The complex interplay between *Plasmodium* and the hepatocyte: Potential therapeutic targets

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Abstract

Malaria is one the most life-threatening infectious diseases in humans, affecting 219 million people worldwide in 2017. The disease is caused by the genus *Plasmodium* and transmitted between hosts by the female *Anopheles* mosquito. While many of the previous attempts to reduce the amount of malaria cases have been unsuccessful, the recent approval of the RTS,S vaccine meant a big step towards eradication. Although this malaria vaccine is expected to have a significant effect on improving child survival, its efficacy is only partial and short-lived, highlighting the need for further research into other potential vaccine targets. Despite the rapid expansion the malaria parasite undergoes during the liver stage, developing thousands of merozoites from only a few sporozoites, this phase of the parasite's infection cycle is clinically silent. Hence, inhibition of successful liver stage development will prevent onset of disease, making this part of the parasite's life cycle an ideal target for therapeutic intervention. To identify potential therapeutic targets, it is important to fully understand the complex interplay between *Plasmodium* and the hepatocyte. This thesis therefore elaborates on the various host factors that are utilized by the malaria parasite to complete liver stage development, from proteins required for hepatocyte invasion towards host-parasite interactions necessary to evade the process of autophagy and colocalization with host cell organelles. Furthermore, an insight will be given on how these interactions enable therapeutic intervention.

Layman's summary

Malaria is one of the most life-threatening infectious diseases in the world, causing thousands of deaths per year and mainly affecting the African population. The disease is caused by the *Plasmodium* parasite, which is transmitted between hosts by a female mosquito. Malaria parasites are injected into the human skin by a mosquito bite. The parasites first migrate to the liver, where they infect liver cells and grow at a very high rate. Following their release into the bloodstream, the parasites start infecting red blood cells, which results in the establishment of the first clinical symptoms and can lead to death of the host. Another mosquito bite transfers free parasites present in the bloodstream back to the mosquito, allowing them to be transmitted to a new human host.

Up until now, the many attempts to reduce the amount of malaria cases have largely been unsuccessful. Preventative measures, such as bed nets and insecticides, do not work, and there is an increase in resistance among *Plasmodium* parasites towards existing malaria medication. Although the first malaria vaccine was recently approved by the World Health Organization, and is thought to signficantly improve the chance of survival, there is still need for new therapeutic targets. Because the liver phase of the parasite's infection cycle is clinically silent, inhibiting the malaria parasite to complete liver stage development will prevent onset of disease. This makes the liver stage a very interesting target for vaccination. To identify potential therapeutic targets, it is important to fully understand the complex interactions that take place between the malaria parasite and liver cell.

First, successful entry of the malaria parasite into liver cells requires the binding of a specific parasite protein to sugars present on the outer surface of the host cell. One of the major challenges that the malaria parasite faces within a liver cell is the process of autophagy, which specifically targets the pathogen for elimination. While indeed a lot of the parasites are killed, the majority is able to escape this destruction mechanism. Moreover, it appears that the malaria parasite uses the autophagy pathway to obtain nutrients required for growth and development. Research has also shown that the *Plasmodium* parasite is able to form close interactions with liver cell organelles, intracellular components that the cell uses to maintain homeostasis. In fact, it seems that these connections with the host are necessary for the malaria parasite to survive, probably because they allow the pathogen to secure building blocks and nutrients. The liver stage of the parasite's infection cycle thus contains lots of possibilities for therapeutic intervention. By, for example, preventing the malaria parasite to invade liver cells or inhibiting evasion of autophagy, the parasite will not be able to survive the liver stage and cause disease. Further research into these mechanisms could lead to the development of vaccines and other drugs that will enable us to fully eliminate malaria from the human population.

Chapter 1: Introduction to malaria

With 219 million cases and 435,000 deaths worldwide in 2017, malaria is one of the most life-threatening infectious diseases in humans. It is mostly prevalent in (sub)tropical areas; Sub-Saharan Africa in particular accounts for more than 90% of the cases and deaths. Malaria is a vector-borne disease, caused by parasites of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito[1,2]. Only six *Plasmodium* species are able to infect humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallickeri*, *Plasmodium malariae* and *Plasmodium knowlesi*[1–3], with *P. falciparum* being the leading cause of malaria in young African children[1]. Up until now, the many attempts to reduce the amount of malaria cases have largely been unsuccessful. Bed nets and insecticides are not effective enough, and even though antimalarials are a common way to treat the disease, drug resistance of the parasite is a rising problem[4]. Additionally, the long-term co-evolution between *Plasmodium* and the human population has pressured the parasite to become highly adaptable to its host, making malaria very hard to eradicate[5,6]. However, a crucial step towards elimination was made recently, when the first malaria vaccine was officially approved by the World Health Organization (WHO)[7]. It has been shown that the RTS,S vaccine reduces the risk of contracting severe malaria up to 28% in children between the age of 5 and 17 months[7,8]. Although the vaccine is expected to have a significant effect on improving child survival, research into the parasite's infection cycle and establishing new therapeutic targets remains important to be able to fully eradicate malaria from the human population.

1.1 Life cycle of the malaria parasite and pathogenesis

The life cycle of the malaria parasite (Figure 1) starts when a female *Anopheles* mosquito injects infective sporozoites from its salivary gland into the human skin. The sporozoites migrate to the liver, where they infect hepatocytes and undergo the first round of asexual replication. The resulting multinucleated exo-erythrocytic schizont contains tens of thousands of merozoites, each able to infect a single red blood cell (RBC) upon their release into the bloodstream. Within the RBC, the second round of schizogony occurs, leading to the release of freshly developed merozoites that start a new intraerythrocytic cycle[3,9,10]. The sexual stage of the malaria life cycle begins when a small proportion of merozoites differentiates into female and male gametocytes, which remain in the blood circulation until another bite transfers them from the human host to the midgut of a mosquito[3,10]. The gametocytes use proteases to exit the RBCs and fuse to become a zygote[3,10,11], which converts into an ookinete that is able to penetrate the wall of the mosquito's midgut and develop into an oocyst[3,10]. Following oocyte rupture, the through sporogony produced sporozoites migrate to the salivary gland, allowing them to be transmitted to a new human host, where a new *Plasmodium* life cycle begins[1,3,10].

Out of the six *Plasmodium* species that are able to infect humans, *P. falciparum* causes the deadliest form of malaria[1,10]. The most detrimental symptoms of the disease arise during the blood stage. Sequestration of infected erythrocytes to host tissues via various endothelial receptors can cause obstruction in the microvasculature, leading to severe complications such as cerebral malaria[9,12–14]. Despite the rapid expansion the malaria parasite undergoes during the liver stage, developing enormous amounts of merozoites from only a few sporozoites, this phase is clinically silent[3,10,15]. Inhibition of successful liver stage development will therefore prevent onset of disease, making this part of the parasite's infection cycle a very interesting target for vaccination.

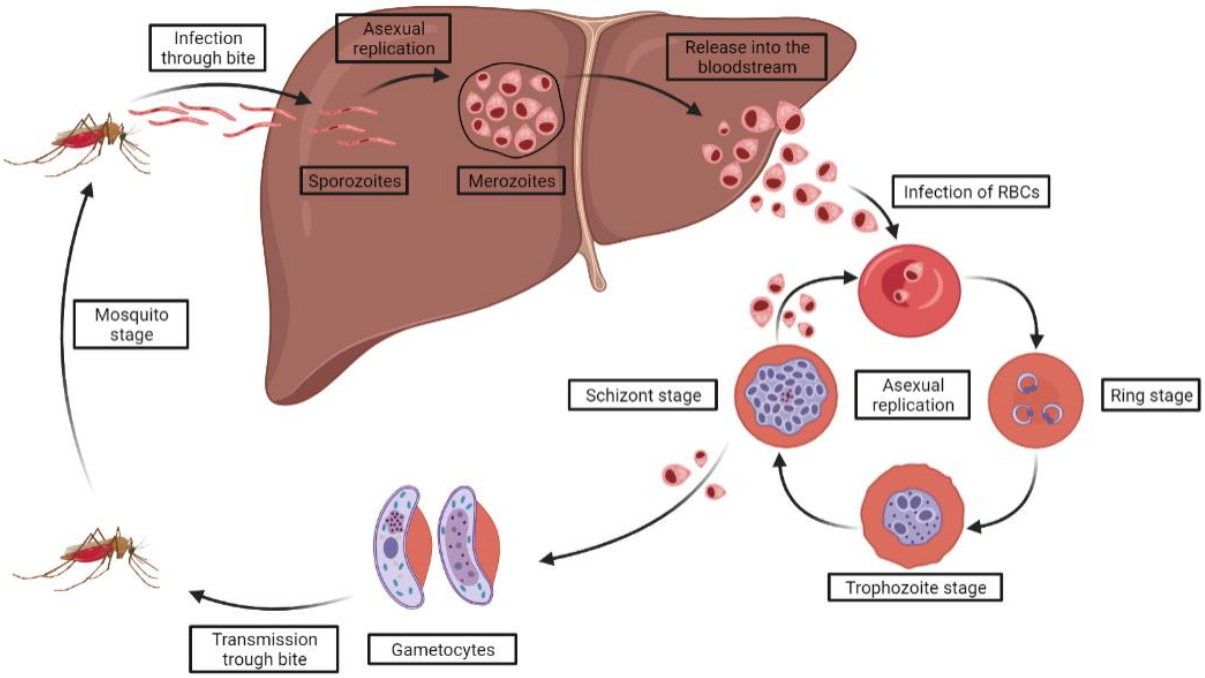

Figure 1: Life cycle of the malaria parasite. The infection cycle starts when a female Anopheles mosquito injects infective sporozoites into the human skin. The sporozoites migrate to the liver, where they invade hepatocytes and undergo the first round of asexual replication, resulting in the production of thousands of merozoites. Following their release into the bloodstream, each merozoite is able to infect a single RBC. Here, the second round of schizogony occurs, and the parasite sequentially passes through the ring, trophozoite and schizont stage. The freshly developed merozoites start a new intraerythrocytic cycle. The sexual stage of the malaria life cycle begins when a small proportion of merozoites differentiates into gametocytes, which remain in the blood circulation until another bite transfers them from the human host to a mosquito. Within the mosquito, the gametocytes fuse to become a zygote, which eventually develops into an oocyst. Following oocyte rupture, the through sporogony produced sporozoites migrate to the salivary gland, allowing them to be transmitted to a new human host, where a new Plasmodium life cycle begins. [Generated with BioRender].

1.2 The formation and composition of the parasitophorous vacuole

To identify potential therapeutic targets, it is important to elucidate the interplay between the malaria parasite and its host cell. The invasion of hepatocytes by *Plasmodium* sporozoites is accompanied with invagination of the host cell membrane, eventually pinching off and creating a parasitophorous vacuole (PV) that functions as an intrahepatocytic niche for the parasite[16,17]. The majority of the interactions between the parasite and hepatocyte will therefore be mediated by the parasitophorous vacuole membrane (PVM).

Two important factors involved in PV formation are the sporozoite 6-cysteine proteins p36 and p52, indicated by the observation that the small proportion of *p36/p52*-deficient parasites that successfully infected liver cells *in vitro*, failed to develop a PV[18]. Interestingly, a PV was still present following hepatocyte infection with single mutant sporozoites[19], suggesting that p36 and p52 can compensate for each other's absence. The first parasite protein identified to localize to the PVM is exported protein 1 (EXP1)[20]. The interaction between the cytosol-facing C-terminal domain of EXP1 and the host plasma protein apolipoprotein H (apoH) appears to be important for parasite survival during the liver stage[21]. As apoH has been shown to be involved in the clearance of liposomes from the cytoplasm[22], the hypothesis is that it mediates the transfer of cholesterol and other lipids towards the parasite through a direct association with EXP1[21]. Two other important intramembrane PVM proteins are upregulated in infective sporozoites 3 (UIS3)[23] and 4 (UIS4)[24]. While *uis3*$^{-}$ mutant *Plasmodium* sporozoites were able to invade hepatocytes *in vitro*, they lacked the capability to develop into mature schizonts[23]. Moreover,

rats intravenously injected with *uis3*$^{-}$ mutants did not show any signs of blood stage parasitemia[23], suggesting that the presence of UIS3 in the PVM is essential for *Plasmodium* to complete the liver stage. Likewise, late liver stage parasites were absent in mice injected with *uis4*$^{-}$ sporozoites[24], showing that UIS4 is as important to successfully produce merozoites and proceed to the intraerythrocytic cycle. It is worth mentioning that on top of the ones listed here above, many more proteins are located within the parasite's PVM, several of them regulating parasite survival through interaction or colocalization with host cell components[16,25,26].

1.3 *Plasmodium* species use host factors to successfully survive the liver stage

To be able to infect and successfully develop within hepatocytes, *Plasmodium* species take advantage of numerous host factors. Both the first attachment of *Plasmodium* sporozoites to liver cells as well as the subsequent invasion is mediated by host cell surface receptors[27–29]. One of the major challenges that the malaria parasite faces within the hepatocyte is the process of autophagy, and it appears that the ability of *Plasmodium* to evade this defense system is dependent on the canonical interaction of UIS3 with the host microtubule-associated protein 1 light chain 3 (LC3)[25]. But malaria parasites do not just interact with host proteins, they are capable of hijacking entire host cell organelles, such as the endoplasmic reticulum (ER)[30], the Golgi network[26] and host mitochrondria[31], in order to confiscate their required building blocks and nutrients.

This thesis focusses on the various host factors that are utilized by the malaria parasite to complete liver stage development and gives insight into how these interactions enable therapeutic intervention. It should be kept in mind that a lot of research regarding malaria is carried out using rodent *Plasmodium* species, such as *P. berghei* and *P. yoelii*. Fortunately, the life cycles of the human and rodent malaria parasites are comparibale[32], which may be explained by the 85% gene overlap between the different *Plasmodium* species[33]. While it cannot be excluded that potential therapeutic targets established in rodent *Plasmodium* species fail to translate to malaria infections in humans, this shows that malaria parasites infecting rodents are amongst the most representative models for human malaria parasites.

Chapter 2: Hepatocyte invasion by *Plasmodium* sporozoites

2.1 The transition from a motile to an invading sporozoite

Following injection into the human skin and successful passage through the blood circulation, *Plasmodium* sporozoites reach the liver sinusoidal vein[10,34]. Here, the parasites cross the sinusoidal wall via a mechanism called cell traversal, which is characterized by disruption of the host cell membrane and leads to free sporozoites in the cytoplasm[17]. The cell traversal ability of *Plasmodium* sporozoites requires the presence of two sporozoite microneme proteins essential for cell traversal: SPECT1 and SPECT2[35,36], the latter carrying a membrane attack complex/perforin-related domain[36]. Other proteins containing this highly conserved motif include the human complement component C9 and human perforin[36], strongly suggesting that malarial SPECT2 forms pores in the host cell membrane, allowing the sporozoites to cross.

After reaching the host's liver cells, *Plasmodium* sporozoites first pass through several hepatocytes before invading one[17]. In contrast to cell traversal, the process of invasion is not accompanied with membrane damage and the sporozoite subsequently develops intracellularly inside a PV[17]. The direct interaction between the sporozoite and hepatocyte is mediated by the binding of the circumsporozoite surface protein (CSP), the major surface protein of the parasite, to heparan sulfate proteoglycans (HSPGs) on the host cell surface[27]. It has previously been demonstrated that the sulfation level of the glycosaminoglycan (GAG) chains within HSPGs regulates the transition from a motile to an invading sporozoite[28]. Fibroblasts and endothelial cells contain undersulfated HSPGs, enabling sporozoites to traverse these cells, but preventing them from invading[28]. Upon encountering HSPGs with highly sulfated GAG chains on liver cells, CSP is crosslinked via its NH_2-domain, triggering the activation of an intracellular calcium-dependent protein kinase-6-dependent signaling pathway[28,37]. The consequent release of a cysteine protease leads to cleavage of CSP, thereby revealing a thrombospondin type 1 repeat domain that binds with high affinity to the sulfated HSPGs, initiating a signaling cascade inside the hepatocyte that enables the sporozoites to invade[28,37].

2.2 Different *Plasmodium* species use alternative host cell entry pathways

2.2.1 CD81 and scavenger receptor class B type I

Two important host proteins involved in regulating *Plasmodium* sporozoite invasion are CD81 and scavenger receptor class B type I (SR-BI). CD81 is a member of the tetraspenin protein family, which consists of integral membrane proteins involved in various cell mechanisms such as migration and adhesion[38]. Tetraspenins are able to interact with each other, resulting in the formation of tetraspenin-enriched microdomains in the plasma membrane[38,39]. The localization of CD81 to such domains is regulated by cholesterol and seems to be required for sporozoite entry[39]. Up until now, no ligand has been identified for CD81[40,41], fueling the hypothesis that instead of a direct interaction with the parasite, CD81 associates with a yet unknown sporozoite receptor inside the cholesterol-dependent microdomains[39,41]. SR-BI on the other hand functions as a high-density lipoprotein (HDL) receptor[42], mediating the transfer of lipids from HDL to the intracellular environment, and in addition, is able to bind low-density lipoprotein (LDL), including its modified isotypes, such as oxidated and acetylated LDL[43]. Considering, it is not surprising that SR-BI is mainly expressed in tissues that are involved in lipid metabolism, such as the liver[42].

Interestingly, the different *Plasmodium* species vary in their dependence on CD81 and SR-BI to successfully invade hepatocytes (Figure 2). Both *P. falciparum* and *P. yoelii* enter liver cells via a CD81-dependent pathway, indicated by the observation that monoclonal antibodies (mAbs) targeted against the extracellular domain of CD81 inhibit the development of exo-erythrocytic forms (EEFs) within primary human hepatocytes and Hepa1-6 cells, respectively[40]. In contrast, anti-SR-BI antibodies were not able to prevent hepatocyte invasion by *P. falciparum* and *P. yoelii*[41,44], suggesting that SR-BI does not contribute to host cell entry by these two *Plasmodium* species. However, as BLT-1, a blocker of lipid transfer, has been shown to inhibit infection of primary human hepatocytes by *P. falciparum* sporozoites[45], it cannot be fully excluded that SR-BI is required for *P. falciparum* invasion. Different from *P. falciparum* and *P. yoelli*, host cell entry by *P. vivax* seems to be independent of CD81 and is mediated by SR-BI[41]. In the case of *P. berghei*, CD81 and SR-BI appear to be redundant. Even though *P. berghei* sporozoites were capable of causing blood stage parasitemia in *CD81*$^{-/-}$ mice[40], the CD81 mAb MT81 has been shown to block liver cell infection *ex vivo*[46], indicating that this rodent *Plasmodium* species can invade hepatocytes both via a CD81-dependent and an CD81-independent pathway. Moreover, where polyclonal anti-SR-BI antibodies were able to decrease the number of *P. berghei* EEF-infected HepG2 cells, this was not the case anymore when these cells were genetically modified to express CD81[41]. Indeed, only simultaneous blockage of SR-BI and CD81 on HepG2/CD81 cells reduced infection[41], strongly suggesting that *P. berghei* can use either SR-BI or CD81 to invade liver cells. SR-BI also appears to support the intracellular development of *P. berghei* sporozoites[45]. Because SR-BI acts as a receptor for lipoproteins[42,43], and has been shown to be expressed in the proximal periphery of the PV[45], it is not unlikely that SR-BI is involved in the transfer of indispensable cholesterol and other lipids towards the intracellular parasite.

Additionally, p36 and p52 have been identified to be involved in hepatocyte invasion, but it appears that only p36 defines which host cell entry route is utilized by the parasite[41]. Where p36 from *P. yoelii* solely mediates sporozoite entry via the CD81-dependent pathway, Pbp36 supports invasion via both CD81 and SR-BI[41].

2.2.2 Aquaporin-9

Another receptor mediating *P. falciparum* invasion of liver cells is aquaporin-9 (AQP9)[29]. Aquaporins are a family of transmembrane channels, acting as transporters for water, glycerol and other solutes, thereby influencing cell motility, morphology and volume[47,48]. Although AQP9 and CD81 are both required for host cell invasion by *P. falciparum*, they seem to work independently, as small interfering RNAs (siRNAs) targeting either AQP9 or CD81 resulted in reduced sporozoite entry into primary human hepatocytes[29]. The rodent malaria parasites *P. yoelii* and *P. berghei* rely differently on the presence of AQP9. Where RNA silencing of AQP9 significantly impaired hepatocyte invasion by *P. berghei ex vivo*, infection by *P. yoelii* was not altered[29]. However, when *AQP9*$^{-/-}$ mice were injected with *P. yoelii* sporozoites, EEF development was partly inhibited, although to a lesser degree than seen using *P. berghei*[29]. These data indicate that *P. yoelii* is, to some extent, dependent on AQP9 for hepatocyte invasion, and imply that *P. berghei* requires AQP9 for efficient host cell entry.

2.2.3 Ephrin receptor A2

As Eph receptors and their ligands are known to regulate cell-cell contact, Ephrin receptor A2 (EphA2) was considered a potential candidate to mediate host cell entry by *Plasmodium* sporozoites[49,50]. However, there are some contradictory statements regarding the contribution of this receptor to

hepatocyte invasion. Kaushansky *et al.*[50] demonstrated that treatment of Hepa1-6 cells with D4A2, a mAb targeting the extracellular domain of EphA2, results in a decreased level of infection by *P. yoelii* relative to treatment with an IgG control antibody. Furthermore, *Eph*$^{-/-}$ mice were significantly less susceptible to *P. yoelii* infection in comparison to wild type (WT) mice[50], suggesting that EphA2 mediates hepatocyte invasion. This hypothesis was counteracted by Langlois *et al.*[51], who showed that RNA silencing of EphA2 does not impair the development of *P. yoelii* EEFs in Hepa1-6 cells. Besides, reduced expression of EphA2 on HepG2 cells had no effect on the level of *P. berghei* invasion, indicating that EphA2 is not involved in regulating host cell entry via an CD81-independent pathway[51]. The authors claim that the observed effect of D4A2 by Kaushansky *et al.* was due to nonspecific events of glycerol and/or sodium azide present in the antibody formulation[51]. To confirm their hypothesis, *Langlois et al.* compared the ability of D4A2 to hamper *P. yoelii* EEF development in Hepa1-6 cells to that of a control IgG antibody that was prepared under the same conditions as D4A2, and detected a similar level of inhibition[51]. To further explore the role of EphA2 in host cell entry, it may be useful to look at the effects of other anti-EphA2 antibodies, such as 1C1, which is able to bind to the extracellular domain of EphA2 and induce internalization of this receptor[52], on hepatocyte invasion by *Plasmodium* species *in vitro* as well as *in vivo*.

2.3 Chapter overview

While the initial binding process of *Plasmodium* sporozoites to liver cells, mediated by the interaction between CSP and HSPGs, seems to be highly conserved, different *Plasmodium* species engage in alternative pathways to enter host cells (Figure 2). However, the wide variety by which CD81, SR-BI and AQP9 contribute to hepatocyte invasion makes them very interesting targets for host-directed therapy.

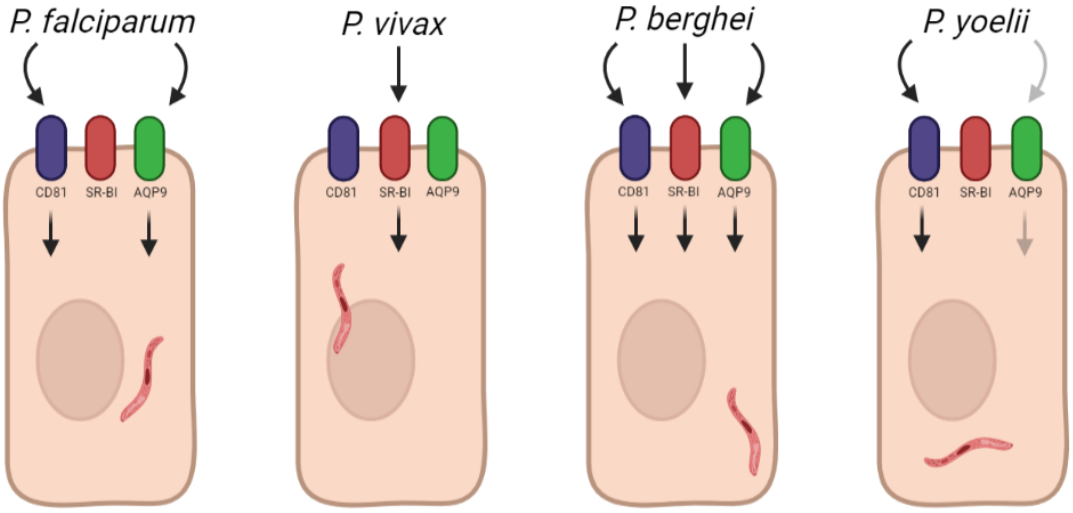

Figure 2: The contribution of CD81, SR-BI and AQP9 to host cell entry differs between Plasmodium species. P. falciparum requires both CD81 and AQP9 to successfully invade hepatocytes. It is thought that CD81 and AQP9 work independently, as they cannot compensate for each other's absence. Host cell entry by P. vivax is mediated by SR-BI, independent of CD81. In the case of P. berghei, CD81 and SR-BI serve redundant functions during hepatocyte invasion. Additionally, P. berghei appears to require APQ9 to enter hepatocytes. P. yoelii sporozoite invasion is mediated by CD81 and although to a lesser extent than seen in P. berghei, AQP9 also contributes to host cell entry. [Generated with BioRender].

Chapter 3: The malaria parasite can evade autophagy

3.1 Canonical and selective autophagy

As soon as a pathogen infects a host cell, it becomes exposed to several host innate immune defense mechanisms that try to detect and eliminate the pathogen. One of these mechanisms is autophagy[53]. Under nutrient deprived conditions, canonical autophagy mediates the turnover of cytoplasmic molecules and organelles to maintain cellular homeostasis[53,54]. Recyclable or undesirable proteins and organelles are engulfed by an isolation membrane, resulting in the formation of an autophagosome. Through fusion with lysosomes, the autophagosome matures into an autolysosome, which contents are degraded, thereby foreseeing the cell in its required nutrients[53,54].

While canonical autophagy is a non-specific destruction process, pathogens induce a selective form of autophagy that specifically targets the pathogen for elimination[53,54]. Although canonical and selective autophagy fundamentally differ in their initiation process, canonical autophagy is induced by kinases and selective autophagy is activated through cell surface receptors, they use the same core machinery[55]. Both canonical and selective autophagy are regulated by autophagy-related (ATG) proteins, involve the formation of membranes around either damaged organelles or intracellular pathogens[53,54], and are often characterized by a process called LC3-associated phagocytosis (LAP)[56,57]. LAP is initiated through the cleavage of the full-length precursor LC3 by the cysteine protease ATG4, generating LC3-I[58]. The newly exposed C-terminal glycine on LC3-I acts as a recognition site for the E1-like activating enzyme ATG7[59,60]. Activated LC3-I is transferred to the E2-like enzyme ATG3[59,61], which is able to conjugate LC3-I to phosphatidylethanolamine (PE) with the help of the ATG12-ATG5-ATG16L1 complex, thereby producing LC3-II[57,59]. Anchored lipidated LC3-II in the autophagosomal membrane is recognized by host lysosomes, resulting in the destruction of the captured cargo[57,62].

3.2 *Plasmodium* sporozoites induce a novel form of autophagy

Similar to other pathogens, *Plasmodium* sporozoites are labeled with host LC3 following their invasion into hepatocytes (Figure 3)[57,63]. It has been suggested that interferon-γ (IFN-γ) mediates this process, as IFN-γ treatment of the HC04 cell line resulted in increased colocalization of LC3 with *P. vivax*[64]. Interestingly, intracellular *P. berghei* sporozoites do not become surrounded by an autophagosomal membrane, and LC3 is directly incorporated into their PVM[57,63]. Ubiquitin and sequestosome 1, two markers of selective autophagy, have also been shown to associate with the PVM of *P. berghei* liver stage parasites[57]. However, as selective autophagy is characterized by the formation of an autophagosomal membrane[53,54,57], it appears that *Plasmodium* induces a novel form of autophagy.

3.3 Evasion of selective autophagy

The amount of colocalized LC3 seems to be dependent on the parasite's developmental stage[57,65]. Where LC3 was shown to be closely associated with *P. berghei* parasites during the sporozoite and early schizont stage, LC3-positive aggregates were much more distributed throughout the cytoplasm at the time of the mature schizont stage[57,65]. It appears that LC3 is progressively moved from the PVM into the tubulovesicular network (TVN), membranous structures that extend from the PVM into the periphery, and eventually buds off into the cytoplasm[65]. This suggests that the malaria parasite is able to clear its PVM from autophagy-mediating proteins throughout liver stage development, thereby preventing further events that could lead to degradation. Indeed, *P. berghei* schizonts that remained in close

contact with LC3 were smaller in comparison to the parasites that were cleared from LC3, supporting that failing to remove LC3 from the PVM is accompanied with growth arrest and elimination of the parasite (schematically depicted in Figure 3)[57].

During early liver stage development of *P. berghei*, lysosomes fuse with the parasite's PVM, demonstrated by the colocalization of the lysosomal marker lysosomal-associated membrane protein-1 (LAMP-1) with UIS4[57,66]. The majority of the successfully developed *P. berghei* liver stage parasites progressively cleared LAMP-1 from their PVM, and their PV remained free from the lysosomal protease cathepsin D (CTSD) and acidification[57]. Similar to what was observed in the case of LC3, the PVM of arrested liver stage parasites stained positive for LAMP-1 as well as CTSD[57]. In addition, the PV of these parasites showed clear signs of acidification, suggesting that they were being eliminated[57]. Taken together, this indicates that although some *Plasmodium* parasites are phagocytosed upon their invasion into hepatocytes, the majority is able to escape this innate immune defense mechanism. What exactly determines the fate of a parasite is not completely known, but it is possible that the surviving parasites fuse with milder endocytic vesicles, as amphisomes can be detected in close proximity to the intracellular parasite as well[63,66]. It has also been proposed that some parasites are able to neutralize the acidification of their PV with the presence of a pH buffer, thereby preventing destruction[66].

The ability of a malaria parasite to escape autophagy seems to depend on the PVM protein UIS3. Infection of HepG2 cells with *uis3*$^{-/-}$ *P. berghei* parasites significantly decreased the level of EEF development in comparison to infection with *uis3*$^{+/+}$ control parasites[25]. However, autophagy arrest induced via RNA silencing of either ATG5 or Rab7 restored the infectivity of *uis3*$^{-/-}$ mutants *in vitro* and *in vivo*[25], suggesting that UIS3 mediates autophagy resistance. Through co-immunoprecipitation experiments, it became apparent that LC3 is able to form a canonical interaction with the C-terminal domain of UIS3[25]. Under nutrient deprived conditions, LC3 binds to autophagy-mediating proteins, such as p62 and Rab7, via its LC3-interacting region (LIR)-binding surface, thereby enabling autophagic flux[25,67,68]. Although UIS3 does not contain the LIR motif[25], the LIR-binding surface of LC3 is still involved in the interaction with UIS3, arguing that through competition with canonical LC3 ligands, UIS3 can abolish the autophagic process and thereby prevent parasite removal[25].

3.4 The autophagy pathway serves as a nutrient source

While the lysosomal components of the autophagy pathway are responsible for degradation of unwanted intracellular pathogens, they also function as a great source for metabolites and seem to support parasite development. Nutrient starvation in mice, thereby inducing the canonical autophagy pathway, resulted in an increased *P. berghei* parasite load and size relative to the control group[57]. Furthermore, sequestration of cholesterol in late endosomes impaired the growth of *P. berghei* in human hepatoma Huh7 cells, while subsequent cholesterol release rescued their development[69]. These data propose that *Plasmodium* parasites are able to confiscate the late endocytic pathway, rich in cholesterol and other lipids, and use it as a nutrient source to stimulate their maturation during the liver phase.

3.5 Chapter overview

Following their invasion into hepatocytes, *Plasmodium* sporozoites induce a novel form of autophagy that specifically targets the parasite for elimination. While some parasites are indeed destroyed, the majority has the ability to escape this innate immune defense mechanism (Figure 3). However, what exactly determines the fate of a parasite remains elusive. The malaria parasite also seems to be able to hijack the canonical autophagy pathway, using it as a nutrient source to stimulate its own growth and development during the liver stage.

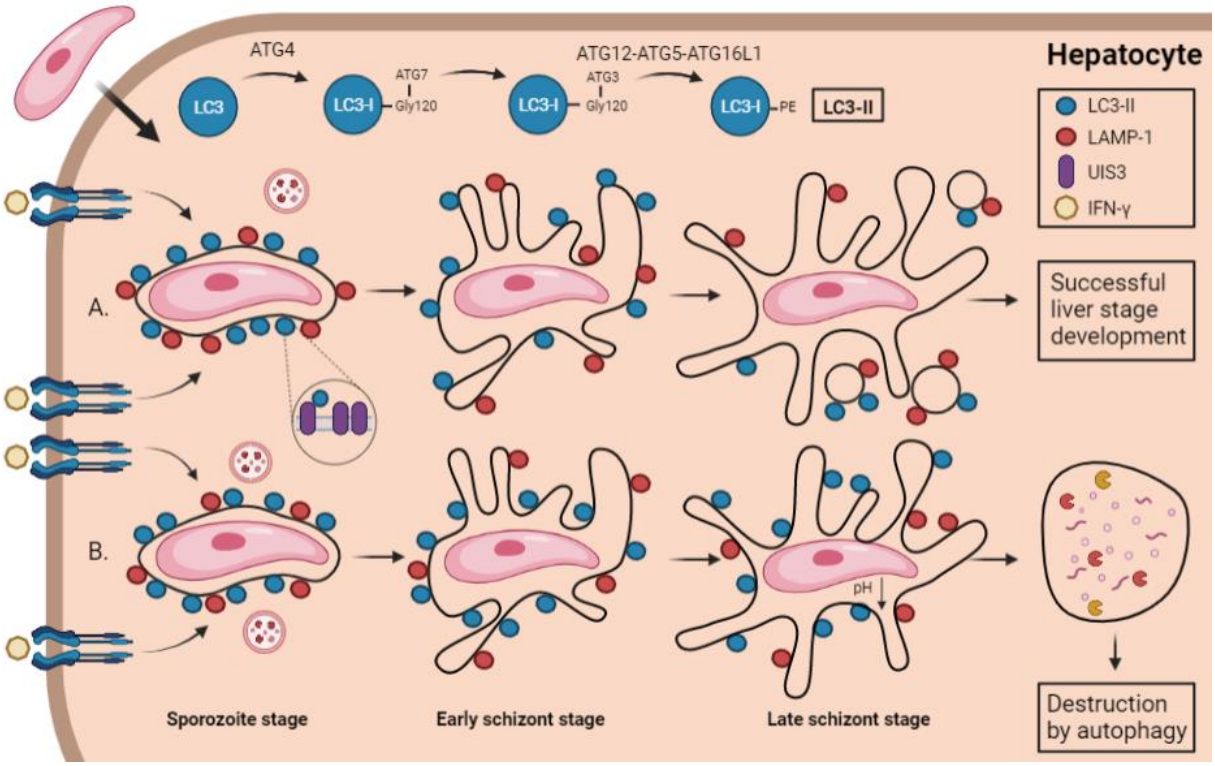

Figure 3: An overview of the major mechanisms involved in targeting Plasmodium parasites for elimination and their strategy to evade autophagy. Following invasion into hepatocytes, Plasmodium sporozoites become labeled with the lysosomal protein LAMP-1 and host LC3-II, a process that is stimulated by IFN-γ. LC3-II is produced from the full-length precursor LC3 and its canonical interaction with the C-terminal domain of UIS3 seems to be required for a parasite's ability to evade autophagy. Where successfully developed liver stage parasites (A) are able to clear LC3 and LAMP-1 from their PVM, these proteins remain closely associated with arrested parasites (B). It appears that by progressively moving autophagy-mediating proteins from its PVM towards the TVN, where they eventually bud off into the host cytoplasm, the parasite is able to protect itself from destruction by autophagy. In parasites that fail to clear LC3 and LAMP-1 from their PVM during liver stage development, autophagic flux is induced, resulting in acidification of the cytoplasm and degradation of proteins. [Generated with BioRender].

Chapter 4: *Plasmodium* parasites hijack host cell organelles

4.1 The interplay between *Plasmodium* and host cell organelles

After entrance into the hepatocyte, *Plasmodium* parasites make use of the host microtubule network to move into the center of the cell, where the majority of the mammalian organelles, such as the ER and Golgi apparatus, are also localized[30,32]. Research has shown that the PV is larger in diameter when the parasite is located close to the nucleus rather than distant from it[30], suggesting that a juxtanuclear position is more ideal. These observations led to further investigation of the possible interactions between *Plasmodium* and host cell organelles, which could be beneficial for the parasite to complete the liver stage.

4.1.1 Endoplasmic reticulum

Liver cells are highly involved in lipid production and to coordinate this, they contain sufficient amounts of both smooth and rough ER[70]. Using fluorescence microscopy, it could be observed that the ER of *P. berghei*-infected cells accumulates around the parasite's PVM[30]. On a critical note, this data was obtained in human foreskin fibroblasts and not in a hepatocyte cell line. As *Plasmodium* species show an exponential growth rate during liver stage schizogony, colocalization with the host ER could provide the parasite with the necessary lipids to expand the PVM. The ER is also the major working machine behind the production of new proteins[71], including major histocompatibility complexes (MHCs) that are important for pathogen elimination. It is therefore not unlikely that the association with the ER is used by the parasite to modulate the host cell immune response[30].

The ability of the ER to respond to metabolic requirements is important to maintain cellular homeostasis. Conditions that challenge ER function, such as the aggregation of unfolded proteins, initiate the unfolded protein response (UPR), which involves the X-box binding protein 1 (XBP1)-mediated activation of a signaling pathway that eventually restores the protein folding capacity of the ER, and regulation of the liver metabolic pathway via the hepatocyte specific cAMP responsible element-binding protein (CREBH)[72]. Upon the infection of Hepa1-6 cells with *P. berghei* sporozoites, the transcriptional regulation of several ER stress factors, including Atf3, Atf6 and Trib3, was remarkably increased[73]. Besides, various ER proteins involved in correct protein folding and ER-associated protein degradation were upregulated[73], indicating that *Plasmodium* parasites activate the UPR in response to ER stress. It has been shown that tunicamycin, an inducer of ER stress, increases the number of *P. berghei* EEFs 24h after the start of hepatocyte infection *in vivo*[73], raising the idea that UPR activation supports liver stage development of *Plasmodium*. Disruption of the *Xbp1* gene in *P. berghei* indeed resulted in a decreased amount of EEFs in the mouse liver[73]. As XPB1 is involved in gene expression regulation of proteins mediating fatty acid synthesis[74], the malaria parasite most likely depends on XBP1 to confiscate its required lipids. Likewise, RNA silencing of CREBH resulted in reduced parasite load in primary mouse hepatocytes in comparison to the WT group[73]. Taken together, it seems that the induction of ER stress via both the XBP1 and CREBH pathway supports parasite survival, suggesting that targeting the ER with the purpose to resolve ER stress could alleviate malaria liver stage infection.

4.1.2 Golgi apparatus

The Golgi apparatus consists of membrane enclosed disc-like structures, called cisternae, and plays an important role in the host secretory pathway. Newly synthesized proteins in the ER are received at the

cis-Golgi network, become further processed, e.g. through glycosylation, in the cisternae and are finally sorted by the *trans*-Golgi network for either secretion or intracellular localization[75]. For some time it was thought that the ER was the only host cell organelle to form a close association with the *Plasmodium* parasite[30]. However, de Niz et al.[26] showed that already 2h after infection of HeLa cells or primary mouse hepatocytes with *P. berghei*, an interaction between the parasite and the *cis*- and *trans*-Golgi could be detected[26]. This colocalization remained and became even more close throughout liver stage development[26]. Where shortly after infection, there were still parts of the Golgi network that were associated with the TVN or not at all in contact with the parasite, nearly the whole host cell Golgi was localized at the parasite's PVM 48h post infection (pi)[26]. The interaction between *Plasmodium* parasites and the Golgi complex seems to induce Golgi fragmentation, as 50% of the *P. berghei*-infected HeLa cells displayed sufficient amounts of G-elements 2-12h after the start of infection, only increasing with time[26]. Moreover, the proportion of infected cells containing Golgi fragments directly correlated with the proximity of the Golgi network to the PVM of the parasite[26].

Strikingly, *P. berghei* parasites lacking contact with the host cell Golgi showed morphological signs of death[26], such as loss of membrane integrity and fragmentation of the parasite, indicating that interacting with the Golgi network helps the malaria parasite to successfully survive the liver stage. Hampering vascular trafficking through the expression of double negative Arf and Rab1a mutants, two GTPases involved in ER-Golgi transport[76,77], has been shown to impair *P. berghei* EEF development in HeLa cells[26]. Although to a lesser extent, infection of HeLa cells expressing the Rab11a double mutant also resulted in growth delay and decreased parasite numbers[26]. However, these cells did not display an increase in the amount of G-elements upon infection with *P. berghei*, arguing that this GTPase is required for the parasite to induce Golgi fragmentation[26]. Altogether, this shows that *Plasmodium* most likely uses its colocalization with the host cell Golgi to scavenge for building blocks and nutrients.

4.1.3 Mitochondria

Like in many other organisms, key metabolic functions of the *Plasmodium* parasite, including energy metabolism and amino acid degradation, depend on enzyme complexes that require lipoic acid (LA) as a covelently bound co-factor[31]. Identified *P. berghei* enzymes involved in the catalyzation of lipoylation are LipA, LipB, lipoic acid protein ligase A1 (LplA1) and LplA2[31]. As lipoylated proteins can be detected in the parasite's mitochondrion as well as the apicoplast[31], another organelle of endosymbiotic origin, these enzymes are most likely located here. In fact, PbLipA and PbLipB can be found in the apicoplast[78], PbLplA1 only in the mitochondrion[78] and PbLplA2 in both these organelles (Figure 4)[79]. Whereas lipoylation of proteins in the apicoplast is dependent on *de novo* LA synthesis, the mitochondrion needs to scavenge LA from the host[78,80]. The compound 8-bromo-octanoic acid (8-BOA) can serve as a substrate ligand for LplA1, thereby specifically inhibiting the lipoylation of mitochondrial target proteins (schematically depicted in Figure 4)[31,80]. 48hpi, the *P. berghei* parasites in 8-BOA-treated HepG2 cells were significantly smaller in comparison to the parasites in the control cells, indicating that lipoylation of proteins is essential for the parasite to survive[31]. This was confirmed by the observation that 8-BOA treatment prevented the majority of the *P. berghei* parasites to complete liver stage development[31]. The addition of exogenous LA rescued the inhibitory effect of 8-BOA on parasite growth in HepG2 cells[31], suggesting that 8-BOA impairs liver stage development by blocking mitochondrial LA scavenging. It is important to note that the effect of 8-BOA was mainly visible when applied 24-48hpi and dispensable earlier or later during the liver stage[31]. At the time of schizogony, the parasite grows at an extraordinary

rate[10,31], requiring enormous amounts of nutrients. It is therefore not surprising that this stage is the most sensitive to the inhibition of enzymes dependent on LA, which are involved in DNA and protein synthesis. Although not as extensively as observed in the case of the apicomplexan *Toxoplasma gondii*[30], host cell mitochondria are closely associated with the PVM and mitochondrion of malaria parasite[30,31], implying that they are the source of *Plasmodium*-scavenged LA.

4.2 Chapter overview

Besides interacting with single host receptors or intracellular proteins, the *Plasmodium* parasite is thus also able to hijack entire host cell organelles. Colocalization with the host ER, Golgi apparatus and mitochondria seems to provide the parasite with required lipids and proteins, thereby promoting its survival. While this makes these host cell organelles interesting drug targets to treat malaria, more research is still needed on their role in *Plasmodium* parasite growth and development.

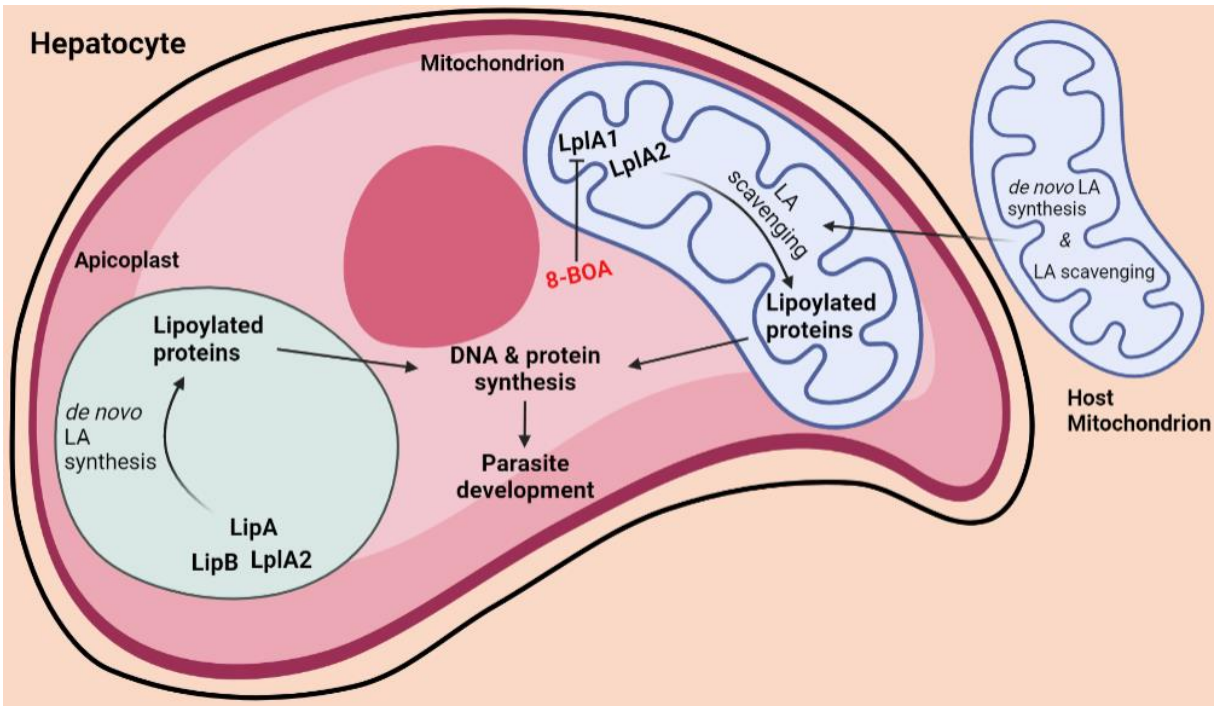

Figure 4: Malaria parasites depend on LA scavenging for successful liver stage development. Key metabolic functions of the Plasmodium parasite depend on enzyme complexes that require LA as a co-factor. Proteins involved in the catalyzation of lipoylation, including LipA, LipB, LplA1 and LplA2, are located in the parasite's mitochondrion, apicoplast or in both these organelles. Lipoylation of proteins in the apicoplast is dependent on de novo LA synthesis, whereas the mitochondrion needs to scavenge LA from the host. Lipoylated proteins are mainly involved in DNA and protein synthesis, which is required for parasite development. Plasmodium-scavenged LA most likely comes from host cell mitochondria, which are closely associated with the PVM and mitochondrion of the parasite. The chemical compound 8-BOA can serve as a substrate ligand for LplA1, thereby specifically inhibiting the lipoylation of mitochondrial target proteins and hampering the malaria parasite to complete the liver stage. [Generated with BioRender].

Chapter 5: Metabolite and lipid acquisition by *Plasmodium* parasites

5.1 *Plasmodium* parasites depend on their host to obtain required metabolites and lipids

During the liver phase of their infection cycle, malaria parasites expand at an exponential rate, producing thousands of merozoites from a couple of dozen sporozoites[3,10]. To successfully complete the liver stage, the parasite therefore requires tremendous amounts of building blocks. Besides containing several *de novo* synthesis pathways[78,81], *Plasmodium* depends on the supply of metabolites and nutrients by their host cell to meet these high demands.

5.1.1 Biotin

Biotin, also called vitamin B7, is an essential metabolite, acting as a CO_2 carrier in (de)carboxylation reactions[82]. The only protein known to be biotinylated in the malaria parasite is Acetyl-CoA carboxylase (ACC)[83,84], which is located in the apicoplast[83] and important for the production of fatty acids[85]. While ACC expression in the apicoplast can be detected in both the liver and blood stage of *P. berghei* as well as *P. falciparum*, it is only biotinylated, and thereby activated, during the liver phase[83]. Holocarboxylase synthethase 1 (HCS1) and 2 (HSC2) are the two biotin ligases responsible for this activation by transferring biotin to ACC[83]. Infection of HepG2 cells with *P. berghei* containing a knock out construct of *HSC1* resulted in reduced growth of late liver stage parasites and a decreased production of merozoites relative to the control group[83]. Furthermore, loss of HSC1 led to depletion of biotinylated proteins during the liver phase and delayed the onset of blood stage infection *in vivo*[83]. Remarkably, hepatocytes infected with malaria parasites and grown in biotin depleted medium were shown to produce fewer merozoites compared to hepatocytes infected with the *HSC1* mutants and in the presence of biotin[83]. These data propose that *Plasmodium* species scavenge biotin from the host and that this is required for successful development during the late liver stage. Both the mammalian hepatocyte and malaria parasite cannot synthesize biotin *de novo*[84], implying that the parasite is dependent on the nutrient status of the host.

5.1.2 Phosphatidylcholine

Lipidome analysis comparing *P. berghei*-infected Huh7 cells to non-infected cells revealed an upregulation of the major membrane lipid phosphatidylcholine (PC) following infection, while other phospholipids, such as PE, phosphatidylserine and phosphatidylinositol were downregulated[86]. RNA silencing of enzymes involved in the *de novo* PC synthesis pathway of the host cell resulted in reduced *P. berghei* EEF numbers, not EEF size, in Huh7 cells[86]. As no decrease in parasite load was observed the first 6hpi[86], it appears that PC is only important for hepatocyte infection and not invasion by *Plasmodium* parasites. Even though the malaria parasite is able to produce PC itself[87], these data indicate that it also requires PC from the host cell for successful liver stage development. Confocal imaging revealed that host PC localizes to various membranous structures of the parasite, such as the PVM, its plasma membrane and the ER[86]. Moreover, primary mouse hepatocytes deficient for *de novo* PC synthesis harbored *P. berghei* parasites with a reduced amount of UIS4 in their PVM[86], a colocalization that is required for parasite development[24]. It has been proposed that PC could be important for the correct positioning of proteins in the PVM and through its association with the ER, is involved in the transport of indispensable proteins towards the parasite's plasma membrane[86].

5.1.3 Liver-fatty acid binding protein

The liver-fatty acid binding protein (L-FABP) family consists of proteins that are involved in the binding and transport of (un)saturated fatty acid chains, thereby influencing the concentration of cytosolic fatty acids[88,89]. Through co-immunoprecipitation experiments on cell extracts from yeast strains, a direct interaction could be detected between the C-terminal region of *P. yoelii*'s UIS3 and mouse L-FABP[90]. Similarly, ELISA assays revealed a linkage between UIS3 of *P. falciparum* and the human homologue of L-FABP[91]. To investigate the role of L-FABP during liver stage development of the malaria parasite, Huh7 cells were treated with L-FABP siRNA and using qPCR, the amount of 18S rRNA of *P. berghei* was measured. Reduced L-FABP expression decreased the amount of parasite rRNA up to 80%[90], suggesting that L-FABP is important for *Plasmodium* liver stage development. This was further verified with immunofluorescence microscopy, which showed that parasites in Huh7 cells with a decreased L-FABP concentration displayed signs of a fragmented PVM and significant reduction in growth[90]. The other way around, overexpression of L-FABP induced parasite infection[90]. There thus seems to be a direct correlation between the level of L-FABP expression and parasite liver stage development. Although the parasite contains a *de novo* fatty acid synthesis pathway in the apicoplast[81], this is probably not sufficient enough to support the enormous growth of the parasite during the liver stage, therefore also requiring host-derived lipids. It is thought that the delivery of host lipids to the intracellular parasite is regulated by the established L-FABP-UIS3 interaction[90].

However, nuclear magnetic resonance (NMR) spectroscopy did not reveal any kind of interaction between human L-FABP and the C-terminal domain of PfUIS3[92]. It was also shown that PfUIS3 is unable to bind lipids, probably explaining why no lipid transportation from human L-FABP to PfUIS3 could be detected[92]. The authors speculate that the previously observed interaction between L-FABP and UIS3 was due to loss of structural integrity of UIS3, exposing amino acids able to bind to L-FABP[92]. NMR spectroscopy has the advantage to retain structural conformation[92], more closely mimicking possible interactions occurring *in vivo*. Although this research suggests that it is more likely that L-FABP provides lipids to the PVM without associating with UIS3, it cannot be fully excluded that the *in vivo* environment is more suitable for an interaction between L-FABP and the PVM protein. To further explore the binding possibilities between L-FABP and UIS3 or other PVM proteins *in vivo*, the next step could be to perform co-immunoprecipitation of L-FABP on the cell lysates of primary mouse hepatocytes.

5.1.4 AQP3

Even more than AQP9, the expression of AQP3 is elevated in HepG2 cells following *P. berghei* infection[93]. The earliest localization of AQP3 into the PVM of *P. berghei* could be detected from 28hpi[93]. Similar observations were made in the case of *P. vivax* hypnozoites, the dormant stage of the parasite that can remain in the body for years and cause relapse infections[10,94], and mature schizonts[94]. As AQP3 regulates glycerol transport[47], it is thought to be involved in the delivery of glycerol to the intracellular parasite. Here, AQP3 is probably used to produce phospholipids, which are incorporated into the growing PVM[93]. The gold-based glycerol transport inhibitor auphen, which works on the active cysteine residues of aquaporins, indeed inhibited *P. berghei* and *P. vivax* schizont growth[93,94]. Interestingly, auphen was also observed to inhibit *P. vivax* hypnozoite development[94], indicating that dormant malaria parasites still retain a low level of metabolism. Altogether, it appears that AQP3 is essential for *Plasmodium* liver stage development, and more importantly, inhibition of this aquaglyceroporin by the chemical compound auphen impairs the growth and development of various *Plasmodium* species at different time points during the liver phase (Figure 5).

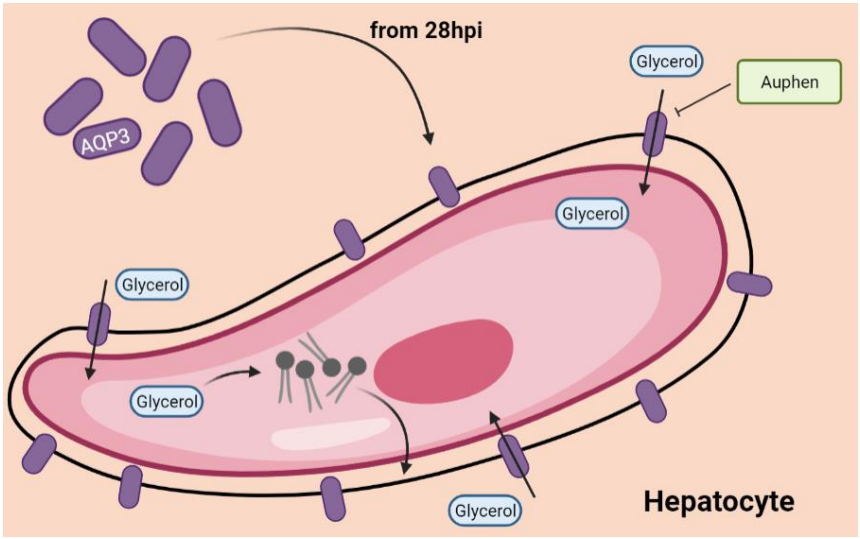

Figure 5: Plasmodium depends on host AQP3 for successful liver stage development. From 28phi, host AQP3 is incorporated into the PVM of the malaria parasite. It is thought that this aquaglyceroporin transports glycerol towards the intracellular parasite. Here, AQP3 is used to produce phospholipids, which are incorporated into the expanding PVM, allowing the parasite to grow. The chemical compound auphen acts on the cysteine residues of AQP3, thereby preventing glycerol transport and inhibiting parasite growth and development during the liver stage. [Generated with BioRender].

5.2 Chapter overview

The malaria parasite is thus partly dependent on the host cell to meet its high demands for metabolites and lipids during the liver phase. Not only is the nutritional status of the host important for parasite survival, host cell synthesis of PC and L-FABP is also essential for successful liver stage development. Like shown for AQP3 using auphen, targeting the transport routes of these molecules to the intracellular parasite could be a way to prevent or minimize the onset of blood stage infection by *Plasmodium*.

Chapter 6: Discussion

The enormous burden of malaria on the human population has put it amongst the top priorities of many health care programs and organizations. After years of research, the WHO recently approved the first malaria vaccine[7,8]. The RTS,S vaccine consists of a truncated form of *P. falciparum* CSP, including 19 of the NANP repeating units and the C-terminal domain that harbors various T-cell epitopes, fused to the primary surface antigen of the hepatitis B virus. The vaccine construct automatically assembles into virus-like particles, with CSP expressed on the outer surface, and is administered together with the AS01 adjuvant[95,96]. Although the RTS,S vaccine is capable of inducing high amount of antibody levels and sufficient T-cell responses[97–99], a phase III clinical trial showed that its efficacy is only partial and short-lived, even after multiple booster vaccinations[8]. While the current RTS,S/AS01 vaccine elicits a protective antibody response against the repeat domain, immunodominance of these NANP repeat units hinders the production of antibodies against other regions of the vaccine[100]. Recent research revealed that mice immunized with CSP9, a CSP construct containing only nine NANP repeat units and both the N- and C-terminal domain of CSP, produced significantly more antibodies against the N- and C-terminal domain of CSP in comparison to mice immunized with a nearly full-length CSP molecule[100]. More importantly, parasite burden in the mouse liver was significantly reduced following immunization with CSP9[100]. These data indicate that second generation RTS,S vaccines containing a minimal amount of NANP regions could elicit a broader antibody response and increase protection against malaria. Although the recently approved RTS,S vaccine is expected to have a significant effect on improving child survival, its limitations highlight the need for further research into other potential vaccine targets and possibilities for therapeutic intervention.

As the liver phase of the parasite's infection cycle is clinically silent[3,9], inhibiting the parasite to complete liver stage development will prevent onset of disease. The complex interplay between the parasite and hepatocyte that was reviewed here, revealed a lot of potential therapeutic targets. For example, the host cell surface receptors CD81, SR-BI and AQP9 are used by various *Plasmodium* species to invade liver cells[29,40,41], meaning that by blocking these proteins, hepatocyte infection and consequent blood stage parasitemia might be prevented. Indeed, mAbs targeting CD81 have already been shown to inhibit *P. falciparum* infection of primary human hepatocytes[40]. The advantage of host-directed therapy (HDT) over targeting the malaria parasite is that there is no selective pressure on the parasite to develop resistance[101]. On the other hand, one big limitation of HDT is the possibility of unwanted off-target effects[101]. For example, SR-BI is involved in the transfer of lipids towards the hepatocyte[42], meaning that blocking SR-BI could be detrimental for host cell homeostasis. Before mAb therapy against host cell surface receptors can be considered a potential malaria prophylactic, several other aspects need further investigation. It is still not exactly known how CD81, SR-BI and AQP9 mediate hepatocyte by *Plasmodium* sporozoites. In the case of CD81, the hypothesis is that it mediates host cell entry through an interaction with a yet unknown sporozoite receptor in the host cell membrane[39,41]. Identifying which proteins interact with these host cell receptors through co-immunoprecipitation experiments could therefore be a good next step towards the discovery of an effective antimalarial drug. Moreover, in the case of COVID-19, the costs of mAb therapy are 50-100 times higher than a two-dose vaccine[102]. Additionally, the absence of immunological memory following mAb administration might mean that the duration of protection is relatively short, especially for individuals living in highly endemic area's[102]. Together this asks for thorough evaluation of the cost-effectiveness of mAb therapy in comparison to other malaria prophylactics. It should also be kept in mind that, because of the various pathways that *Plasmodium*

species can utilize to infect liver cells, a combination therapy targeting multiple host cell surface receptors might be required to fully inhibit hepatocyte invasion by malaria sporozoites. It will therefore be relevant to assess whether it is safe and possible to administer multiple mAbs at once.

Because subunit vaccines appear to induce suboptimal protection, whole organism vaccines containing genetically attenuated parasites, also called GAP vaccines, are now under high investigation[15,103]. *p36*$^{-}$*/p52*$^{-}$*/sap1*$^{-}$ *P. falciparum* parasites were unable to complete liver stage development and seem to be fully attenuated[103], emphasizing the potential of this GAP vaccine to prevent disease. It has also been proposed that *uis3* deletion could significantly improve any attenuated whole-organism vaccine against malaria[25], as this protein is involved in various important parasite mechanisms, such as PVM formation[23] and autophagy resistance[25]. While GAP vaccines are thought to induce long-lasting protective immunity[104,105], there are also several limitations to keep in mind. One of the major disadvantages of attenuated vaccines is the chance of genetic reversion[106], which, in the case of *p36*$^{-}$*/p52*$^{-}$*/sap1*$^{-}$ parasites, could abolish liver stage arrest and lead to blood stage parasitemia. While adding additional genetic deletions, such as *uis3*$^{-}$, to the knock-out construct could be a way of minimizing the chance of obtaining a virulent pathogen, this should not result in reduced parasite viability. Moreover, assuming that sporozoites injected through a mosquito bit are as infective as sporozoites administered through a vaccination, it was estimated that around 10^4-10^5 sporozoites are needed for a GAP vaccine to cause protection[104,107]. While improvements have been made regarding the production of sporozoites in a mosquito[107], it should be evaluated whether it is feasible to efficiently generate this amount of parasites.

A lot of the current malaria therapeutics, including the RTS,S vaccine, target *P. falciparum*. Although *P. vivax* infections rarely have a fatal outcome, this *Plasmodium* species still causes a tremendous burden on the human population[1]. The development of effective therapeutics against *P. vivax* is heavily challenged by the dormant stage of the parasite, and to this date[108], only two drugs that target elimination of *P. vivax* hypnozoites are on the market[101]. Some potential lies in the small molecule C4, which works on the interaction between LC and UIS3 and is able to inhibit liver stage development of *Plasmodium in vitro*[109]. It is thought that C4 induces autophagic flux in the host liver cell by disrupting the interaction between LC3 and UIS3, resulting in destruction of the parasite[109]. As C4 did not affect intrinsic host cell autophagy[109], treatment with C4 could be accompanied with limited unwanted off-target effects. Another option would be to impair the supply of metabolites, such as biotin or glycerol, towards intracellular *Plasmodium* parasites. As the glycerol transport inhibitor auphen, which targets AQP3, is able to inhibit both *P. vivax* hypnozoite and schizont development[94], it is not unlikely that impaired metabolite transport also hinders latent malaria parasites.

Several studies have shown that *Plasmodium* parasites closely associate with liver cell organelles, such as the ER, Golgi apparatus and host mitochondria[26,30,31]. While it has been hypothesized that host cell mitochondria are the source of *Plasmodium*-scavenged LA[31], there is no direct evidence that supports this. Looking at the effect on liver stage development of the malaria parasite following inhibition of *de novo* LA synthesis in host cell mitochondria could provide further insights into the role of these host cell organelles in LA scavenging. The compound 8-BOA can function as a substrate ligand for LiplA1, thereby inhibiting LA scavenging by the malaria parasite[31,110]. Where 8-BOA was able to impede the growth and development of liver stage *P. berghei in vitro*, it did not abolish protein lipoylation in host cell mitochondria[31], highlighting its potential as a malaria therapeutic. As both *P. falcipar*um and *P. vivax* carry a LplA1 homologue[110], 8-BOA might be able to treat infection caused by either of these human

Plasmodium species. An important factor to take into account is that 8-BOA only appears to be effective between 28-48h after the parasite has invaded liver cells[31], meaning that the time of infection must be known for the treatment to be successful. Moreover, 8-BOA alone is not able to completely induce liver stage arrest[31]. Due to a different substrate specificity of LplA2 in comparison to LplA1[110], LplA2 might be insensitive to 8-BOA treatment. It is therefore not unlikely that a proportion of the parasites start to use LplA2 for mitochondrial LA scavenging and protein lipoylation, thereby compensating for the unavailability of LplA1 and increasing their chance at survival.

While already a lot of progress has been made over the years regarding the development of malaria vaccines and prophylactics, there is still a long way to go before the disease is fully under control. One of the main challenges in malaria vaccine development seems to be the low immunogenicity of the antigens that are under investigation, which was initially also the case for the RTS,S vaccine[95,111]. Moreover, widespread implementation of antimalarial drugs in developing countries is often hindered by poor infrastructure and economic instability[112]. It is also worth mentioning that research into human *Plasmodium* species requires a high level of safety and is only possible *in vitro* in primary human hepatocytes and *in vivo* in immunocompromised apes, making it very difficult to study their life cycle[32]. Specifically the liver phase of the parasite's infection cycle has been overlooked for a long time, even though the bottleneck in parasite load and clinical silence make this stage an ideal drug target. This thesis has highlighted that the interplay between the hepatocyte and malaria parasite allows for numerous ways of therapeutic intervention during the parasite's liver stage. Further research into these potential therapeutic targets would be a great next step towards the elimination of malaria from the human population.

Abbreviations

ACC	Acetyl-CoA carboxylase
apoH	apolipoprotein H
AQP	aquaporin
ATG	autophagy-related
BLT	blocker of lipid transfer
CREBH	cAMP responsible element-binding protein
CSP	circumsporozoite surface protein
CTSD	cathepsin D
EEF	exo-erythrocytic form
EphA2	ephrin receptor A2
ER	endoplasmic reticulum
EXP1	exported protein 1
GAG	glycosaminoglycan
GAP	genetically attenuated parasite
HDL	high-density lipoprotein
HDT	host-directed therapy
HSC	holocarboxylase synthethase
HSPG	heparan sulfate proteoglycan
IFN-γ	interferon-γ
LA	lipoic acid
LAMP-1	lysosomal-associated membrane protein-1
LAP	LC3-associated autophagy
LC3	microtubule-associated protein 1 light chain 3
LDL	low-density lipoprotein
L-FABP	liver-fatty acid binding protein
LIR	LC3-interacting region
Lpl	lipoic acid protein ligase
mAb	monoclonal antibody
MHC	major histocompatibility complex
NMR	nuclear magnetic resonance
PC	phosphatidylcholine
PE	phosphatidylethanolamine
P. falciparum	*Plasmodium falciparum*
pi	post infection
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
RBC	red blood cell
siRNA	small interfering RNA
SPECT	sporozoite microneme protein essential for cell traversal
SR-BI	scavenger receptor class B type I
TVN	tubulovesicular network
UIS	upregulated in infective sporozoites
UPR	unfolded protein response

WHO	World Health Organization
WT	wild type
XBP1	X-box binding protein 1
8-BOA	8-bromo-octanoic acid

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