph, rely more on mitochondrial ATP production (MacRae et al. 2013; Lamour et al. 2014; Srivastava et al. 2016). Metabolomic assays have demonstrated the differences in the TCA cycle substrate between sexual and asexual stages, revealing that inhibition of this cycle in the asexual stages does not affect viability, whereas in the gametocyte, it arrested development and killed the transmissible parasite (MacRae et al. 2013). In vitro, DSM265 did not show gametocidal activity, contrary to blood and liver-stage activity, which is in line with the drug's effect prior to schizogony (Phillips et al. 2015). Atovaquone has demonstrated gametocidal effects in vitro at 1 μM, while being known for not possessing gametocidal activity in vivo (Reader et al. 2015; Allman et al. 2016). Since gametocytes are dependent on TCA cycle ATP production, particularly in early- to mid-stage gametocytes, drugs that target this pathway could be promising antimalarials and should be metabolically investigated further (Lamour et al. 2014; Srivastava et al. 2016; Murithi et al. 2020).

Isoprenoid biosynthesis

Another organelle that differentiates the parasite from the host cells is the apicoplast (Wu et al. 2015; Gisselberg et al. 2018; Kennedy et al. 2019; Swift et al. 2020; Tewari et al. 2021b). Specific to the Apicomplexan, and hence the filo name, it is responsible for various metabolic functions. The sole essential metabolites to be produced in the apicoplast are the isoprenoid precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). These are synthetized through the 7-enzyme methylerythritol phosphate (MEP) pathway, essential in the blood-stage malaria infection, as proven by recovery through the presence of IPP (Gisselberg et al. 2018; Kennedy et al. 2019; Swift et al. 2020; Zimbres et al. 2020). IPP and DMAPP can be further transformed through farnesyl/geranylgeranyl

diphosphate synthase to longer prenyl chains, as happens with ubiquinone, cyclized/conjugated by a variety of prenyltransferases, or originate *cis*-polyisoprenoids in the cytosol (Gisselberg et al. 2018; Zimbres et al. 2020). These metabolites are vital for the posttranslational modification of proteins in the *Plasmodium* and are involved in many cellular tasks, including signaling, replication, and in membrane structures (Wu et al. 2015; Imlay et al. 2015; Gisselberg et al. 2018; Kennedy et al. 2019; Swift et al. 2020; Tewari et al. 2021b).

Isoprenoid biosynthesis is not limited to Apicomplexan; it also exists in bacteria. As such, repurposed antibiotics with antiplasmodial activity mostly target this process. Examples of antibiotics are doxycycline, clindamycin, and fosmidomycin (Wu et al. 2015; Cobbold et al. 2016a; Gisselberg et al. 2018; Kennedy et al. 2019; Murithi et al. 2020; Swift et al. 2020). Human cells also synthetize isoprenoids through a different pathway. However, homology exists between certain human and parasitic enzymes of this pathway (Wu et al. 2015; Gisselberg et al. 2018). Consequently, toxicity assays are important to verify cross-activity and it is recognized that broad inhibition of these system's enzymes could lead to toxicity in patients (Gisselberg et al. 2018).

Plasmodial isoprenoid biosynthesis can be targeted at multiple points, and it has been linked with disturbances at multiple cellular pathways (Gisselberg et al. 2018). All enzymes of the MEP pathway can be potential targets, as is the case for the IspC and IspD, targeted by fosmidomycin and MMV08138, respectively. Inhibition of the MEP is characterized by diminished levels of isoprenoid precursors, 1-deoxy-D-xylulose-5-phosphate and 2-C-methylerythritol-2,4-cyclodiphosphate, and downstream compounds (Kennedy et al. 2019; Swift et al. 2020). Precisely due to the branching of isoprenoid uses, downstream metabolic effects can also be affected, e.g., in variations of hemoglobin-derived peptides and amino acids, nucleotides, and lipids (Swift et al. 2020).

The antibiotics doxycycline and clindamycin are known to inhibit prokaryotic transcription and translation in bacteria. They also interfere with the plasmodial apicoplast genome, inhibiting protein synthesis, thus being active antimalarials.(Wu et al. 2015) Because they present the "delayed death" phenotype, they are used in combination therapies as slow acting partner drugs. The "delayed death" signature happens when parasitic death is only achieved by the second lifecycle (Kennedy et al. 2019; Tewari et al. 2021b). Fosmidomycin acts by inhibiting the IspC of the MEP, bypassing the "delayed death" profile (Tewari et al. 2021b). Despite immediate onset, with sudden arrest in IPP biosynthesis, it has a short half-life and, consequently, high recrudescence rates when used in monotherapy (Tewari et al. 2021b). Despite acting in the same life stage, the trophozoite, when most prenylation reactions occur, fosmidomycin acts immediately in blocking this process, while delayed-death drugs lead to gradual depletion of IPP and eventual loss of prenylation (Wu et al. 2015; Gisselberg et al. 2018; Kennedy et al. 2019). This is thought to lead to the transmission of defective apicoplast to the merozoites during replication, which leads to unviable parasites in subsequent cycles (Kennedy et al. 2019; Swift et al. 2020; Tewari et al. 2021b).

Metabolically speaking, when these drugs are used, there are no significant changes in the first lifecycle because the parasite is able to adapt its metabolism to compensate the isoprenoids deficit and successfully complete its lifecycle (Wu et al. 2015; Kennedy et al. 2019). A multiple omics study by Tewari et al. looked into what compensation mechanisms the *P. falciparum* used to assure survival when treated with 1 μM of fosmidomycin, its half-inhibitory concentration (IC_{50}) (Tewari et al. 2021b). The study revealed adjustments through the polyamine metabolism in which enhanced purine-recycling and decreased phosphatidylcholine metabolism let the parasite maintain synthesis of purine nucleotides by suppressing phospholipid synthesis (Tewari et al. 2021b). This had already been hinted by the Swift et al. metabolomic study, where variations in lipid and sphingolipid metabolism would suggest a linked compensation mechanism for the loss of the apicoplast (Swift et al. 2020).

Resistance to fosmidomycin has been described in vitro by metabolomics as an enzymatic amplification that induces the parasitic glycolysis flux and, consequently, the metabolite intermediates available for the isoprenoid biosynthesis pathway. As precursors become increasingly available, the inhibition of IspC by fosmidomycin becomes less important and the MEP is not totally arrested (Gisselberg et al. 2018; Dumont et al. 2019).

Innovative compounds that target isoprenoid biosynthesis are still pertinent, seeing the multitude of target options available that would be parasite-specific. However, compounds would have to present faster killing effectivity and longer half-lives (Gisselberg et al. 2018). Kennedy et al. used a metabolomic study to describe the molecular mechanism of the "delayed death" profile (Kennedy et al. 2019). To do so, indolmycin, an antibiotic inhibitor of the apicoplast tryptophanyl-tRNA synthetase in *P. falciparum*, was used (Kennedy et al. 2019). Similarly to other antibiotics that target translational processes, it induced "delayed death" that can be rescued with IPP in vitro (Kennedy et al. 2019). It was found that no metabolic variations occurred during the 1st intraerythrocytic asexual lifecycle (Kennedy et al. 2019). Afterwards, severe disruption of the morphology of the DV could be observed, along with diminished hemoglobin-derived peptides levels, and it was deduced aberrant hemoglobin uptake could be one of the mechanisms that kills the parasites (Kennedy et al. 2019). Additionally, isoprenoid precursors and downstream species levels decreased,

consistent with a defective apicoplast that does not produce isoprenoids (Kennedy et al. 2019). Promising compounds like MMV-08138 have been studied, but showed a similar profile (Wu et al. 2015). Omics research that elucidates MoA of promising isoprenoid biosynthesis inhibitors could prove beneficial for the development of innovative antimalarial therapies (Gisselberg et al. 2018). The apicoplast is a necessary structure throughout the intraerythrocytic lifecycle; thus, it could present important blocking transmission and chemoprophylaxis activity that is worth pursuing (Wu et al. 2015; Gisselberg et al. 2018; Zimbres et al. 2020).

Fatty acid metabolism

The *Plasmodium* apicoplast also harbors enzymes necessary for lipid metabolism, namely de novo fatty type II acid synthesis (FASII) and elongation (Srivastava et al. 2016; Kennedy et al. 2019; Beri et al. 2019). Despite the similarities in this pathway with the host cells', structural differences between eukaryote and prokaryote enzymes make this pathway noteworthy as a potential target (Yoo et al. 2020). Fatty acids, phospholipids and lipids are required at multiple *Plasmodium* lifecycle points for proliferation, protein trafficking, hemoglobin degradation, signaling, and host interaction (Lamour et al. 2014; Gulati et al. 2015; Ghosh et al. 2016; Srivastava et al. 2016; Tewari et al. 2020; Yoo et al. 2020) Phosphoenolpyruvate is a precursor of the TCA cycle, glycolysis, and fatty acid synthesis. In the latter case, it is transported into the apicoplast where it is transformed into pyruvate by pyruvate kinase 2 and into acetyl CoA by pyruvate dehydrogenase (Srivastava et al. 2016). Acetyl CoA is the precursor for FASII synthesis that will generate the key building blocks of phosphatidylcholine, phosphoethanolamine, or phosphatidylinositol 4-phosphate, involved in signaling and in membrane structures of the vacuolar system and development of merozoites (Gulati et al. 2015). Parallelly, the parasite can scavenge fatty acids from the serum to be incorporated into parasitic membranes by modifying them through elongases and desaturases, thus optimally adapting its resources (Gulati et al. 2015; Ghosh et al. 2016). Interestingly, lipidic metabolites have been linked to various host interactions that are known to be relevant players in malaria pathology. Specifically, the metabolomics study by Beri et al. demonstrated how the lipid metabolism of the *P. falciparum* is deeply intertwined with the host, going as far as interfering with RBC deformability (Beri et al. 2019). Accumulation of membrane precursors/degradation metabolites (choline, ethanolamine and glycerol 3-phosphate) and decrease of choline-containing lysolipids were consistent with RBC membrane rigidity and composition changes caused by the *Plasmodium* infection (Beri et al. 2019). Additionally, a more recent metabolomics assay by Tewari et al. revealed the increase of eicosanoid-signaling precursors in iRBC, which once released can modulate inflammation, further confirming the role of lipids in host immune-modulation (Tewari et al. 2020).

This pathway is not essential for all *Plasmodium* species or life stages. Some species, like *P. berghei* or *P. lophurae*, are known to scavenge CoA from the host cells during the asexual stages, while *P. falciparum*, being dependent on mature RBC, lacks external supplies and is instead dependent on de novo synthesis in the apicoplast and mitochondria (Srivastava et al. 2016). Debate exists on the extent to which the *P. falciparum* scavenges fatty acids from the host, while parallelly synthesizing them de novo. A lipidomic study by Gulati et al. analyzed 304 lipids found in *P. falciparum* blood stages and in non-infected and iRBC (Gulati et al. 2015). The study used GW4869, an inhibitor of parasitic ceramide production, that failed to achieve 100% growth inhibition in vitro, even at 170 times its IC_{50} (Gulati et al. 2015). Since the RBC possesses up to 10 times the parasite's level of ceramide, survival could be due to scavenging it from the host, revealing that even though GW4869 has potent antiplasmodial activity in vitro (IC_{50} of 6 nM), the inhibition of the hydrolysis of sphingomyelin is not unsurmountable for the parasite (Gulati et al. 2015). In the same study, Orlistat, a triacylglycerol lipase inhibitor, revealed a lower IC_{50} (0.9 μM), but a better profile as antimalarial, as it targets the metabolic pathway of a lipid that cannot be scavenged without cannibalizing the RBC (Gulati et al. 2015). Likewise, in this study, phosphatidylglycerol, acyl phosphatidylglycerol, lysophosphatidylinositol, bis(monoacylglycero)phosphate, monosialodihexosyl-ganglioside, and diacylglycerol were found to also be unsalvageable and to require likely de novo synthesis (Gulati et al. 2015). These results show that some pathways might, hence, be promising targets for antimalarial therapies, while others could be bypassed through host metabolite importation. Consequently, it is theorized that blood stages are not as reliant on general fatty acid synthesis and this could prove relevant when developing inhibitors of this pathway (Ghosh et al. 2016). Another way to affect the asexual blood stage could be through preventing merozoite invasion, which is theorized to be possible if the membrane lipid composition is affected (Koch et al. 2019).

Concerning the gametocyte stage, the same lipidomics analysis by Gulati et al. showed that the lipid environment can induce gametocytogenesis (Gulati et al. 2015). Supplementation with serine, which increases levels of phospholipids and sphingolipids, and N,N-dimethyl-sphingosine, which decreases ceramide catabolism, were shown to augment gametocytemia (Gulati et al. 2015). A previous ^{1}HNMR-based lipidomics study by Lamour et al. had already hinted at the correlation between depletion of lipid moieties and gametocyte maturation, and the same study by Tewari et al. referred above also denoted the high abundance of polyunsaturated fatty acids in the iRBC promoted

gametocytogenesis (Lamour et al. 2014; Tewari et al. 2020). These results indicate that lipids play a part in signaling the gametocytogenesis commitment and development and could, hence, be used to block transmission.

Contrary to the blood stages, mosquito stages are known to be reliant on fatty acid synthesis. A metabolomic and genomic study by Srivastava et al. gave insight on this theory by studying both in vitro and in vivo *P. berghei* carbon metabolism (Srivastava et al. 2016). Indeed, the carbon metabolism was proven essential in the mosquito stages, which showed high sensitivity to variations in the TCA cycle, CoA synthesis, and glutamine catabolism (Srivastava et al. 2016). Additionally, FASII was shown to be essential for complete sporogony of *P. Falciparum* in the mosquito midgut (van Schaijk et al. 2014; Srivastava et al. 2016). This points to the important role of inhibitors of the FASII or of the CoA synthesis in blocking transmission.

Although no traditional antimalarials directly affect this metabolism, it is possible it is disturbed indirectly through the disruption of the apicoplast or the glycolysis pathway (Srivastava et al. 2016; Kennedy et al. 2019). Promising compounds that target the *plasmodium* lipid metabolism have been metabolomically investigated. A study by Creek et al. aimed to determine the MoA of 100 antimalarial compounds from the Malaria Box collection through untargeted metabolomics in *P. falciparum* iRBC in vitro (Creek et al. 2016). In this study, three compounds were found to disturb unique aspects of parasite fatty acid metabolism: C7, E4, and P2_A3. C7 showed a tenfold depletion of traumatic acid, a product of the plant-like α-linolenic acid pathway whose role in the *Plasmodium* parasite remains unknown (Creek et al. 2016). Still, C7 (IC_{50} of 448 nM) had a unique signature, with no changes in α-linolenic acid precursors or other end products, proving there might be merit in targeting this metabolic branch (Lakshmanan et al. 2012; Creek et al. 2016). E4 also diminished traumatic acid levels, but to a lesser degree, although with a promising IC_{50} (215.5 nM) (Creek et al. 2016). P2_A3 induced a decrease of linoleic acid specifically and, uniquely, with no other changes (Creek et al. 2016). Considering linoleic acid is an upstream precursor of the α-linolenic acid metabolism pathway, its MoA could be an indirect effect, e.g. in fatty acid uptake, since *P. falciparum* might not synthesize linoleic acid (Lakshmanan et al. 2012; Creek et al. 2016). Overall, the three compounds point to the α-linolenic acid pathway as a potential antimalarial target. Another metabolomic assay by Yoo et al. sought to study Sal A, a natural compound produced by a *Salinispora* sp. bacterium with a promising IC_{50} (50 nM) against *P. falciparum* and a selectivity index > 1000 over many mammalian cell lines (Yoo et al. 2020). Accumulation of acylglyceride metabolites, and profile resemblance with Orlistat, revealed that Sal A inhibits α/β serine hydrolases, which lead to incomplete merozoite formation and failure in egression from iRBC (Yoo et al. 2020). Overall, these three compounds revealed metabolomic profiles specific of fatty acid metabolism disruption, including traumatic acid, linoleate, lysophospholipids, sphingolipids, glycolipids, monoacylglycerol, and others, as referred thus far. However, it cannot be discarded that compounds targeting this pathway may show changes at other levels, like the TCA cycle, central carbon metabolism, glycolysis, and even hemoglobin catabolism (Srivastava et al. 2016; Creek et al. 2016; Beri et al. 2019; Tewari et al. 2020).

Folate biosynthesis

Folate biosynthesis is an essential pathway for any replicating biological system, as it is directly responsible for the production of purines. For the *Plasmodium*, this need is even higher, as the parasites undergo schizogony every 48 h or 72 h. In order to meet such demands, this genus adapted to synthetize folate metabolites de novo. To do so, two metabolites are key: *p*-aminobenzoic acid (pABA), which can be obtained through the shikimate pathway or by importing it from the host, and glutamate, diverted from hemoglobin catabolism or the host, as described in Fig. 3 (Rijpma et al. 2016; Choudhary et al. 2018; Krishnan et al. 2020). Folate biosynthesis can be described in three major axes: the generation of pteridine metabolites, the building blocks of purine metabolism; the synthesis of dihydrofolate; and the pyrimidine synthesis cycle (Rijpma et al. 2016). The first axis is the chain conversion of GTP to hydroxymethyl dihydropteridine pyrophosphate, which relies on multiple enzymes. The second axis depends on pABA and hydroxymethyl dihydropteridine pyrophosphate components to be joined into dihydropteroate, which will then be converted to dihydrofolate, dependent on glutamate. Finally, the third axis involves the cycle that converts dihydrofolate to tetrahydrofolate, to methylene tetrahydrofolate, and back to dihydrofolate. All reactions of this pathway are catalyzed by enzymatic complexes passible of interreference by drugs that would effectively block parasite replication (Rijpma et al. 2016).

Several antimalarials act as inhibitors of this pathway. Sulfonamides, as sulfadoxine, are analogs of pABA, effectively blocking the second axis (Rijpma et al. 2016; Khan et al. 2019; Murithi et al. 2020). Pyrimethamine and cycloguanil are known inhibitors of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Cobbold et al. 2016a; Allman et al. 2016). DHFR-TS is an important complex of the third axis that mediates the folate-dependent conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), while using NADPH as a cofactor (Allman et al. 2016). Other drugs are known to possess antimalarial activity through this MoA, such as the antibiotic trimethoprim, WR99210, and methotrexate, a structural

analog of folate (Rijpma et al. 2016; Allman et al. 2016; Khan et al. 2019). Folate antagonists might interfere at other stages, like preventing the formation of dihydropteroate or of dihydrofolate. Unfortunately, resistance to antifolates by the *Plasmodium* is well described in the field after accumulation of point mutations (Khan et al. 2019; Ross and Fidock 2019; Kamchonwongpaisan et al. 2020). Parallelly, it is possible that the parasite upregulates genes that increase the flux of upstream substrates, which make downstream substrates more abundant, thus counteracting enzymatic inhibition. This has been reported for the GTP-cyclohydrolase, the first enzyme of the folate biosynthesis pathway, for example (Khan et al. 2019). Still, cycloguanil derivatives have shown that structural modifications are able to bypass antifolate drug transmission on the basis that the parasite is limited to maintain a functional DHFR-TS (Kamchonwongpaisan et al. 2020).

A metabolomics screening by Allman et al. successfully demonstrated the clustering of antifolate drugs and reported on their profiles (Allman et al. 2016). DHFR-TS inhibitors showed increased dUMP and NADPH as a unique signature, which also clustered compounds P218 and MMV667487 (Allman et al. 2016). This profile also displayed decreased downstream intermediates, dTMP and deoxythymidine triphosphate (Cobbold et al. 2016a; Allman et al. 2016). In a multi-omics study, genetic deletion of a parasitic membrane transport in *P. falciparum* blood-stage schizont allowed for the identification of resistant factors through untargeted metabolomics. It was described that depletion of folate created parasites resistant to methotrexate, while maintaining sensitivity to pyrimethamine (Rijpma et al. 2016). This assay successfully demonstrated that this metabolic pathway is reliant on more structures besides the DHFR-TS, like specific membrane transporters, for which new antimalarials may be developed. Disruption of this biosynthesis affects multiple intraerythrocytic stages, although the most active in DNA synthesis are the trophozoite late stages and during schizogony, which is successfully blocked by this class of compounds (Rijpma et al. 2016). Other studies are underway to determine what key targets could make antifolates potential antimalarials, as this pathway still offers multiple specific inhibition targets.

Homeostasis

The *Plasmodium*, as an obligatory intracellular parasite, faces the challenge of maintaining homeostasis (Park et al. 2015; Beri et al. 2017, 2019). At one level, the parasite introduces morphological and chemical changes in the iRBC and has to ascertain its integrity, including membrane potential, osmolarity, free radicals, and pH. On a second level, the parasite itself goes through multiple changes throughout its lifecycle, imposing other threats to this fragile system (Park et al. 2015). Thus, the parasite needs powerful systems to assure homeostasis. Amongst them is a potent antioxidant system that relies on superoxide dismutase, thioredoxin, and glutathione. Additionally, by alternating between scavenging and de novo synthesis, while maintaining redundancy of some metabolites and metabolic pathways, the *Plasmodium* ascertains a wide safety net for its development (Bullard et al. 2015; Creek et al. 2016; Pretzel et al. 2016; Beri et al. 2017, 2019). These systems are robust and known to be powerful in countering antimalarial effectivity, alongside point mutations and other resistance factors.

All metabolic pathways are intertwined and co-dependent in a complex network. For example, pyruvate from glycolysis can be fermented into lactate directly, enter the TCA cycle in the mitochondria, or be imported into the apicoplast to serve as the precursor of isoprenoid or fatty acid biosynthesis. Hence, when a metabolic pathway is overwhelmingly stressed, and the parasite cannot compensate it, a cascade that affects multiple systems will ensue (Cobbold et al. 2016a; Beri et al. 2019). For example, a disruption of the apicoplast can lead to changes in lipidic and isoprenoid metabolites, but it can go as far as disturbing hemoglobin uptake and digestion, a process that occurs in an altogether different section of the parasite. In addition to the problems that have been discussed so far, such a disruption would precipitate osmotic difficulties, which speed parasite death (Lee et al. 2018; Kennedy et al. 2019; Murithi et al. 2020). This death cascade can be perceived as a general metabolic shut down and pleiotropic antimalarials often arise this kind of profile in omic studies. Various metabolomic assays analyzed the profile of antimalarials with this kind of MoA, such as dihydroartemisinin, primaquine, and tafenoquine (Allman et al. 2016).

Dihydroartemisinin, the active metabolite of artemisinin, is a potent pleiotropic antimalarial, whose unconventional resistance mechanism forced a reform on how antimalarials were used in therapy (Cobbold et al. 2016a; Siddiqui et al. 2017; Ross and Fidock 2019; Yang et al. 2019). It is thought to be activated upon endoperoxide bridge opening, possibly in the DV by hemoglobin-derived Fe^{2+}-heme, therefore releasing free radicals (Mok et al. 2021). These will interact mainly with proteins, but its oxidative damage has other ramifications, causing lipid peroxidation and maybe depolarization of the mitochondrial and plasma membranes, thus altering homeostasis in multiple fronts (Cobbold et al. 2016a; Siddiqui et al. 2017; Khan et al. 2019; Ross and Fidock 2019; Yang et al. 2019). Artemisinin resistance is a known example of how omics studies successfully uncovered both the resistance mechanism and the MoA that had eluded the scientific community for years. This resistance phenotype differs from others, in which the parasites tolerate high drug concentrations and instead show development deviations (Khoury et al. 2020). Several characteristics are

now known to be associated with this resistance: *Pf*Kelch13 mutations, the dominant determinant of resistance; redox detoxification, and early-ring dormancy (Khoury et al. 2020; Mok et al. 2021). Omics helped establish the *Pf*Kelch13 gene, and subsequent effects, as the artemisinin-resistance factor and demonstrated how changes in protein folding disable an otherwise potent antimalarial (Siddiqui et al. 2017; Yang et al. 2019). Specifically, untargeted metabolomic studies successfully identified the decrease of the *Pf*Kelch13 protein and hemoglobin-related peptides and the increase of glutathione and gamma-glutamylcysteine. These variations confirm these metabolites as biomarkers of resistance and dihydroartemisinin's link with heme activation and oxidative damage (Siddiqui et al. 2017). A genomic study confirmed the role of a parasite endoplasmic reticulum enzyme, the *Plasmodium* eIF2α Kinase PK4, that begins the cascade of latency and leads to recrudescence post artemisinin treatment (Zhang et al. 2017). This assay found that inhibition of this enzyme successfully blocks this cascade and assures artemisinin treatment, giving leads to partner drugs to use alongside artemisinin-derivatives (Zhang et al. 2017; Yang et al. 2019). Additionally, another study identified PI3Ks as an artemisinin target and found lipidic product phosphatidylinositol-3-phosphate levels to increase in resistant strains (Mbengue et al. 2015). Further omics studies demonstrated that this metabolite is involved in a vesicular system that neutralizes artemisinin interactions with the proteome and ensues modifications that increment resistance and enable recrudescence (Bhattacharjee et al. 2018; Ross and Fidock 2019). In silico models based on experimental data were used to predict metabolic differences between artemisinin-resistant and sensitive parasites (Carey et al. 2017). This study pointed to unique genes differentiating pyrimidine and folate biosynthesis and mitochondrial reactions in resistant parasites, beside other changes mentioned thus far (Carey et al. 2017). More recently, a study focused on integrating multiple omic tools to ascertain the intricate mechanisms through which the *Plasmodium* resists artemisinin treatment. This ambitious assay by Mok et al. discovered new functionalities of the *PfKelch13* gene and validated others suggested thus far (Birnbaum et al. 2020; Mok et al. 2021). Notably, the *PfKelch13* gene was shown to interfere with pyruvate and glutamate-linked carbon metabolism, with alterations in glycolysis/gluconeogenesis, the TCA cycle, and purine metabolism (Mok et al. 2021). Specifically, the low levels of malate and 2-ketoglutarate, TCA intermediates, point to ring stages that mimic conditions of anoxia and lower the mitETC complex III activity (Mok et al. 2021). Additionally, variations in phosphoenolpyruvate, glutamine synthetase, and NADP-specific glutamate dehydrogenase reveal resistant parasites that rely more on reverse glutaminolysis (Mok et al. 2021). This energy rewiring might be involved in the recovery after treatment, as had been suggested before (Peatey et al. 2015; Mok et al. 2021). Parallelly, the *PfKelch13* showed to facilitate this energetic shift while augmenting the proteostatic capacity of the resistant parasite by facilitating the elimination of damaged proteins (Mok et al. 2021). Generally, resistant parasites are predicted to display higher metabolic flexibility, but metabolomic assays should be performed to confirm this.

Primaquine and tafenoquine are prodrugs metabolized by the hepatocytic CYP2D6, which awards them hepatic schizonticide activity (Reader et al. 2015; Pewkliang et al. 2018). Their exact MoA is unknown, but similarly to dihydroartemisinin, they are considered to induce oxidative stress (Reader et al. 2015). Because of their metabolization and rapid onset of action, they are effective in preventing relapses, which limits their use, as the hepatic stage is asymptomatic and not all *Plasmodium* sp. create hypnozoites (Khan et al. 2019). Primaquine is reported to be toxic in glucose-6-phospate dehydrogenase-deficient individuals and to have low drug tolerance, which further compromises its clinical utilization (Pewkliang et al. 2018; Roth et al. 2018).

Commonly, not only pleiotropic antimalarials can generate a general death cascade profile. This is because, as discussed thus far, metabolic pathways are interconnected, and if one chain link fails, the others fail too. A metabolomic study by Cobbold et al. assessed the MoA of front-line antimalarials, including dihydroartemisinin, atovaquone, Torin 2, and others, in order to compare their metabolomic fingerprints (Cobbold et al. 2016a). In it, pleiotropic drugs clearly separated from one-target mechanistic drugs. Compound 3361 is a selective inhibitor of the *P. falciparum* plasma membrane hexose transporter. Incubation with [^{13}C]-U-glucose labeling revealed disturbances in carbon metabolism (Cobbold et al. 2016a). Unsurprisingly, 3361 disrupted glycolysis, but not exclusively (Cobbold et al. 2016a; Hapuarachchi et al. 2017). TCA cycle and purine alterations are probable downstream consequences of the loss of energy production and carbon skeletons (Cobbold et al. 2016a). Moreover, hemoglobin-derived peptides were also disturbed, demonstrating how the lack of ATP can interfere with DV acidification or transporters, which would prevent correct hemoglobin catabolism (Cobbold et al. 2016a). This profile was significantly different from dihydroartemisinin, which affected hemoglobin digestion within 1 h of treatment, consistent with reports that drug activation occurs inside the DV (Cobbold et al. 2016a). Compound 3361 ultimately led to nonrecoverable parasites, indicating the likelihood of downstream damage that prevented parasite replication (Cobbold et al. 2016a). Similarly to 3361, 2-deoxyglucose is known to competitively inhibit the conversion from glucose to glucose-6-phosphate in glycolysis (Srivastava et al. 2016; Allman et al. 2016; Sakata-Kato and Wirth 2016). This compound has been studied extensively in multiple assays, including metabolomics, as is the case

by Allman et al. and Srivastava et al. The first study pointed to a widespread metabolic collapse profile in blood-stage *P. falciparum* (Allman et al. 2016). The second focused on finding how dependent gametes are on glycolysis, finding that *P. berghei* male gametogenesis was completely reliant on this pathway, pointing towards transmission blocking (Srivastava et al. 2016). Regrettably, RBC and other cells, likewise to the parasite, rely heavily on the glycolysis pathway for energy. Hence, secondary effects are expected, unless specific parasitic targets can be focused, as is the case of compound 3361, which does not inhibit glucose import to the RBC (Cobbold et al. 2016a; Srivastava et al. 2016).

Other targets can also ensue homeostasis disruption. Compounds that target the parasite formate nitrite H^+-transporter (*Pf*FNT), responsible for lactate efflux and pH maintenance, also demonstrate a profile of metabolic collapse. This inhibition leads to hemoglobin metabolism and pyrimidine biosynthesis disruption, besides arising cytosolic alkalinization (Hapuarachchi et al. 2017). It is the MoA of two compounds, MMV007839 and MMV000972, found in the metabolomics study by Hapuarachchi et al. (2017).

Additionally, the previous study mentioned by Allman et al. (2016) also included in the homeostasis cluster KAE609, (+)-SJ733 and KAF246, compounds that target the *Pf*ATP4, a Na^+/H^+-ATPase in the parasite plasma membrane that is restricted to apicomplexan parasites.(Allman et al. 2016; Dennis et al. 2018) *Pf*ATP4 inhibitors prevented Na^+ efflux, that would occur in exchange of H^+ intake, disturbing the parasite's ionic gradient, pH, and osmolarity (Allman et al. 2016; Dennis et al. 2018). In the same study, 2-deoxyglucose displayed a different metabolomic profile from *Pf*ATP4 inhibitors, evidencing that compounds targeting glycolysis develop a unique print (Srivastava et al. 2016; Allman et al. 2016; Sakata-Kato and Wirth 2016). Creek et al. goes further in this analysis, and clusters dihydroartemisinin with *Pf*ATP4 inhibitors, suggesting that this metabolomic profile derives from potent and fast-acting antimalarials that disrupt multiple metabolomic pathways simultaneously, rather than a specific target (Creek et al. 2016). Further studies with KAE609 and others with the same MoA showed that the Na^+ uptake provokes swelling of both parasite and iRBC, increasing these cell's osmotic fragility and killing them through this mechanism (Dennis et al. 2018). KAE609 underwent one phase 2 clinical trial that ended in 2019 to adjust the dose in regard to reported hepatotoxicity and is undergoing another to evaluate its intravenous efficacy and safety in severe malaria patients, set to end in 2024 (registered with ClinicalTrials.gov, NCT03334747 and NCT04675931, respectively) (White et al. 2014; Ashley and Phyo 2020).

Lastly, peroxide antimalarials, like ozonides, are known to target homeostasis systems by disrupting redox processes. A recent proteomics and targeted LC–MS-based thiol metabolomics assay by Siddiqui et al. sought to describe ozonides MoA (Siddiqui et al. 2022). To do so, the protein and thiol changes were assessed after dihydroartemisinin and artefenomel treatment, an ozonide clinical candidate (Siddiqui et al. 2022). Artefenomel provoked disproportionate alkylation of proteins involved in redox homeostasis, confirming that this group of antimalarials might act similarly to dihydroartemisinin (Siddiqui et al. 2022).

Homeostasis is a fragile balance to maintain for the blood-stages, which opens the way for pleiotropic drugs like dihydroartemisinin or *Pf*ATP4 inhibitors like KAE609 to be fast and effective antiparasitic agents. Generally, the metabolomic profile associated with homeostasis disturbance displays variances in thioredoxin reductase; glutathione precursor cysteine; oxidized glutathione and cysteine-glutathione disulfide; deoxyribonucleotides; ribonucleotides; carbon metabolites, such as glucose-6-phosphate and other glycolytic intermediates; and hemoglobin-derived peptides, demonstrating the extent of this perturbance (Bullard et al. 2015; Cobbold et al. 2016a; Allman et al. 2016; Beri et al. 2019). Overall, the parasitic homeostasis is a fragile balance that requires enormous amounts of energy and multiple structures to maintain it, which makes it a valuable antimalarial route. However, careful selective targeting of these systems is necessary to avoid toxicity.

Unknown and others

Lastly, the attained metabolomic profile might not have statistical sturdiness to cluster with other classes and, in that case, the MoA remains uncertain (Creek et al. 2016; Allman et al. 2016). In antimalarial drug discovery, compounds usually start by being screened in vitro against *P. falciparum*, in which the IC_{50} is used to rank activity. Thus, when compounds are further explored through metabolomics and their profile is ambiguous, it should not be linked to a lack of activity, but to other factors. The compound might target metabolic pathways inactive or non-essential in the tested stage, have a slow time of action, and require metabolomic activation from the host or higher concentrations (Creek et al. 2016; Allman et al. 2016). Lastly, the altered metabolites might not be detected or reproducibly quantified, which would leave gaps in the attained results (Creek et al. 2016; Allman et al. 2016).

Alternative profiles have emerged in metabolomic studies that correlate to innovative targets that do not yet have validated clinical efficiency. Such is the case of MMV007571 and MMV020439 that were shown through untargeted metabolomics to inhibit new permeation pathways (NPP), along with pyrimidine synthesis and the mitETC (Dickerman et al. 2016; Rawat and Verma 2020). Since NPP may be redundant in importing and exporting nutrients and waste products, it is difficult to link a metabolomic fingerprint to

this particular pathway, more so since the compounds also interfered with other metabolomic pathways. However, NPP are recognized potential antimalarial targets, as they are induced by the parasite and important for its viability (Dickerman et al. 2016; Rawat and Verma 2020). Another alternative profile that has emerged is associated with the acetyl CoA anabolism. This pathway has been successfully targeted by pantothenamide bioisosteres, not only in the asexual stages, but also in sexual phases, consequently blocking transmission (Schalkwijk et al. 2019). Pantothenate is the precursor of CoA, and its phosphorylation by the *P. falciparum* pantothenate kinase is the first step in CoA biosynthesis (De Villiers et al. 2017). The inhibitor profile was established by detection of converted pantothenamides to CoA-pantothenamides, along with a reduction in the levels of acetyl CoA and other downstream metabolites, consistent with findings that these inhibitors are likely activated by pantothenate kinase, whose products will inhibit other downstream enzymes or reactions or behave as antimetabolites (De Villiers et al. 2017; Schalkwijk et al. 2019). Preclinical characterization of the antimalarial pantothenamide MMV693183 was recently described, further ascertaining this promising antimalarial target (de Vries et al. 2022).

Many other antimalarial targets are still expected to be discovered, especially through the implementation of omics or multiomics studies in compound screenings. Although metabolomic profiling is not always easy, metabolic fluxes and interactions are reliable indicators of parasitic death mechanisms and will never fail to give a glimpse on drug's MoA.

Conclusion

Metabolomics is a comprehensive omics field that transformed and continues to innovate the way diseases and therapies are studied. In fact, its application in the malaria field has enabled the characterization of both established and potential antimalarials, thus contributing to a rational drug design. By providing an in-depth view of the metabolic reactions of the asexual parasite when exposed to antimalarials, accurate and interesting phenotypic profiles have been discovered that will contribute to the development of innovative antimalarials. More precisely, extraction with a proportion of methanol:water analyzed through LC–MS seems to be most recurring method towards antimalarial drug discovery, as revealed by pioneer studies (Hapuarachchi et al. 2017; Kennedy et al. 2019; Schalkwijk et al. 2019; Birrell et al. 2020; Murithi et al. 2020). However, metabolomics inherent complexity exposes the lengthy path ahead before it can become a routine technique. The *Plasmodium* poses obstacles in the variety of host cells it parasitizes across multiple life stages, and the broad metabolic interactions and modifications intrinsic to each of them. It would be interesting if omic sciences could be broadly implemented to study all life stages and other species, such as *P. vivax*. This would certainly shed light on stage and species specificity, thus predicting differential antimalarial activity and guiding drug development successfully.

Despite the increasing number of assays in the field, standardization of experimental procedures and data processing and analysis are lacking. This, in turn, compromises study's reproducibility and interpretation, leading to an uncertain link of causality between the original design question and the attained results. Particularly, techniques such as HILIC-based stationary phase chromatography, although pertinent, do not have the capability to thoroughly analyze the lipidome. This represents a significant mishap, since the lipidome is a promising source of information in the malaria context that appears to have been neglected in most metabolomic studies. The coupling of analytical platforms such as LC–MS with GC–MS or NMR, though intricate, has been shown to complement the information attained and seems to be an important strategy towards new target discovery. Additionally, isotope tracing and fluxomics should be explored and be more vulgarly used, as the obtained information would add dimension to malarial discovery. Methodologies still need to evolve in order to allow a true holistic view and complete metabolic fingerprint. These data can then be used to build metabolomic databases based on concrete characteristics, like the retention time, mass, or NMR spectra, which will ease interpretability of further studies and improve the predictability power of in silico models. Additionally, the integration of multiple omics, as demonstrated by the cited studies, presents invaluable advantages in result interpretation and should be encouraged. Software and statistical tools that can easily analyze such complex datasets are also readily needed, as they would facilitate interpretation and make this research field more accessible.

Lastly, it would appear there is a delimited number of essential pathways vulnerable to pharmacological intervention that can be explored in the context of the malaria disease; however, new metabolomic studies are portraying innovative targets. This would seem to indicate that chemotherapy can now be expanded through the help of powerful and robust omic technologies that will shed light on innovative antiparasitic MoA. Additionally, an in-depth characterization of *Plasmodium*'s biological functions would prove invaluable towards the disclosure of resistance before implementation of new therapies. As metabolomics could close the gap between clinical studies and experimental research, proper chemotherapy design could be achieved faster and to a greater result in eradicating malaria. Overall, metabolomics proves to be a promising field for the exploration and comprehension of pathology intricacies.

Author contribution Allison Ledoux, Pascal De Tullio, Joëlle Quetin-Leclercq, and Michel Frédérich had the idea for the review. Lúcia Mamede and Fanta Fall did the bibliographic search. Lúcia Mamede wrote the main manuscript text and prepared the figures. Fanta Fall, Matthieu Schoumacher, Allison Ledoux, Pascal De Tullio, Joëlle Quetin-Leclercq, and Michel Frédérich read the manuscript and participated in the preparation of the final version of the manuscript. All authors reviewed and approved the final manuscript.

Funding This work was supported by the Belgian National Fund for Scientific Research (FNRS, grant PDR T.0092.20).

Data availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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