Trends in Parasitology

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Review

The nutrient games – *Plasmodium* metabolism during hepatic development

Aparajita Lahree [1], João Mello-Vieira [2,3] and Maria M. Mota [4,*]

Malaria is a febrile illness caused by species of the protozoan parasite *Plasmodium* and is characterized by recursive infections of erythrocytes, leading to clinical symptoms and pathology. In mammals, *Plasmodium* parasites undergo a compulsory intrahepatic development stage before infecting erythrocytes. Liver-stage parasites have a metabolic configuration to facilitate the replication of several thousand daughter parasites. Their metabolism is of interest to identify cellular pathways essential for liver infection, to kill the parasite before onset of the disease. In this review, we summarize the current knowledge on nutrient acquisition and biosynthesis by liver-stage parasites mostly generated in murine malaria models, gaps in knowledge, and challenges to create a holistic view of the development and deficiencies in this field.

Highlights

Plasmodium parasites must invade, develop, and replicate inside hepatocytes into thousands of daughter parasites before progressing to the blood and causing malaria.

Plasmodium scavenges certain nutrients from the hepatocyte (e.g., glucose, amino acids) while synthesizing other biomolecules (e.g., fatty acids) to putatively achieve a high replication rate.

Hepatocyte metabolism is remodeled to facilitate parasite development and includes elevation in the uptake of host extracellular nutrients, diversion of host endocytic traffic, and interaction with host organelles.

Low infection efficiency limits the number of infected hepatocytes available and the detection of infection-specific events. Temporal and spatial single-cell analysis, possibly combined with parasite and host mutants, may revolutionize the field by overcoming this limitation.

Liver stage of *Plasmodium* infection

Parasitism is a complex form of biological interaction in which one organism (the parasite) derives its nutrients from another (the host). Parasitism is a highly successful and inherent mode of life – parasites are found in organisms of all species [1], and over 50% of all living organisms are parasitic during at least one point in their lives. Particularly interesting are intracellular pathogens, which invade individual host cells and utilize their intracellular machinery to acquire the necessary resources. One such parasite is *Plasmodium*, the causative agent of malaria, a serious public health concern, with an estimated 247 million cases and 619 000 mortalities in 2021 [2].

Plasmodium spp. are obligatory parasites from the phylum Apicomplexa and have a complex and intricate life cycle between a mosquito and a vertebrate host. The infection with *Plasmodium* in vertebrates is initiated upon the bite of an infected female *Anopheles* mosquito, which delivers sporozoites (the liver infectious form of the parasite) into the host during its blood meal. In mammals, the parasite first undergoes a single round of development in liver hepatocytes, which may last between 48 h (in rodents) to around a week (in primates) for different plasmodial species. It is accompanied by rapid asexual replication, giving rise to thousands of daughter parasites termed merozoites [3], which are eventually released in the bloodstream to infect erythrocytes cyclically (Figure 1). In humans, the blood-stage infection causes mild febrile illness which may progress to severe pathologies compromising organ function, including coma and eventually death [4]. Two human-infectious species, ***Plasmodium vivax*** (see Glossary) and *Plasmodium cynomolgi* can form dormant forms (**hypnozoites**) during the liver stage that can be reactivated at later time points [5].

Within hepatocytes, the parasite resides inside a **parasitophorous vacuole** (**PV**) (Figure 1). The **parasitophorous vacuole membrane** (**PVM**) is formed during hepatocyte invasion through the invagination of the host plasma membrane around the invading sporozoite. The PVM is gradually decorated with parasite proteins that enable interaction with host organelles and facilitate the development of the parasite **exoerythrocytic form** (**EEF**) [6]. Evidence of the malarial parasite

[1]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[2]Institute of Biochemistry II, School of Medicine, Goethe University Frankfurt, Frankfurt am Main, Germany
[3]Buchmann Institute for Molecular Life Sciences, Goethe University Frankfurt, Frankfurt am Main, Germany
[4]Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal

*Correspondence:
mmota@medicina.ulisboa.pt (M.M. Mota).

infecting liver cells prior to infecting red blood cells (RBCs) was produced in 1948 by Garnham and Shortt [7] and since has been under investigation, especially to develop interventions that could block the onset of the blood stage altogether.

While all *Plasmodium* species go through a phase of replication inside nucleated cells prior to infecting red blood cells and causing malaria, only mammalian-infectious parasites preferentially target the liver and replicate inside hepatocytes at an extraordinary rate to generate tens of thousands of erythrocyte-infectious merozoites [6]. EEFs can also undergo complete development in mammalian skin cells, albeit they are much smaller than in hepatocytes [8]. Avian and reptile malaria parasites, by contrast, infect macrophages near the bite site and differentiate into only dozens of erythrocyte-infectious merozoites [9]. The reason behind the high replication rate achieved by mammalian-infectious parasites inside hepatocytes – as key to guaranteeing the establishment of infection by overcoming the bottleneck of malaria transmission caused by a low sporozoite inoculum – remains utterly unexplored. Hepatocytes are metabolically active cells producing proteins, cofactors, and other micronutrients, while also storing several types of biomolecules [10], making them suitable for rapidly proliferating intracellular pathogens. The molecular factors driving the selection of hepatocytes by *Plasmodium* are yet to be fully understood.

For many years, the use of rodent malaria parasite species (like ***Plasmodium berghei***) has been obligatory to dissect the mechanisms underlying *Plasmodium* liver-stage biology. Albeit the relevance of such studies to human infections is always a lingering question that has become more frequently addressed with the development of new *in vitro* and *in vivo* tools that allow ***Plasmodium falciparum*** and *P. vivax* liver-stage infections to be studied.

In this review, we compile the existing knowledge on how *Plasmodium* EEFs acquire or synthesize biomolecules, their relevance in parasite development, differences between the rodent and human infectious forms, and the open questions and challenges in this area of research.

Glucose fuels *Plasmodium* liver-stage infection

The development of *P. berghei* EEFs depends on glucose availability *in vitro* [11] and *in vivo* [12]. Glucose concentration lower than 10 mM impaired *P. berghei* EEF development *in vitro* although excess glucose did not linearly increase growth [11], suggesting that glucose utilization for parasite metabolism in hepatocytes has an upper limit. *Plasmodium* parasites take up glucose via a plasma membrane hexose transporter (HT), which can import D-glucose, D-mannose, and D-fructose (with lower affinity) [13] (Figure 2). HT is expressed in *P. falciparum* and *P. berghei* (entire life cycle) [14,15] and inhibition of *Pb*HT during the liver stages impairs parasite growth [15]. Around 12 h post-infection (hpi), Albuquerque and colleagues observed an enrichment of carbohydrate metabolic process in infected hepatocytes [16], as the parasite drains hepatocyte glucose during the first 28 hpi. Afterwards, the infected hepatocyte almost doubles its glucose uptake [12] by translocating its surface glucose transporter 1 (GLUT1) to the plasma membrane [12] (Figure 2). Similar to HT, inhibition of GLUT1 activity reduces the burden of liver infection [12]. Hepatocytes express several GLUT isoforms [17], with knockdown of GLUT2, GLUT4, and GLUT9, but not GLUT3, leading to a reduction in parasite burden in Huh7 cells [12]. Interestingly, GLUT1 is preferentially expressed in pericentral hepatocytes [18], where Afriat and colleagues found a significant proportion of viable *P. berghei* EEFs in mid- to late-infected hepatocytes [19]. Similarly, pericentral hepatocytes (high glutamine synthetase, low glucokinase) are more permissive to *P. falciparum* EEF development, where host glutamine synthetase (GS) is imported into the parasite [20]. Liver-stage infection appears to be regulated by hepatocyte metabolic zonation, yet molecular mechanisms facilitating this are an exciting area of investigation.

Glossary

Apicoplast: a nonphotosynthetic organelle of *Plasmodium* parasites (and other Apicomplexans) responsible for synthesizing fatty acids, isoprenoids, and heme, all crucial during the liver stage of infection.
Exoerythrocytic form (EEF): a form of *Plasmodium* parasite that develops during the liver stages.
Fatty acid synthesis II pathway (FASII pathway): the metabolic pathway for the synthesis of fatty acids that is not present in animal cells. In *Plasmodium*, nine enzymes are responsible for synthesizing fatty acids from acetyl-CoA and malonyl-CoA.
Hypnozoite: dormant exoerythrocytic forms of *P. vivax* or *P. cynomolgi*, which can reactivate to finalize liver-stage infection and yield blood-infectious merozoites.
Parasitophorous vacuole (PV): a vacuole, in which *Plasmodium* parasites develop during their intracellular developmental stages.
Parasitophorous vacuole membrane (PVM): the membrane of the parasitophorous vacuole between the plasma membrane of the parasite and the cytosol of the host cell.
***Plasmodium berghei*:** a species of *Plasmodium* which infects rodents.
***Plasmodium falciparum*:** a species of *Plasmodium* which infects humans and is responsible for most of the malaria-associated deaths.
***Plasmodium vivax*:** a species of *Plasmodium* which infects humans. During the liver stage, *P. vivax* can halt its development and remain in a dormant form called a hypnozoite.

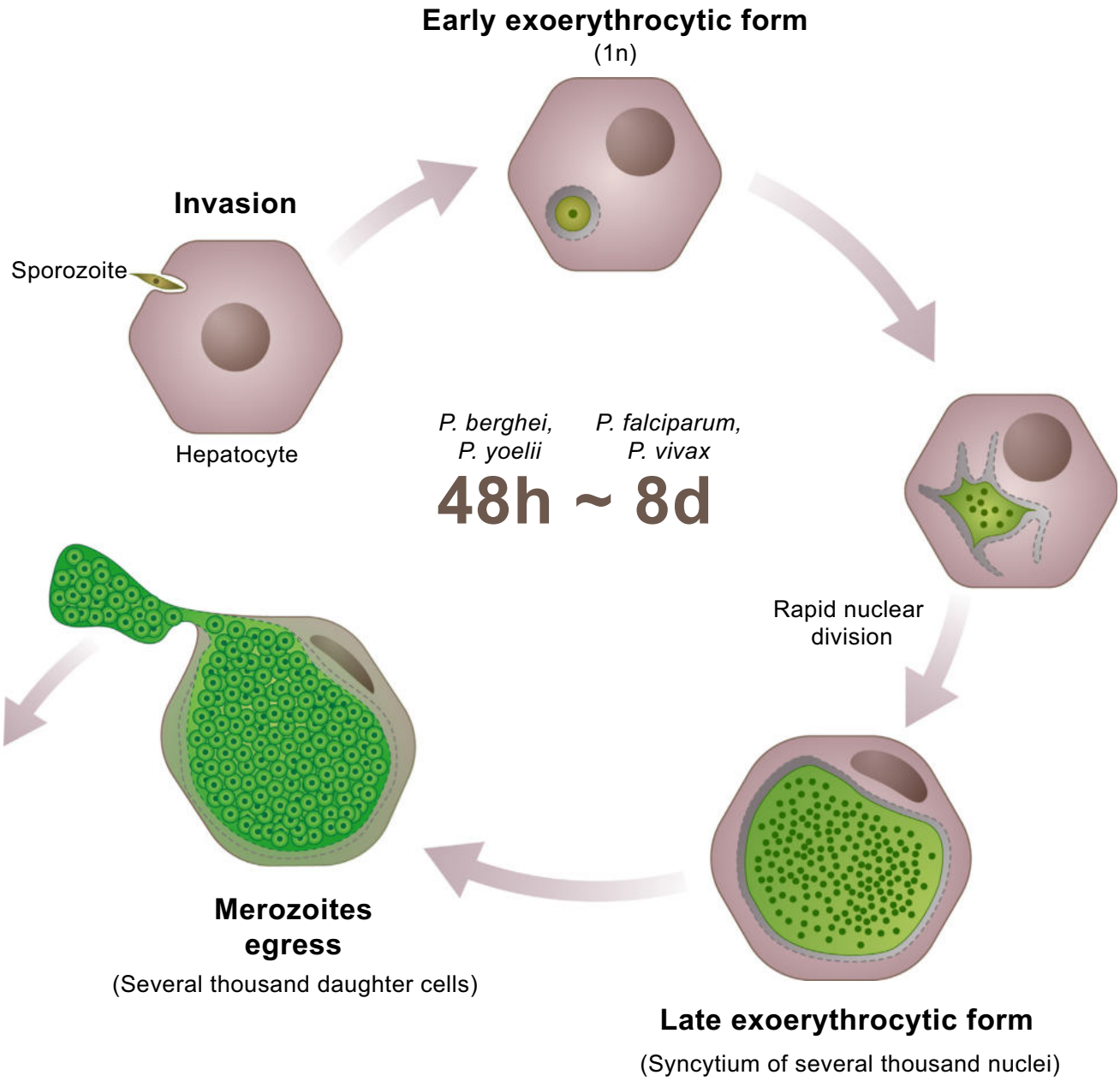

Figure 1. Schematic representation of the life cycle of *Plasmodium* spp. infecting mammalian hosts. Hepatocyte infection is established upon successful invasion by the sporozoite, which invaginates a part of the host plasma membrane during the invasion step. This membrane is called the parasitophorous vacuole membrane (broken line around the parasite) and acts as a platform for host–parasite interactions. Parasites in the hepatocytes are referred to as exoerythrocytic forms (EEFs) (light green) and, depending on the species, can have a different duration of this stage, from 2 days for rodent malaria parasites to 8 days for primate malaria parasites. The common factor is that all EEFs undergo several rounds of asexual reproduction to generate thousands of daughter parasites (merozoites) that are released into the host bloodstream through an eventual disintegration of the infected hepatocyte. Merozoites are infectious to erythrocytes and initiate the blood-stage infection leading to malarial pathology. *Plasmodium* demonstrates an intriguing metabolism during this stage and replicates at a very high rate compared to other eukaryotic cells.

Hepatocytes store glycogen which supplies glucose under starvation, although its utilization during parasite development remains unexplored. Furthermore, unlike blood-stage forms, we lack knowledge of the primary route of energy production using glucose in EEFs (Table 1).

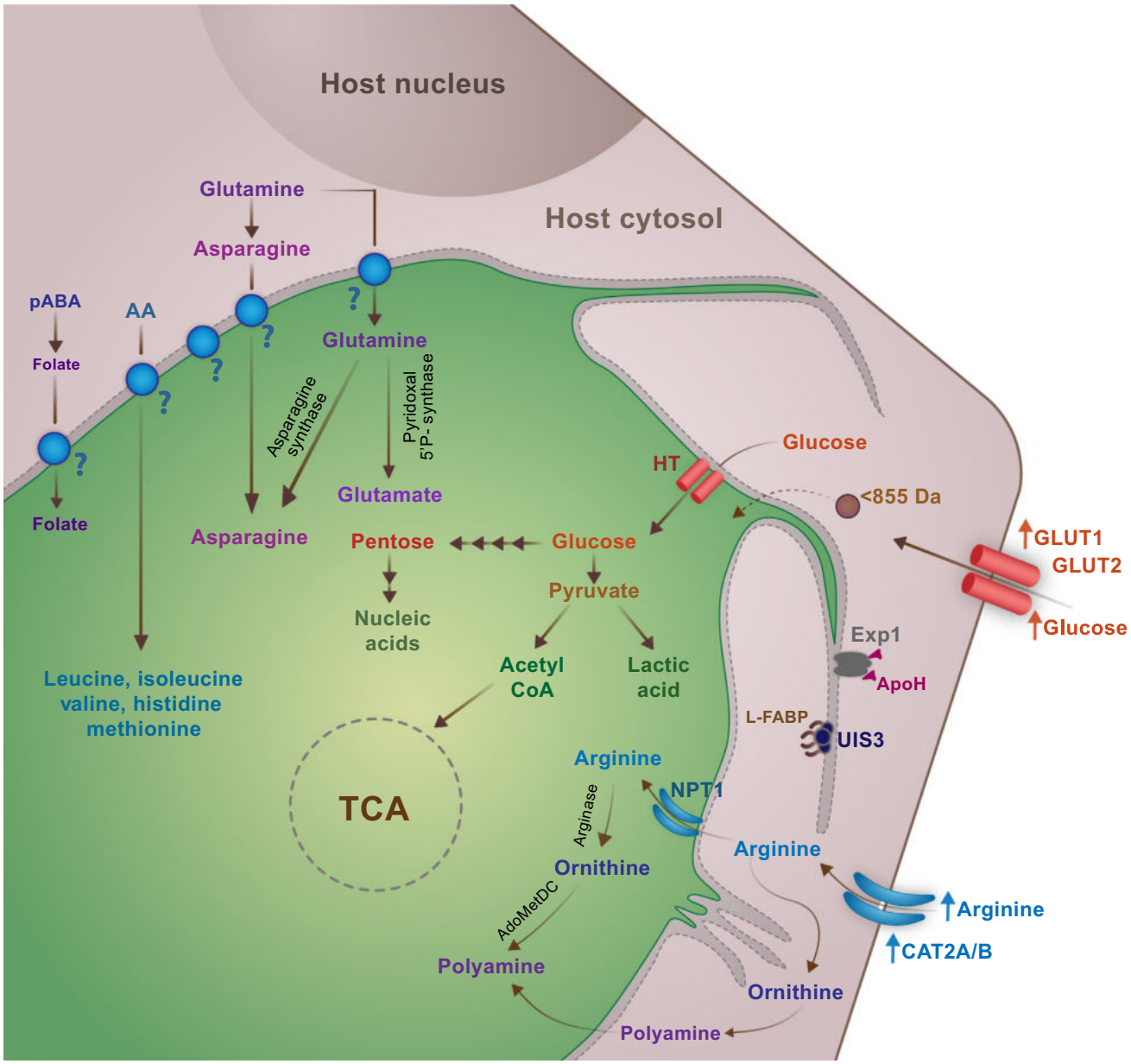

Figure 2. Amino acid and glucose uptake and metabolism by a *Plasmodium* exoerythrocytic form (EEF) in an infected hepatocyte. Molecules of less than 855 Da can pass through the parasitophorous vacuole membrane (PVM, broken line around parasite) (shown in *Plasmodium berghei*). Glucose import into the EEF is mediated by a hexose transporter (HT, orange) at the parasite plasma membrane (shown in *P. berghei*). The infected host upregulates its surface expression of glucose transporters (GLUT), increasing glucose influx (shown in *P. berghei*). Glucose is used in the glycolytic pathway and the tricarboxylic acid (TCA) cycle for energy production and nuclei acid synthesis through the pentose phosphate pathway (PPP). While several amino acids are obtained from the host cell, there is limited knowledge on all classes of parasite transporters regulating the import of different amino acids. Arginine is imported via Novel Putative Transporter 1 (NPT1, blue crescent-shaped) residing on the parasite's plasma membrane and used for the synthesis of polyamines (shown in *P. berghei*). Certain amino acid transporters are upregulated in infected hepatocytes, such as CAT2A and CAT2B which import arginine (shown in *P. berghei*). Glutamine is used by the parasite for glutamate and asparagine synthesis. Lipoprotein and lipids may be scavenged via interaction between the PVM proteins Exp1 (gray) with host apolipoprotein-H (dark red) and, UIS3 (dark blue) with L-FABP (brown) at the PVM (shown in *P. berghei* and *Plasmodium yoelii*, respectively). Abbreviations: AA, amino acid; pABA, para-aminobenzoate.

Table 1. Similarities and differences between blood-stage and liver-stage metabolism[a]

Nutrient	Blood stages (*Pf* and/or *Pb*)	Liver stages (*Pb* only)
Glucose uptake by the infected cell	Upregulated 100-fold in infected RBCs [99]	Upregulated twofold in infected hepatocytes [12]
Parasite energy metabolism	Principally glycolytic [19]	Evidence lacking
Amino acid source	RBC hemoglobin is the primary source of amino acids for the parasite [100]	Some amino acids are synthesized by the parasite [24,33,35] while auxotrophic amino acids are scavenged from the host [26,31,34]. Exact substrates remain unknown
Effect of inhibiting host uptake of cationic amino acids	Does not alter parasite load *in vivo* [26]	Impairs parasite load *in vivo* [26]
Folate synthesis	Derived from uptake of host pABA or *de novo* folate synthesis [35]	Folate directly taken from the host, independently of pABA [35]
Lipid synthesis and procurement	Parasite lipid synthesis is dispensable; most lipids are scavenged from the host cell	Parasite lipid synthesis pathway is essential for EEF development [45]
Lipid transporters	Two putative lipid transporters identified [76–78], awaiting validation	Transcripts also identified in the liver stage [79,80], awaiting validation.
Lipoic acid source	Scavenged from host to parasite mitochondria [54]	Similar to blood-stage development [43]
Heme procurement	Obtained from host RBCs Hemoglobin and synthesized by parasite [81,82]	Synthesized by parasite [81,82]
Vitamin metabolism	Parasite synthesized vitamins B1 and B6 and scavenges B5 from the host RBCs. Vitamin B7 is dispensable for development [91]	No information regarding vitamins B1, B5, and B6. Host-derived vitamin B7 (biotin) is crucial for EEF development and merosome release [91]

[a]Abbreviations: EEF, exoerythrocytic form; KD, knock-down; pABA, para-aminobenzoic acid; *Pb*, *Plasmodium berghei*; *Pf*, *Plasmodium falciparum*; *Pf*SAMS, *Plasmodium falciparum* S-adenosylmethionine synthase; RBCs, red blood cells.

Among the scant information, the upregulation of *Plasmodium* glyceraldehyde-3-phosphate dehydrogenase at 12 hpi may provide a starting point to explore the regulation of glycolytic enzymes, especially during replicative bursts. As for glucose storage by *Plasmodium* liver-stage parasites, there is no evidence of glycogen synthesis enzymes nor glycogen itself in the parasite [21], indicating that glucose is continually exhausted during development. *Plasmodium* expresses the first enzymes of the pentose phosphate pathway (PPP) [21]. This pathway is a source of ribose and deoxyribose, essential for RNA and DNA synthesis, respectively, which may be crucial for the rapidly dividing *Plasmodium* in hepatocytes (Figure 2), although it remains only speculation due to lack of evidence.

Apart from energy metabolism, carbohydrates in *Plasmodium* are also employed to generate amino sugars required for synthesizing glycoconjugates, such as glycosylphosphatidylinositol (GPI)-anchored proteins [22].

Amino acids are diversely obtained by liver-stage parasites

Plasmodium shares metabolic pathways and relic organelles with plants, but unlike plants, it cannot synthesize all the 20 amino acids: the parasite genome has lost components for nine pathways [23], and the amino acids whose biosynthetic components are encoded include asparagine, aspartate, glutamine, glutamate, glycine, and proline [23] (Figure 2). *Plasmodium* scavenges amino acids principally via hemoglobin degradation during asexual development in RBCs (Table 1), albeit mechanisms of sourcing amino acids during liver-stage development remain poorly understood.

Arginine is key for EEF development as a source of polyamines

As a metabolic substrate, arginine generates nitric oxide, polyamines (putrescine, spermine, and spermidine, essential for eukaryotes [24]), glutamate, and nonproteogenic amino acids (citrulline and ornithine) [25]. *P. berghei*-infected hepatocytes increase arginine uptake [26] by upregulating the expression of the host cationic amino acid transporter (CAT) family – SLC7A2 (both CAT2A and CAT2B isoforms) which transport arginine across the host plasma membrane [16]. SLC7A2 depletion reduces *P. berghei* parasite load *in vivo* [26]. Arginase is also expressed in *Plasmodium* parasites and participates in polyamines synthesis [27] in which the rate-limiting enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) exist as a bifunctional unit, regulating parasite polyamine abundance [28,29] (Figure 2). Polyamines are essential for *P. falciparum* blood-stage development [30] (Table 1), but in liver stages, the knockdown of host arginase or ODC did not impair *P. berghei* intrahepatic development. Depletion of *Plasmodium* arginase, however, presented two parasite populations: one with impaired development and the other without [26]. Additional depletion of host arginase or ODC in cells infected with arginase knockout parasite impaired the development of the fraction of parasites that otherwise were unaffected in wild-type hepatocytes [26]. Taken together, this suggests that the *P. berghei* liver stage depends on polyamines, which can be sourced from the hepatocyte directly, or synthesized by the parasite from arginine obtained from the hepatocyte. A novel putative transporter 1 (NPT1) (Figure 2) may be a promising candidate for the arginine transporter, as *P. berghei* NPT1 was shown transport arginine [31], localizes around the parasite plasma membrane in blood stages, and is abundantly expressed during liver stages [32].

Asparagine is necessary for liver-stage development

Asparagine is synthesized by the parasite using an isoform of asparagine synthetase (AS) that utilizes aspartate as substrate [33] (Figure 2). Depleting AS in *P. berghei* reduces sporozoite production and delays blood patency upon liver-stage infection [33]. Further depletion of asparagine in the host infected with AS-deficient parasites severely reduced liver parasite load, indicating that host-derived asparagine can also sustain liver-stage development [33]. Transporters involved in asparagine import from the host are yet to be explored.

Glutamine is synthesized and scavenged by *Plasmodium* EEFs

Plasmodium parasites are capable of *de novo* glutamine synthesis [23] but may scavenge host glutamine or utilize host GS for glutamine production (Figure 2). As mentioned earlier, *P. falciparum* parasites were shown to preferentially develop in host GS-expressing subsets of hepatocytes, with host GS localizing inside mature EEFs [20]. In mammals, host GS is abundant in pericentral hepatocytes, a region where *P. berghei* EEFs were reported as more viable [19].

Nonbiosynthesized amino acids are scavenged to enable liver-stage development

Apicomplexans lack pathways for *de novo* synthesis of leucine, isoleucine, valine, histidine, and methionine. As such, they must be scavenged from the host. Methionine serves to provide homocysteine and S-adenosylmethionine (SAM), both of which are substrates necessary to regulate cellular homeostasis [34]. However, the sourcing of any of these amino acids has not been explored (Figure 2).

Nonproteogenic amino acids are required by EEFs for alternate metabolic pathways

Among the nonproteogenic amino acids, *Plasmodium* can synthesize para-aminobenzoate (pABA) and δ-aminolevulinic acid (ALA). pABA is a precursor for folate synthesis (which plays a critical role in DNA synthesis) and is essential for *P. berghei* blood stages but not for liver stages [35]. Folate can also be scavenged from the host by both blood- and liver-stage parasites (Table 1 and Figure 2) [35]. ALA is necessary for heme biosynthesis and production of cytochrome; it is

synthesized in the parasite mitochondria from glycine and succinyl coenzyme-A (CoA) by the action of δ -aminolevulinate synthase (ALAS) [36,37] (discussed in 'Micronutrients – cofactors during the liver stage').

Lipid metabolism: how can one parasite synthesize so many membranes?

In *Plasmodium* most of the knowledge regarding lipid synthesis comes from a unique Apicomplexan organelle known as the **apicoplast**, which is a relic nonphotosynthetic plastid [38]. Like the plant and cyanobacterial plastids, the apicoplast houses the **fatty acid synthesis II** (**FASII**) pathway, and the nonmevalonate isoprenoid precursor synthesis pathways [39] (Figure 3). Additionally, the FASII pathway is interconnected with lipoic acid biosynthesis as the latter generates the lipoate-cofactor required for the functioning of FASII-associated machinery [39] (Figure 3). There is growing evidence regarding lipid scavenging mechanisms by *Plasmodium* from hepatocyte to satiate the needs for its development, similar to blood-stage parasites that extensively scavenge lipids from the host RBCs (Table 1) [40].

FASII pathway is central for lipid biosynthesis in EEFs of rodent malaria parasites

Fatty acids generated in the apicoplast via the FASII pathway are employed for lipoic acid (LA) biosynthesis, and as precursors for membrane lipids. Interestingly LA indirectly regulates the FASII pathway through the synthesis of lipoate cofactor in the apicoplast, which is necessary for the pyruvate dehydrogenase (PDH) complex function, [41,42]. LA can be scavenged from the host into the mitochondria of *P. berghei* during liver-stage development [43] (Figure 3, inset 2), and lipoylated protein can be found in the apicoplast and the mitochondria [44].

The FASII pathway shows differential requirement in *Plasmodium* spp. It is dispensable during blood stages of *P. falciparum, Plasmodium yoelii* [45], and *P. berghei* [46], but essential for the late liver stages of *P. berghei* and *P. yoelii* (Table 1). Depletion of these enzymes in *P. falciparum* arrests its development in the mosquito stage, thus impeding functional explorations into its liver stages [45,47]. The apicoplast FASII machinery comprises nine enzymes spread across reactions which may be segregated into 'preparation', 'initiation', and 'elongation' steps and, together with the acyl carrier protein (ACP), generate saturated fatty acids [39] (Figure 3, inset 2).

During the preparation step, phosphoenolpyruvate (PEP) is transported to the apicoplast from the parasite cytoplasm through plastidic phosphate transporters (pPTs, phosphorylated carbon compounds, and inorganic phosphate antiporters) [48], followed by the conversion of PEP to pyruvate by pyruvate kinase and the formation of ATP [42]. pPTs were localized to the outermost and innermost apicoplast membranes in *P. falciparum*, and only the latter in *P. berghei* [40]. In the final stage of the preparation step, PEP is converted to acetyl-CoA by the PDH complex with the release of carbon dioxide [48]. Acetyl-CoA carboxylase (ACC) is the first enzyme of the initiation step, that converts acetyl-CoA to malonyl-CoA [49]. The malonyl group is then transferred to ACP (catalyzed by malonyl-CoA: ACP transacylase, FabD), which ultimately leads to the condensation of malonyl-ACP with acetyl-CoA through the action of β-ketoacyl-ACP synthase III (FabH) [50–52]. The end of the initiation pathways leads to the generation of CoA, carbon dioxide, and acetoacetyl-ACP, paving the way for the elongation step. During the elongation stage, acetoacetyl-ACP grows by the addition of two carbon moieties per reaction cycle to yield acyl-ACPs [53]. The fatty acids synthesized are necessary for membrane biosynthesis, although there are no described exporters to transport fatty acids out of the apicoplast.

Lipoic acid crucially complements liver-stage development

The canonical enzymes (LipB, LipA, and Lp1A2) catalyze the synthesis of lipoic acid (LA) from a derivative of the FASII pathway (octanoyl-ACP) and SAM in the apicoplast [41]. In *Plasmodium*,

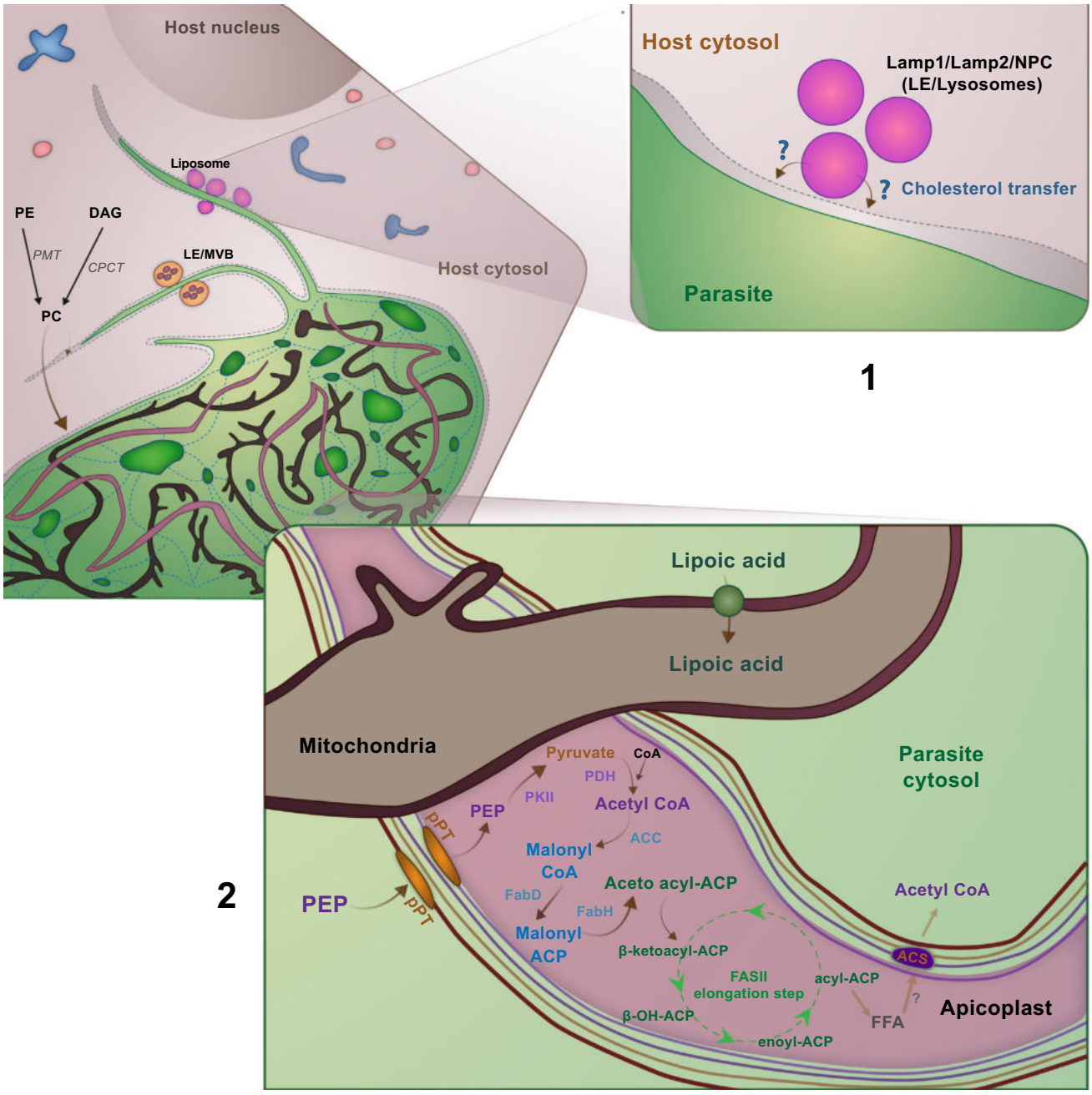

Figure 3. Simplified schematic of lipid uptake and metabolism by a *Plasmodium* exoerythrocytic form (EEF) in an infected hepatocyte. The phospholipid-phosphatidylcholine (PC) is obtained by the EEF from the host, which itself synthesizes it from either phosphatidylethanolamine [PE, catalyzed by phosphatidylethanolamine methyl transferase (PMT)] or from diacylglycerol [DAG, catalyzed by choline phosphate cytidyltransferase (CPCT)] (shown in *Plasmodium berghei*). Inset 1. *Plasmodium* EEF (light green) obtains cholesterol from host late endosomes (LEs) and lysosomes (magenta) at the parasitophorous vacuole membrane (PVM) via the lysosomal Niemann–Pick type C (NPC) and lysosome-associated membrane proteins (LAMP 1 and 2) (shown in *P. berghei*). Inset 2. Fatty acid synthesis in the parasite occurs via the fatty acid synthesis II (FASII) pathway in the apicoplast. Starting with the preparation phase (purple), phosphoenol pyruvate (PEP, purple) is imported to the apicoplast via plastidic phosphate transporters (pPTs, orange) (shown in *P. berghei* and *Plasmodium falciparum* blood stages) and converted to pyruvate by pyruvate kinase (PKII) followed by the action of the pyruvate dehydrogenase (PDH) complex to generate acetyl-CoA. The initiation phase (blue) proceeds with the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC) followed by the transfer of the malonyl group acyl carrier protein

(Figure legend continued at the bottom of the next page.)

LA is a cofactor for the apicoplast PDH complex and some mitochondrial enzymes [43] (Figure 3). LipB (octanoyl-ACP: protein N-octanoyl transferase) enzyme deletion was not lethal during *P. falciparum* and *P. berghei* blood stages; however, an acceleration in asexual replication was reported upon *Pf*LipB deletion [54]. A significant decrease in the lipoylation of mitochondrial enzymes was observed upon LipB deletion in *P. falciparum* and *P. berghei* [54,55]. LipB deletion in *P. berghei* EEFs led to defects during late liver-stage development [55]. The second enzyme in lipoic acid synthesis, LipA (lipoyl synthase), acts as a sulfur donor to the octanoyl moiety bound to the PDH complex [44]. LipA is refractory to deletion and has limited structural and functional information [56]. The third enzyme, Lp1A2, can also possess residual octanoyl transferase activity, enabling it to catalyze, alternatively, the first step in LA synthesis [54]. *Pf*Lp1A2 activity studies produced incongruent observations regarding its role as a lipoate protein ligase [54]. The apicoplast Lp1A2 is an ATP-dependent lipoate protein ligase, and its deletion in *P. berghei* compromises mosquito stage development [57]. Lp1A2 also localizes to the mitochondria, which are involved in the scavenging of lipoic acid [57].

Plasmodium unceremoniously obtains lipids from the hepatocyte

Interestingly, *P. berghei* and *P. yoelii* mutants lacking critical FASII pathway proteins develop normally during the first 40 h of the liver stage, leading us to question whether most of *Plasmodium* development is fueled through scavenged lipids.

The PVM is permissive to molecules under 855 Da [58], and the EEF stays close to the endoplasmic reticulum (ER) [58], the Golgi [59], and the apical domains of the hepatocyte (facing the bile canaliculi) [60] throughout the liver stage, all of which play a central role in lipid synthesis and traffic in the hepatocyte. Whether the parasite positions itself to siphon host lipids is an ongoing area of investigation.

Liver-stage parasites use host-derived phospholipids and cholesterol

In rodent parasites, fatty acids generated via the FASII pathway are used in the synthesis of phosphatidic acid (PA), which is a precursor for phospholipid biosynthesis. This involves a three-step reaction comprising two apicoplast enzymes (G3PAT and G3PDH) [61] and one ER-resident enzyme (LPAAT) [46]. Hepatocytes infected with *P. berghei* show upregulated phospholipid and cholesterol synthesis proteins [16], and elevated levels of neutral lipids, sphingolipids, cholesterol, and the phospholipid phosphatidylcholine (PC) [62], while other phospholipids, like phosphatidylethanolamine (PE) and phosphatidylserine (PS) were reduced [62]. The parasite's survival depends on the synthesis of PC by hepatocytes (Figure 3) [62], unlike blood stages which synthesize the majority of their PC [63]. Mice and cells deficient in choline phosphate cytidyltransferase (CPCT, that inserts the choline moiety into diacylglycerol) or phosphatidylethanolamine methyl transferase protein (PMT, that inserts methyl groups into the ethanolamine moiety, converting PE into PC) harbor lower levels of liver infection [62] as do mice fed on a choline-deficient diet [62]. Curiously, increased levels of tri-acyl-glycerides (TAG) and lower levels of free fatty acids (FFAs) in the host do not compromise *P. berghei* hepatic development [64], hinting that the parasite may be self-sufficient for FFAs in this stage.

Also, *Plasmodium* is auxotrophic for sterols, yet it has sterols on its PV and plasma membrane in liver stages [58], as the rodent parasites effectively scavenge hepatocyte cholesterol from low-density lipoproteins and the mevalonate pathway but not from high-density

(ACP) by the enzyme FabD. FabH then catalyzes a condensation reaction with acetyl-CoA to generate β-ketoacyl-ACP. In the elongation phase (green) the acetoacetyl-ACP undergoes cyclic steps to yield acyl-ACP, which serves for free fatty acid (FFA) generation. FFAs can be used for lipoic acid synthesis, although lipoic acid can be scavenged from the host and imported into the EEF mitochondria.

lipoproteins [65] (Figure 3). The parasite can increase cholesterol uptake from either route to circumvent the blockade of one [65]. Cholesterol esterification by the host does not deter *P. berghei* development [62].

Are host late endosomes used for scavenging lipids?

The precise mechanisms of phospholipid and cholesterol uptake from the host are not completely understood. One possible source of lipids could be the host late endosomes (LEs), which, in hepatocytes, are sites of cholesterol salvage and can be found surrounding liver-stage parasites throughout their development [66] (Figure 3, inset 1). Host LEs regulate EEF development in hepatocytes, which is interesting given that these are acidic compartments and fusion can be lethal for the parasite. Blocking LE acidification [66] or cholesterol export from LEs [67] led to smaller hepatic parasites, indicating a role for host LEs in nutrient delivery. The host endocytic pathway is strictly regulated: so how do *Plasmodium* EEFs redirect host endosomes? LEs and lysosomes accumulate around the *P. berghei/P. yoelii/P. vivax* parasites early in infection and act as the cell-independent response to infection [66,68]. Later, during EEF development, the presence of LEs on the PVM is dependent on phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2] there produced by the host kinase PIKfyve [69]. How the parasite determines which host LE/lysosomes are favorable to the parasite is unknown.

Apart from host LEs, cholesterol can be acquired by *P. berghei* EEFs through host Apolipoprotein H (ApoH), which localizes in the parasite [70]. ApoH mechanism of import in the EEF is unclear, although its interaction with the PVM protein EXP-1 (C-terminal domain) may suggest a route for redirecting this host protein [70]. Additionally, the host-liver fatty-acid-binding protein (L-FABP) – which binds to and transports fatty acids in hepatocytes [71] – regulates liver-stage development of *P. berghei* wherein downregulation or overexpression of L-FABP impairs or boosts parasite load, respectively [72]. Conversely, *P. vivax*-infected hepatocytes downregulate L-FABP expression in late liver development [73], and may have entirely different dynamics relative to *P. berghei*. The mechanism of L-FABP acquisition and lipid transfer remain unclear as one study indicated host L-FABP to coimmunoprecipitate with *P. yoelii* PVM-resident protein, UIS3 [72], but an NMR analysis of human L-FABP and *P. falciparum* UIS3 excluded direct interaction between them or a role in lipid transfer [74].

Finally, the parasite might possess transporters to take up lipids, even though they have not been discovered (reviewed in [75]). So far, only two lipid transporters were identified in the blood stages of *P. falciparum*: one protein transports phospholipids (PFA0210c) [76] and the other protein has sequence homology to a cholesterol transporter (*Plasmodium* Niemann–Pick type C1-related protein), but await functional characterization [77,78]. Both these proteins are expressed during blood stages [76–78] and their transcripts were identified in the liver stage (Table 1) [79,80]. Curiously, both proteins localize to the parasite plasma membrane (PPM) and not to the PVM during the blood stage [76–78], thereby questioning how the PPM and PVM coordinate to mediate the uptake of host material.

Micronutrients – cofactors during the liver stage

The proper functioning of several enzymes requires certain small molecules in trace quantities known as micronutrients, and plasmodial enzymes are no exception. The following section summarizes a few micronutrients described so far in liver-stage development.

Heme is essential during liver-stage development

P. berghei parasites lacking enzymes for the early (δ-aminolevulinate synthase), intermediate (porphobilinogen deaminase or uroporphyrinogen III), or late (ferrochelatase) steps of heme

synthesis cannot complete mosquito stage development [81], although exogenous supplementation with δ-aminolevulinic acid (ALA) could rescue the mosquito and liver stages of development in *P. berghei* parasites lacking δ-aminolevulinate synthase, the first step in the heme synthesis pathway [82] (Figure 4, inset 2). Overall, heme is essential throughout *Plasmodium* development, but blood-stage parasites can source it from hemoglobin [82].

Iron and zinc regulate EEF development

Infected hepatoma cells *in vitro* demonstrate the upregulation of the host protein divalent metal transporter-1 (*h*DMT1) [16], which transports the iron released from transferrin in the early endosome [83] (Figure 4). Iron chelation in cultured hepatocytes expectedly reduces parasite load of *P. falciparum*, *P. berghei*, and *P. yoelii* [84], while iron overload favors *P. yoelii* intrahepatic infection [85].

There is no recognized *Plasmodium* iron transporter localizing at the PVM in hepatocytes or erythrocytes. A vacuolar iron transporter (VIT) has been described in *P. berghei*, which is expressed in all parasite life stages and is involved in detoxifying excess iron [86] (Figure 4, inset 1). Another putative iron and zinc transporter called (ZIPCO) was described as necessary for liver-stage development, indicating the parasite's dependence on scavenging zinc from hepatocytes [87] (Figure 4).

Vitamin metabolism – potential therapeutic target?

Vitamin B5 (pantothenic acid) is necessary for CoA synthesis and is imported by the parasite throughout its life cycle. Deleting the pantothenate transporter in *P. yoelii* did not drastically affect asexual blood stages but compromised oocyst formation, arresting the parasite in mosquito-stage development [88]. Vitamin B5 analogs reduce *P. berghei* and *P. falciparum* liver burden *in vitro* [89,90] and are under investigation as antimalarials.

Presently, the role of vitamins B1 and B6 during *Plasmodium* liver-stage development remains unexplored.

Other essential cofactors include biotin (vitamin B7) and folate (vitamin B9 – discussed under the 'Nonproteogenic amino acids are required by EEFs for alternate metabolic pathways' section). Biotin functions as a carbon dioxide carrier in carboxylation/decarboxylation reactions, (i.e., acetyl-CoA to malonyl-CoA conversion). It is predicted that *Plasmodium* has only one biotinylated protein: acetyl-CoA carboxylase (ACC), which localizes to the apicoplast [49]. During the liver stage, the parasite requires exogenous biotin [91] (Figure 4), likely for ACC biotinylation by holocarboxylase synthetase 1 [91]. Biotin seems dispensable for blood-stage growth, with no biotinylated proteins observed during this stage [91].

Are hypnozoites metabolically active?

For some species of *Plasmodium*, not all EEFs complete their designated life cycle. Such EEFs, called hypnozoites, remain quiescent inside hepatocytes for years [6]. Hypnozoites can become reactivated, to finish their liver-stage development and initiate blood-stage infection (relapse) [6]. Only three species of malaria parasite have been shown to be capable of hypnozoite formation: *P. vivax*, *Plasmodium ovale* (infects humans), and *P. cynomolgi* (infects monkeys) [92]. What triggers reactivation of hypnozoites into liver schizonts is presently unknown. Recent projects were able to study the transcription of these stages, and it will be interesting to further understand the contribution of the liver microenvironment in hypnozoite reactivation.

Using either laser microdissection [93] or fluorescence-activated cell sorting [94,95], the bulk transcriptome of hypnozoites from the simian parasite *P. cynomolgi* was analyzed. These

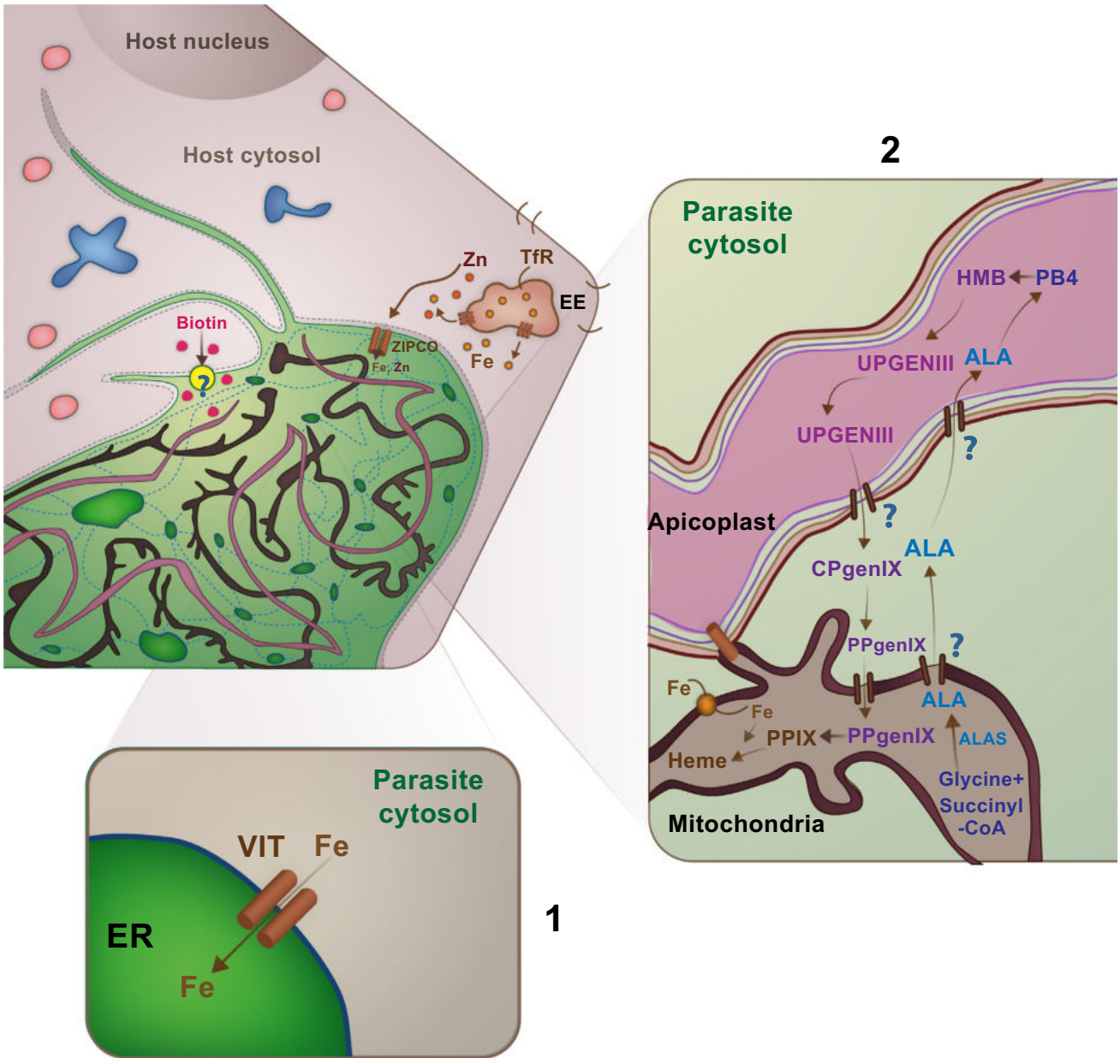

Figure 4. Simplified schematic of micronutrient uptake and metabolism by a *Plasmodium* exoerythrocytic form (EEF) in an infected hepatocyte. Certain vitamins like biotin (pink) and metal ions (iron and zinc) are obtained from the host cell. Main image upper left: Iron (Fe) released from the transferrin–transferrin receptor complex in host early endosomes (EEs) are diverted to the EEF together with host zinc (Zn) and are putatively transported via ZIPCO at the parasite's plasma membrane (shown in *Plasmodium berghei*). Inset 1. Within the EEF, excess Fe is pumped into the parasite's endoplasmic reticulum (ER, dark green) via the vacuolar iron transporter (VIT, brown) to avoid its cytosolic accumulation and toxicity (shown in *Plasmodium berghei*). Inset 2. Heme is an essential metabolite for EEF development and is synthesized from δ-aminolevulinic acid (ALA). ALA is exported from the parasite's mitochondria (dark brown) and imported into the apicoplast (pink), where it is processed to generate intermediates, which are then exported from the apicoplast and imported into the mitochondria where they pass through a series of reactions in the presence of iron (Fe) (shown in *P. berghei*). The relevant mitochondrial and apicoplast transporters of ALA and other pathway intermediates that remain undiscovered are labelled ? in the figure.

quiescent forms express lower levels [93–95] and lower diversity of transcripts [94,95] when compared with developing EEFs. Hypnozoites exhibit a general transcriptional retardation, having only 34% of the possible pathways active at day 6 post-infection [95], and only 19% of active

pathways at day 9 post-infection [94] (the authors defined a pathway as active when more than half of the constituent genes are expressed over 1 mRNA copy per cell). For comparison, hepatic schizonts have 91–93% of possible pathways active between day 6 and 9 post-infection [94,95]. Hypnozoites still express transcripts related to housekeeping functions, such as mitochondrial respiration [95] and fatty acid biosynthesis [94]. Hypnozoites express lower but stable levels of genes related to glucose metabolism [94], indicating that they might require glucose for their basal energic needs.

Mancio-Silva *et al.* demonstrated – using a combination of pharmacological treatment and single-cell transcriptomics in *P. vivax*-infected cells – that they could measure the transcription of both hypnozoites and the infected hepatocytes [73]. Surprisingly, hepatocytes with hypnozoites had reduced metabolic activity themselves, expressing lower levels of genes involved in lipid metabolism and iron transport and storage [73]. An interesting hypothesis from these results could be that *Plasmodium* senses the metabolic status of the hepatocyte and 'decides' to arrest and become an hypnozoite or to continue liver-stage development. The interesting corollary of this hypothesis is that reactivation might be triggered by an increase in the metabolic flux of the hepatocyte, that reactivates the hypnozoite.

Alternatively, it could be argued that the hypnozoite itself causes this reduction of host metabolism. *P. cynomolgi* hypnozoites express genes related to protein export [94]. These parasitic proteins may be exported to the hepatocyte where they influence hepatocyte metabolism as a means to protect the parasite from toxic substances like reactive oxygen species.

Regardless of the precise mechanism, we require more information to understand the metabolism of this elusive stage in order to be able to target it. For example, Voorberg-van der Wel and colleagues identified two putative copper transporters that are highly expressed in *P. cynomolgi* hypnozoites and were able to decrease the number of *P. cynomolgi* hypnozoites *in vitro* using a copper chelator [95]. This opens the opportunity for metabolism to be a target for hypnozoite clearance.

Concluding remarks

Seventy-five years after the discovery of the liver stage of *Plasmodium* infection [7], our knowledge of the metabolism (and biology in general) of liver stages remains hindered by the inability to produce or purify large quantities of liver-stage parasites – which limits the diversity of experiments. Advances in 3D culture systems and liver-humanized mice models [96], which produce systems closer to the infection of a human liver hepatocyte, cannot rescue the low yields of infected hepatocytes.

An elegant way of circumventing this limitation and increasing the ability to identify parasite genes with key roles in liver-stage development was performed by scaling up experimental genetics using a resource of >2900 individually barcoded gene knockout vectors available for *P. berghei* [22]. The authors generated pools of these blood-stage-viable knockout mutants and analyzed their phenotypes throughout the entire parasite life cycle, including the liver stage of infection. They combined the data of the genetic screen with a previous liver-stage transcriptome [79] and generated the first liver-stage metabolic model for *P. berghei*. This study determined that seven metabolic pathways should be essential during the liver stage: the FAS II pathway, the fatty acid elongation pathway, the shikimate pathway, the tricarboxylic cycle, amino sugar metabolism, the heme pathway, and the lipoate pathway. However, such studies using reverse genetic approaches come with a limitation: *Plasmodium* parasites are amenable to genetic modification only during the blood stage. This makes it hard to study genes during the liver stage that are essential during the blood stage, which is the case for the tricarboxylic acid (TCA) cycle. Novel

Outstanding questions

How do *Plasmodium* parasites achieve such high replication rates within a single hepatocyte?

What are the distinct carbon sources and pathways of energy generation throughout the liver stage of parasite development and replication?

Which metabolites are derived from the host organelles, and how are such biomolecules transferred between the host and parasite compartments?

What are the impacts of the different hepatocyte metabolic states on the parasite replicative fate – active or dormant (hypnozoites)?

What is the metabolic status of a hypnozoite? Which metabolic cues trigger their reactivation and therefore relapse of infection?

What are the roles of other nonparenchymal liver cells (stellate cells, cholangiocytes, Kupffer cells, endothelial cells) and the liver microenvironment itself on the outcome of infection?

strategies of conditional knockout have been used to study the invasion of sporozoites [97,98] but this has not yet been applied to metabolic studies.

Single-cell analysis has just recently kicked in and it certainly has the potential to be the most helpful strategy to overcome some of the limitations of studying this key and obligatory stage of the *Plasmodium* life cycle. Afriat and colleagues [19] used single-cell transcriptomics to analyze infected murine liver hepatocytes throughout parasite liver-stage development to uncover dynamic changes in the infected host hepatocytes and the parasites within them. While this initially might not be very different from previous bulk infected cell transcriptomics, the ability to study individual infected cells allowed the realization that the infected cell's location in the hepatic metabolic axis is critical for the infection's success [19]. This study concluded that, at least in mice, infected periportal hepatocytes are more likely than infected pericentral hepatocytes to enter what we call an 'abortive hepatocyte' state and as such a higher probability of an infection that fails. Importantly, *P. falciparum* sporozoite infections of primary human hepatocytes also showed that this parasite has a selective preference for pericentral hepatocytes [20]. Altogether, these findings imply that successful parasite development in the liver is determined largely by the differential metabolic state in particular hepatocyte subtypes.

Despite the many limitations, several discoveries have been made in the past decades and what once was a black box is now full of interesting pieces of a puzzle that is slowly getting assembled. There remain more questions than answers, and the liver is a complex organ (see Outstanding questions). While the field has focused for now on the hepatocyte–*Plasmodium* closed habitat, some of the most pertinent questions are related to the role of other nonparenchymal liver cells and the effect of the liver metabolic microenvironment itself on the outcome of infection. The possibility of unveiling the mechanisms linked to host–parasite interactions within the physiology of a complex tissue and organ at cellular resolution will indubitably be highly informative towards the design of antimalarial strategies targeting liver-stage infection, including hypnozoites, paving the way for malaria elimination.

Acknowledgments

We want to apologize to our colleagues, whose work could not be cited due to space constrains. We want to thank Cygny Malvar for drawing the figures. This work was also financed by la Caixa Foundation (HR17/52150010) to M.M.M.

Declaration of interests

The authors declare that they have no competing interests.

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