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Plasmodium asexual growth and sexual development in the haematopoietic niche of the host

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Abstract

Plasmodium spp. parasites are the causative agents of malaria in humans and animals and they are exceptionally diverse in their morphology and life cycles. They grow and develop in a wide range of host environments, both within blood-feeding mosquitoes, their definitive hosts, and vertebrates, which are intermediate hosts. This diversity is testament to their exceptional adaptability and poses a major challenge for developing effective strategies to reduce disease burden and transmission. Following one asexual amplification cycle in the liver, parasites reach high burdens by rounds of asexual replication within red blood cells. A few of these blood-stage parasites make a developmental switch into the sexual stage (or gametocyte), which is essential for transmission. The bone marrow, in particular the haematopoietic niche (in rodents also the spleen), is a major site of parasite growth and sexual development. This Review focuses on our current understanding of blood-stage parasite development and vascular and tissue sequestration, which is responsible for disease symptoms and complications, and when involving the bone marrow, provides a niche for asexual replication and gametocyte development. Understanding these processes provides an opportunity for novel therapies and interventions.

[H1] Introduction

Malaria is one of the major life-threatening infectious diseases in humans and is particularly prevalent in tropical and subtropical low-income regions of the world. According to the World Health Organization (WHO), 219 million malaria cases and 435,000 deaths worldwide were reported in 2017, with >90% of both cases and deaths in sub-Saharan Africa[1]. The past decade has seen a drastic reduction in malaria cases and deaths worldwide, however this stunning progress has now been halted by widespread emergence of drug resistance in both parasite and vector species. Whereas *Plasmodium falciparum* dominates in sub-Saharan Africa,

Plasmodium vivax is responsible for most cases in many regions of Asia. At least 4 additional species can infect humans: *Plasmodium malariae*, *Plasmodium knowlesi, Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri.* Many other species of *Plasmodium* have been reported to cause malaria in vertebrates including non-human primates (for example, *Plasmodium cynomolgi* in macaques and *Plasmodium reichenowi* in chimpanzees), rodents (for example, *Plasmodium berghei* and *Plasmodium yoelli*), birds (for example, *Plasmodium gallinaceum*, *Plasmodium relictum* and *Plasmodium elongatum*) and reptiles (for example, *Plasmodium mexicanum*).

Malaria parasites have a complex life cycle marked by successive rounds of asexual replication across various stages and tissues, both in the intermediate vertebrate host and the definitive insect host. Sexual stages (gametocytes) are always formed in blood cells in the vertebrate host, whereas gametogenesis **[G]** and meiosis **[G]** require transmission to the insect host. In most *Plasmodium* species, the highest cell numbers are reached during asexual replication in circulating blood cells of the vertebrate host; a small fraction of those asexual parasites differentiates into sexual stages. In the past decade, renewed focus on sexual stages and transmission has unravelled pathways triggering their formation and unique cellular features. Moreover, a series of studies have shown parasite replication and sexual differentiation in the hematopoietic niche of the vertebrate, which adds an unexpected, new feature to the parasite life cycle. In this Review, we will discuss the biology of blood-stage malaria parasites, with a particular focus on recent breakthroughs in our understanding of the sexual stage and its development in the haematopoietic niche. We will put these findings in an evolutionary context and discuss new avenues for identifying drug targets and strategies to block transmission.

[H1] ***Plasmodium*** **life cycle**

The features of the malaria parasite life cycle are largely conserved across *Plasmodium* lineages that infect mammals (**Figure 1**). When an infected mosquito takes a blood meal from a vertebrate, it also injects sporozoites **[G]** into the skin. The motile sporozoite enters the blood stream, which enables it to reach the liver and thereby escape host immunity or drainage through the lymphatic system[2,3]. Once sporozoites have reached the liver sinusoids **[G]** , they cross the sinusoidal barrier and enter into hepatocytes[2], in which they establish a parasitophorous vacuole **[G]** and differentiate in a first round of asexual replication **[Au:OK?]**[4]. Over the course of two to several days (dependent on species) a multinucleated exo-erythrocytic schizont **[G]** (or meront) containing thousands of daughter merozoites **[G]** forms. Some parasite species such as *P. vivax* and *P. ovale* then can enter a period of latency by forming a non-replicating hypnozoite **[G]** instead of a schizont. These hypnozoites enable long-term survival of the parasite and can lead to relapses. Upon egress from the hepatocyte, merozoites are clustered in membrane-bound vesicles called merosomes and released back into the bloodstream via the liver sinusoids[5]. Merozoites invade red blood cells (RBCs) in which a second asexual schizogony takes place. This asexual replication cycle produces up to 32 merozoites over the course of 24-72 hours (both parameters vary between species). Through repeated rounds of invasion and growth the parasite establishes acute and eventually chronic infections. Some species, such as *P. vivax,* are restricted to reticulocytes **[G]**, which make up a small fraction of circulating RBCs, thereby limiting total parasitemia. Others such as *P. falciparum* are not restricted and may infect a high proportion of RBCs leading to high parasite burden, a factor implicated in the capacity of *P. falciparum* to cause severe disease.

The sexual cycle is initiated when a small proportion of asexual parasites commit to produce sexual progeny, that is, gametocytes. Mature gametocytes can circulate in the human blood for several days, which maximizes their chance of transmission to mosquitos. A few minutes after

entering the mosquito midgut, both male and female gametocytes use proteases to exit the RBCs and differentiate into 8 microgametes **[G]** and 1 macrogamete **[G]** , respectively[6], which fuse to produce the zygote **[G]**. The zygote transforms into a motile ookinete **[G]**, which crosses the epithelial layer of the midgut wall to form an oocyst **[G]**. In the oocyst, parasites undergo the third cycle of asexual replication to produce thousands of sporozoites that are released into the haemolymph **[G]**. Sporozoites that reach the salivary glands of the mosquito attach and invade the gland where they remain until transmitted to a new vertebrate host **[**through mosquito bite, to start the cycle again.

[H2] Gametocytogenesis

The rate of commitment to sexual development varies widely between species and is determined by a combination of genetic, epigenetic and environmental factors. Initial studies identified chromosomal deletions that lead to the loss of gametocytogenesis of *P. falciparum* during *in vitro* culture and of *P. berghei* in mice[7,8]. A few years ago, genetic studies in *P. falciparum* and *P. berghei*[9,10] identified an essential transcriptional activator of sexual commitment, *ap2-g*. The *ap2-g* locus is epigenetically silenced in asexual parasites through the cooperative action of heterochromatin protein 1 (HP1)[11] and histone deacetylase 2 (HDA2)[12]. Recently, it was demonstrated that the perinuclear protein gametocyte development 1 (GDV1)[13] directly interacts with HP1 and derepresses the *ap2-g* locus[14], leading to *ap2-g* transcription and sexual commitment in a subset of schizonts. In *P. falciparum*, the rate of sexual commitment is sensitive to environmental factors and can be altered depending on *in vitro* culture conditions[15-17]. Recent work revealed that physiological levels of the human serum phospholipid lysophosphatidylcholine (LysoPC) can repress sexual commitment *in vitro*[17,18]. LysoPC thereby functions as an environmental signal for nutrient availability in the host as its metabolites are required for membrane biosynthesis and hence parasite replication. Whereas

ap2-g is conserved across *Plasmodium* species, the *gdv1* locus and the repressive activity of LysoPC are absent in the subgenus *Vinckeia*, a rodent malaria lineage of *Plasmodium*. Besides *ap2-g,* the earliest detectable transcriptional signature of sexual commitment is an increased expression of a subset of invasion markers [17-20]. A second wave of induced genes encodes many proteins that are exported into the host RBC, the functions of which are discussed below. The process of sexual commitment has been summarized and discussed in detail elsewhere [21,22].

Depending on the species gametocyte development takes 1 – 12 days and results in infectious male and female forms (**Figure 2**). At 9-12 days, *P. falciparum* has the longest (known) gametocyte development, which spans five morphologically distinct phases (stages I to V)[23]. All other studied species from the primate, rodent and avian lineages show subtle morphological changes during gametocyte development and a cycle time between 24 – 60 hours[24]. During gametocyte development in *P. falciparum* a continuous sheath of microtubules assembles. The microtubules are attached to an array of alveolar sacs beneath the plasma membrane of the parasite, which is called the inner membrane complex (IMC). An IMC can also be found in sporozoites and ookinetes, in which it is required for cellular motility and passage across the sinusoidal and epithelial barrier, respectively. In *P. falciparum*, the establishment of the IMC during early gametocyte development coincides with modifications of the cytoskeleton of the infected RBC (iRBC), including integration of exported parasite antigens, and results in a reversible stiffening of the iRBC [25-27]. Consequently, stage II-IV gametocytes are more rigid than stage V gametocytes. Interestingly, the characteristic features of the *P. falciparum* gametocytes (continuous IMC and alterations to RBC cytoskeleton and rigidity) are absent in asexual blood-stage parasites, suggesting fundamental differences in the biology between these two blood stages. It is unclear whether these features are limited to *P. falciparum* gametocytes (and closely related species of the subgenus *Laverania*) or whether they are more conserved across the *Plasmodium* lineage.

[H1] Vascular sequestration

P. falciparum can induce cytoadherence of iRBCs to the endothelial cell lining of capillaries and venules in various tissues (**Figure 3**) and this process is a major pathogenic mechanism in cerebral or placental malaria [28-30]. Only trophozoites and schizonts cause cytoadherence and sequestration of iRBCs, whereas iRBCs containing ring-stage parasites remain in circulation. Uninfected RBCs (uRBCs) and ring-stage iRBCs are biconcave; by contrast, RBCs infected with later asexual stages are spherical, less deformable[31] and more permeable for small solutes[32], and their cytoadherence prevents clearance in the spleen. *In vitro* studies under static and physiological shear flow highlighted the similarities between iRBC cytoadherence and the mechanisms of vascular adherence of leukocytes during an inflammatory immune response after injury[33]. In *P. falciparum* the variant surface antigen *P.falciparum* erythrocyte membrane protein 1 (PfEMP1)[34,35] is the major determinant of cytoadherence. Electron-dense structures called knobs lift PfEMP1 above the dense coat of RBC surface receptors, which facilitates interactions between PfEMP1 and endothelial receptors, such as CD36, intercellular adhesion molecule 1 (ICAM1), chondroitin sulphate A (CSA) and endothelial protein C receptor (EPCR), causing iRBCs to adhere and sequester in the microvasculature and removing them from circulation. Individual PfEMP1 variants have differential binding affinities to host receptors and the organ-specific distribution or activation of these host receptors determines disease development. Binding of PfEMP1 to EPCR[28] and ICAM1[36] is crucial for brain sequestration (and causal for cerebral malaria), whereas the interactions with CSA[37] and immunoglobulin M (IgM)[38] are required for sequestration in the placenta (and causal for placental malaria). PfEMP1 is the major target of host immunity on the iRBC[39] and under strong selection to maximize both its ability to evade immunity and to bind host receptors. The role of other surface antigens, such as repetitive interspersed families of polypeptides

(RIFIN)[40,41] and subtelomeric variant open reading frame(STEVOR)[41,42] in cytoadherence of *P. falciparum* is less clear. However, both variant antigens have been implicated in rosetting, a sequestration mechanism in which iRBCs bind to uRBCs to form clusters that obstruct the microvasculature[42,43]. Parasite-induced modifications of the cytoskeleton and surface of iRBCs, in particular knob structures, increase the likelihood of clearance in the spleen due to altered biophysical properties of the iRBC. Hence cytoadherence of iRBCs actively prevents parasites from being in the circulation thereby passing through the spleen. *P. coatneyi* is the only parasite in the primate malaria lineage that is known to induce knob-like structures and cytoadherence[44]. The parasite determinants are unknown however, as both the major knob component, knob-associated histidine-rich protein (KAHRP), and the major surface ligand, PfEMP1, are limited to *P. falciparum* and other members of the *Laverania* subgenus and therefore absent in *P. coatneyi*. *P. vivax*, on the other hand, increases the deformability of host cells during asexual blood-stage development to facilitate passage through the spleen, and there is no conclusive evidence for parasite accumulation in brain or placenta and associated pathology in this species[45].

[H1] Sequestration in the bone marrow

P. falciparum gametocytes were first identified by the French physician Alphonse Laveran in blood samples of Algerian soldiers in 1881. Marchiafava and Bignami, two Italian pathologists found asexual parasite stages in various tissues, and observed gametocytes only in bone marrow and spleen, suggesting that both asexual stages and gametocyte sequester during their development[46]. Several case studies identified *P. falciparum* and *P. vivax* gametocytes in bone marrow and spleen[47-50], leading to the hypothesis that these organs may represent major sites of gametocyte sequestration. A series of recent studies finally provided quantitative data to confirm these earlier findings (**Figure 4**). A histological and qRT-PCR analysis of asexual and

191 immature gametocyte stages in samples from children who died from *P. falciparum* malaria in
192 Malawi showed that the bone marrow is the only organ with substantial gametocyte
193 enrichment, whereas most asexual parasites were found in spleen, followed by brain, heart, gut
194 and bone marrow[51]. Analysis of blood samples and bone marrow aspirates from children with
195 *P. falciparum* malaria and severe anemia in Mozambique by smears and qRT-PCR showed
196 substantial enrichment of immature gametocytes in the bone marrow compared to blood[52]. The
197 bone marrow is the major haematopoietic organ in adult mammals, birds and reptiles. It
198 constitutes ~4% of total body mass in humans, producing approximately $5x10^{11}$ haematopoietic
199 stem cells **[G]** per day[53-55] (all white blood cells, red blood cells and platelets). Erythropoiesis
200 occurs in the bone marrow parenchyma **[G]**, which is connected to the blood circulation
201 through branched sinusoidal vessels. Terminal erythropoiesis occurs in specialized niches,
202 which are called erythroblast islands and consist of a central macrophage surrounded by
203 nucleated RBC precursors[56]. The final nucleated precursor stage is the orthochromatic
204 erythroblast and once it loses its nucleus, newly formed reticulocytes cross the sinusoidal
205 endothelium to enter the blood circulation. Haematopoiesis outside of the bone marrow can
206 occur both under physiological and pathological conditions, in particular in the red pulp of the
207 spleen and in the liver sinusoids. In contrast to humans and all other mammals, the red pulp of
208 the spleen is the major haematopoietic organ in rodents.

209 In bone marrow samples from children who died from malaria 50 to 90% of all
210 gametocytes were associated with erythroblastic islands. Only young (stage I) gametocytes
211 were found in reticulocytes, suggesting that gametocytes can either form in cells of the
212 erythroblastic island, or home to bone marrow as merozoites or in reticulocytes[51]. This finding
213 represented the first quantitative evidence of an extravascular reservoir of blood-stage
214 *Plasmodium* spp. parasites. Histological analysis in splenectomised non-human primates
215 infected with *P. vivax* confirmed the bone marrow as a primary site of gametocyte enrichment

in *Plasmodium* spp.[57]. Again, most gametocytes were found outside of blood circulation in the bone marrow parenchyma, followed by the liver sinusoids. Moreover a tissue screen of mice infected with *P. berghei* showed enrichment of immature gametocytes in the parenchyma and sinusoids of spleen, bone marrow and liver[58]. Finally, *P. falciparum* infection in immune-deficient mice showed gametocyte accumulation in bone marrow and spleen[59]. The apparent conservation of this trait across different hosts and three *Plasmodium* species that have varying gametocyte maturation times (2 days in *P. vivax* and *P. berghei* versus 12 days in *P. falciparum*) and morphology (roundish in *P. vivax* and *P. berghei* versus elongated in *P. falciparum*) indicates that the sequestration of immature gametocytes in the bone marrow and secondary haematopoietic organs is a ubiquitous feature of *Plasmodium* spp. parasites.

Interestingly, the haematopoietic niche is also a reservoir for asexual parasites. In the autopsy case study of children with malaria, sequestering *P. falciparum* asexual stages were found in blood vessels, sinusoids and in particular the parenchyma of the bone marrow[58]. Moreover, the bone marrow parenchyma and liver sinusoids are the main *P. vivax* reservoir outside of the blood circulation in splenectomised non-human primates[57], whereas spleen, bone marrow and liver are major reservoirs of asexual *P. berghei* parasites in mice [58,60]. It was generally assumed that *P. berghei* and *P. vivax* parasites do not sequester, however low levels of *P. vivax* trophozoites and schizonts compared to ring stages in the blood circulation[57,61,62], and the high frequency of severe anaemia despite low peripheral parasitaemia have already challenged this assumption[63]. Altogether these findings demonstrate that the haematopoietic niche of the bone marrow, and other haematopoietic organs such as spleen and liver (depending on host species) represent a major reservoir of blood-stage malaria parasites, including asexual stages. This finding is of particular relevance for parasites that prefer, or are restricted to, young RBCs found in bone marrow, such as *P. vivax* and *P. berghei*.

An extravascular reservoir of asexual parasites may sustain parasite replication and promote gametocyte formation. This hypothesis is supported by the presence of stage I *P. falciparum* gametocytes in bone marrow reticulocytes[51] and the observation that splenic reticulocytes are the major source of gametocytes in a blood-stage *P. berghei* infection[60]. The conditions in the haematopoietic niche may be conducive for gametocyte commitment. Indeed, levels of the major physiological repressor of gametocyte commitment, the host phospholipid LysoPC, are much lower in bone marrow than blood[17]. In addition, the gametocyte fraction in the BM is about 50% both in *P. falciparum* and *P. vivax* compared to less than 5% in the peripheral blood [51,57]. Alternatively, gametocyte formation may be triggered in the vasculature, possibly through local LysoPC depletion at sites of parasite sequestration, systemically during inflammation[17] or simply in a stochastic manner. In such a scenario young gametocyte forms must be detectable in blood circulation and able to home to the bone marrow before becoming too rigid and cleared by the spleen. Indeed, early gametocyte transcripts including *ap2-g* are present at detectable levels in patient blood both by qRT-PCR and microarray[64-67], enabling quantification of *in vivo* conversion rates [64]. Importantly, experiments with *P. berghei* also provide indirect evidence of homing and extravasation of a subset of merozoites to the bone marrow parenchyma. In these experiments, a series of endothelial receptors were blocked using specific antibodies prior to infection with *P. berghei* merozoites[58]. Inhibition of either P-selectin or a combination of ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 vascular cell adhesion molecule-1) reduced the number of young gametocytes (but not asexual parasites) in early reticulocytes in the bone marrow, suggesting an involvement of these receptors in merozoite extravasation to the bone marrow[58]. Interestingly, human P-selectin has been found *in vitro* to interact with members of the family of merozoite surface protein 7 related proteins (MSRP) in *P. falciparum*, *P. vivax* and *P. berghei*[68]. Moreover, single cell and bulk transcriptome analyses have consistently shown an upregulation of *msrp1,* and a second

merozoite antigen, *dblmsp2*, in sexually committed *P. falciparum* schizonts[17-19]. It is therefore tempting to speculate that these two antigens are involved in extravasation and/or host cell invasion of sexual merozoites in the bone marrow. Interestingly, experiments in the rodent model also provided evidence for homing of uninfected and ring stage-infected RBCs to the bone marrow, including the extravascular compartment[58]. Altogether there is evidence both for a genuine sexual commitment cycle in the bone marrow and for intravascular commitment with subsequent homing to this niche. However, more research is needed to understand the molecular mechanism(s) of parasite entry and exit at the bone marrow interface. Whereas merozoites are invasive stages with the intrinsic ability to invade and possibly migrate across host cells, transmigration of ring stage-infected RBCs is more likely driven by the same machinery that facilitates intravasation of reticulocytes. Furthermore, the relative contribution of sexual commitment in the bone marrow versus in the blood to overall levels of gametocyte formation is unknown, but there is likely variation according to host conditions, intrinsic parasite factors and between parasite species.

[H2] Host cell modifications required for extravascular sequestration. Asexual *P. falciparum* parasites efficiently bind to the bone marrow endothelium. In fact, panning experiments of iRBCs with endothelial cells from various tissues demonstrated that bone marrow-derived endothelial cells (BMECs) share binding properties with those from brain[69]. Given the preferred localization of immature gametocytes among erythroblast islands, this parasite stage may interact with RBC precursors including reticulocytes and/or macrophages, but not with BMECs. Early studies suggested adhesion of immature *P. falciparum* gametocytes to ICAM-1 on BMECs[70]. However, further analysis demonstrated only minimal binding of immature gametocytes to BMECs compared to asexual stages[71,72], arguing against a classical PfEMP1-mediated vascular cytoadherence mechanism. Indeed, both PfEMP1 and KAHRP are

290 epigenetically silenced in gametocytes and these proteins are absent in all gametocyte
291 stages[71,73]. Interestingly, a recent study reported the binding of both asexual stages and
292 immature *P. falciparum* gametocytes to human bone marrow mesenchymal stem cells (hBM-
293 MSCs) in a 3D culture system. This binding was trypsin-sensitive, but independent of PfEMP1
294 and ICAM1, yet the receptor for the interaction could not be identified[74]. In a separate study,
295 no binding to RBC precursor cells was observed[75]. An initial proteomic analysis of immature
296 gametocytes identified a large number of gametocyte-exported parasite proteins (GEXPs) and
297 potential ligands for adhesion[76]. More recently, surface proteomics of immature gametocytes
298 confirmed several of these exported proteins as gametocyte surface antigens, including
299 GEXP07 and GEXP10[77]. These two antigens have independently been characterized in asexual
300 stage parasites and were shown to interact with the human chemokine fractalkine (CX3CL1)[78].
301 CX3CL1 is a transmembrane protein expressed in many endothelial cells, but also in bone
302 marrow stromal cells, including MSCs and macrophages, and it is responsible for retaining
303 monocytes within the bone marrow[79]. As GEXP07 and GEXP10 are CX3CL1 receptor mimics,
304 they may be involved in interactions between asexual and immature gametocytes and cells in
305 the extravascular bone marrow niche, such as MSCs and macrophages. In addition to GEXP07
306 and GEXP10, most gametocyte antigens are also expressed in asexual parasites, suggesting
307 shared functions in host cell interactions[77]. Given the shared binding phenotype to hBM-MSC,
308 it is most likely that such shared antigens are also involved in iRBC interactions in the
309 extravascular bone marrow niche. Interestingly, presence of parasite antigens on the
310 gametocyte-infected RBC surface is limited to stages I and II after which the antigens are
311 gradually removed by as yet unknown processes[77], coinciding with the observed loss of
312 binding[74].

313 Invasion assays with *P. falciparum* and *P. berghei* have demonstrated that the earliest
314 RBC precursor stage that can be invaded and support parasite growth is the orthochromatic

erythroblast[51,60,80], in the final 48 to 72 hours of erythropoiesis. Interestingly, the remaining maturation times of invadable RBC stages and of the asexual parasite stage are similar across all known *Plasmodium* species. Likewise, gametocyte maturation of all *Plasmodium* species investigated so far, except for *P. falciparum*, takes a similar length as the asexual cycle. By contrast, the maturation of *P. falciparum* gametocytes takes 10 to 14 days during which they need to avoid premature release of the iRBC into circulation. The benefit of such an unusually long developmental time is not known, except that it is accompanied by an equally unusual level of host cell remodelling. Immature *P. falciparum* gametocytes become increasingly rigid up to stage IV before reverting into a deformable state at stage V[27,81,82]. The rigidity switch at the onset of stage V gametocytes is preceded by the loss of surface antigens on the iRBC surface between stage II and III gametocytes[77], suggesting that the two processes are part of a coordinated remodelling of the host cell. A rigidity differential may serve the same purpose as in reticulocytes[83], facilitating mechanical retention of the immature gametocytes whereas only the mature infectious stage V is sufficiently deformable to enter the blood circulation. The switch to the deformable stage V appears to be triggered by a drop in the intracellular levels of cyclic AMP (cAMP), and drugs that increase cAMP levels (for example, the phosphodiesterase inhibitor sildenafil citrate) increase the stiffness of mature gametocytes[84]. In mice, sildenafil citrate increased the number of *P. berghei* gametocytes in bone marrow and spleen, supporting the notion that the deformability switch is required for a release of mature gametocytes into circulation[58]. Changes in the parasite cytoskeleton and parasite-induced modifications of the host cell cytoskeleton have been suggested to underlie the deformability switch. *P. falciparum* gametocytes undergo drastic morphological changes during maturation, building up an extensive cytoskeleton of longitudinal microtubules until stage IV, which give these cells the classical elongated shape. Upon maturation to stage V this microtubule cytoskeleton is disassembled, likely contributing to the increased deformability of this stage[25]. The variant

340 STEVOR antigen that localises to the erythrocyte membrane has been implicated in regulating
341 the deformability of gametocytes by interacting with the ankyrin complex, a component of the
342 erythrocyte cytoskeleton[81,85]. Deformability seems to be depend on the phosphorylation status
343 of STEVOR, which is regulated by intracellular cAMP levels, and STEVOR
344 dephosphorylation and internalization renders mature gametocytes deformable[85]. Yet, even
345 STEVOR-negative parasites partially retain rigidity during gametocyte maturation and respond
346 to sildenafil citrate treatment, suggesting that other unknown mechanisms contribute to the
347 deformability switch[85]. Notably, *P. berghei* and *P. vivax* gametocytes do not undergo major
348 shape changes nor build-up of a microtubule cytoskeleton during maturation. As their
349 maturation time is only two days and thus much shorter than for *P. falciparum* gametocytes, it
350 is possible that gametocytes in these species do not need a mechanism for continued
351 extravascular retention, but simply mature and enter the blood together with the host
352 reticulocyte. Importantly, there are differences in parasite distribution within the extravascular
353 bone marrow compartment between *P. falciparum*, *P. vivax* and *P. berghei*: Whereas autopsy
354 data from human *P. falciparum* infections and necropsies of non-human primates infected with
355 *P. vivax* found most asexual and gametocyte stages in the parenchyma[51,58], intravital imaging
356 and necropsy data from infected mice revealed a more even distribution between parenchyma
357 and sinusoids for *P. berghei*[58]. The physiological relevance and underlying host pathogen
358 interactions related to these subtle but notable differences remain to be investigated. Intravital
359 imaging has provided direct evidence of mature *P. berghei* gametocyte intravasation and
360 leukocyte-like movement both in the extravascular and intravascular compartment of the bone
361 marrow and spleen[58]. Similar phenotypes have yet to be investigated in *P. falciparum* and *P.*
362 *vivax*. Mature gametocytes of all species must be ingested by mosquitoes during a blood meal,
363 and hence present in the peripheral blood circulation of the dermis. There has been some

speculation about a mature gametocyte reservoir in the peripheral microcirculation of the skin[86,87], however so far there is no experimental evidence for such a phenotype.

[H2] ***Bone marrow infection as a challenge and opportunity for interventions.*** The unique properties of the bone marrow niche provide both challenges and opportunities for interventions against malaria parasites and other infectious agents (**Figure 5**). Currently used artemisinin combination therapies combine the fast acting and short-lived artemisinin with a long-lasting partner drug such as mefloquine or piperaquine[1]. These treatment regimens are based on bioavailability and efficacy measurements in the vascular blood compartment, which may be inadequate for parasite clearance in the bone marrow. For example, bone marrow-resident RBC precursors are metabolically more active than mature RBCs and therefore intracellular pathogens such as malaria parasites may be exposed to reduced drug concentrations in these host cells due to their increased turnover[88]. In addition, drug availability is reduced in the bone marrow due to limited perfusion [89]. Exposing parasites to sublethal drug concentrations in this compartment mimics current *in vitro* protocols to select for drug resistant parasites[90], and therefore it may increase the likelihood of emergence and spread of drug resistance. Some antimalarials including artemisinin also induce haemolysis and subsequent erythropoiesis in the bone marrow and spleen of infected mice, and hence increase infection levels in the haematopoietic niche[60]. On the other hand, the unique metabolic environment of the bone marrow may also provide opportunities to increase the activity of drugs, as shown recently for the pro-drug primaquine [91]. In addition, loss of mature gametocyte deformability in *P. falciparum*[84] and gametocyte accumulation in bone marrow and spleen in *P. berghei*[58] upon sildenafil citrate treatment provides a proof-of-concept for a transmission blocking drug. Systematic efforts using cellular and target-based high throughput screens are underway to

389 further explore blocking the deformability switch as a drug target[92,93]. Bone marrow infection
390 likely has an effect on the development of immunity, as the environment is naturally immune
391 protected through mechanisms of central tolerance **[G]**[94]. Parasite infection also induces
392 changes in the haematopoietic compartment[95] and loss of B cell populations in the bone
393 marrow[96,97], which may be linked to the slow acquisition of protective immunity in malaria.
394 However, parasite sequestration in the bone marrow may also be an opportunity to induce
395 immunity for vaccine development. Identification of shared iRBC surface antigens that are
396 involved in interactions of asexual parasites and gametocytes with the bone marrow could
397 provide the basis for a new blood-stage vaccine that reduces parasite burden and transmission
398 simultaneously. Indeed, natural human antibodies against such shared antigens correlate with
399 a reduced burden of asexual parasites and mature gametocytes in malaria patients[77].
400 Longitudinal studies are required to identify and validate functional antibodies that could serve
401 as a template for next-generation vaccine development. Persistence and recrudescence **[G]** of
402 a parasite reservoir in the bone marrow and spleen, as described in the rodent model upon drug
403 treatment[60], also creates diagnostic challenges as infections may go undetected. It will be
404 important to identify host and/or parasite markers for bone marrow infection independently of
405 peripheral parasites to define the true parasite reservoir in the population. An asymptomatic or
406 undetected bone marrow parasite reservoir may be particularly relevant for parasite species
407 that are either reticulocyte-restricted or have latent phases — or both, as is the case for *P.*
408 *vivax*[98].
409
410 ***[H1]* Conclusion and future directions**
411 The haematopoietic niche is host to various infectious agents due to its nutrient-rich
412 environment, anti-inflammatory and hence immune-protected state, and its capacity to produce
413 many of the circulating immune cells and blood cells that harbour pathogens[99]. For example,

414 the causative agent of tuberculosis, *Mycobacterium tuberculosis* can enter BM-MSC. The bone
415 marrow may represent a reservoir for latent *M. tuberculosis* infection, as non-replicating yet
416 viable bacteria were successfully isolated from the bone marrow of patients who had undergone
417 antituberculous treatment and been declared disease-free[100]. Likewise, splenic sequestration in
418 visceral leishmaniasis has been reported, although exclusively in the context of active disease
419 [101].
420
421 Identification of the bone marrow as a primary site of gametocyte development and a major
422 reservoir for asexual parasites in *Plasmodium* spp. represents a fundamental shift in our
423 understanding of parasite biology, and it opens up a new research field in the malaria
424 community. There have also been a series of advances in our understanding of bone marrow
425 function and architecture in humans and animals in recent years[102]. In parallel, a series of
426 technical breakthroughs were made, such as bone marrow on a chip models for mouse and
427 human[103,104], humanized mouse models with a human bone marrow niche[105], bone marrow and
428 parasite atlases based on single cell RNAseq[106,107] and advances in tissue imaging, including
429 intravital approaches[58]. We anticipate that these recent developments will enable future
430 research to address some of the major questions. How does parasite infection affect bone
431 marrow function including immunity? What is the molecular basis for parasite phenotypes,
432 such as bone marrow homing and extravasation of young parasites, host-parasite interactions
433 and intravasation of maturing parasites, and mobility of mature gametocytes? Can the first
434 wave of merozoites emerging from the liver schizont directly home to the bone marrow? What
435 is the role of the spleen as a reservoir for asexual parasites and gametocytes in different parasite
436 lineages? Can we develop *in vitro* culture systems for *P. vivax* using bone marrow proxies? Is
437 bone marrow recrudescence an alternative relapse mechanism in *P. vivax* and other parasites

with hypnozoites? Closing these knowledge and tool gaps may translate into novel diagnostics and interventions to eliminate and eventually eradicate these deadly human parasites.

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Author contributions

All authors researched data for the article, wrote the article and reviewed or edited the manuscript before submission.

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Box 1. Evolution of a haematopoietic reservoir in *Plasmodium* spp. and other haemosporidian parasites.

Life cycles of wildlife *Plasmodium* spp. and related haemosporidians are diverse and involve various cell types, in particular during exo-erythrocytic development[108-110]. Many haemosporidians have been detected in the haematopoietic niche[110-112], but the role of this phenotype remains insufficiently understood. For example, all known species of avian *Plasmodium* spp. belonging to subgenus *Huffia* multiply mainly in bone marrow stem cells during exo-erythrocytic development, whereas erythrocytic schizonts predominantly form in bone marrow resident, immature red blood cells (RBCs)[110,111].

Life cycles of haemosporidians share similarities across the different families and genera, which makes avian *Haemoproteus* and *Plasmodium* parasites relevant models for malaria parasites. Notable discoveries based on these models include identification of sexual stages[113], exo-erythrocytic development[114,115] and transmission by mosquitos[116]. Avian malaria parasites were also used for early antimalaria drug screening[117] and attempts for *in vitro* culture[118]. However, there are differences between malaria parasites infecting mammals and those infecting other vertebrates (see figure). All avian and reptile haemosporidians, as well as bat parasites from the genus *Polychromophilus*, have primary and secondary exo-erythrocytic

872 schizogony, often in diverse cell types[109,110]. Multiple secondary exo-erythrocytic replication
873 cycles often produce high tissue parasite burden. Some secondary exo-erythrocytic schizonts
874 produce numerous merozoites, which develop into gametocytes that can reach high peripheral
875 parasitemia. Avian and reptile *Plasmodium* spp. also show erythrocytic schizogony.
876 Merozoites from erythrocytic and exo-erythrocytic schizonts can initiate secondary exo-
877 erythrocytic schizogony, forming phanerozoites **[G]**, which often develop in endothelial cells
878 lining capillaries[110,119,120]. Massive infestation of bone marrow cells by phanerozoites often
879 occurs during infection of birds with *P. elongatum* and some other species[110,111,121]. In these
880 infections, phanerozoites are found in haematopoietic tissues, in particular in haematopoietic
881 stem cells. *P. elongatum* can cause dyserythropoiesis **[G]** and anemia due to damage of bone
882 marrow cells[111,122]. The ability of erythrocytic and exo-erythrocytic merozoites to initiate
883 secondary exo-erythrocytic schizogony in avian and reptile parasites opens opportunities for
884 experiments on tissue stages by inoculation of infected blood, avoiding the use of vectors[119].
885 Blood schizogony is only reported for *Plasmodium* spp. of reptiles, birds and mammals[110,112],
886 and in species of the Garniidae[123]. It is absent in the Leucocytozoidae and Haemoproteidae and
887 in *Polychromophilus*, and it has not been reported in *Nycteria* and *Hepatocystis*. By contrast,
888 gametocytes are always present in cells of the erythroid lineage, and they often reach high
889 parasitemia [108-110,123-125].

890 Major transitions in life history traits during haemosporidian evolution are (I)
891 emergence of erythrocytic schizogony in the Plasmodiidae species of birds and reptiles,
892 resulting in a second source of gametocyte stages; and (II) subsequent loss of the secondary
893 exo-erythrocytic schizogony in Plasmodiidae species of mammals, resulting in erythrocytic
894 schizogony as the only source of gametocytes (see figure). Despite the marked diversity in
895 morphology and life cycles haemosporidians share several features in their gametocyte
896 development. First, the gametocyte is the only parasite stage found in cells of the erythroid

lineage across all known haemosporidians. Second, gametocytes originate from merozoites developing in schizonts (in the Haemoproteidae and Leucocytozoidae families they form only in exo-erythrocytic schizonts; in some members of the Plasmodiidae family (and probably Garniidae family) they form only in erythrocytic schizonts; and in some members of the Plasmodiidae family (and probably Garniidae family) they are formed through erythrocytic and exo-erythrocytic schizogony[110,112]). Interestingly, gametocytes are sexually dimorphic in all known haemosporidians[108-110,112], in contrast to gametocytes in other apicomplexans.

Importantly, the information about life cycles from wildlife haemosporidians is often based on limited observations[108-110]. For example, the absence of blood schizogony in the bat parasite genera *Hepatocystis*, *Nycteria* and *Polychromophilus* requires further investigation[125,126], given its presence in closely related species infecting other hosts[126,127], and lack of an alternative gametocyte source. Blood schizogony either evolved once and subsequently was lost in the above bat parasites or gained independently several times[126,128]. Altogether, distribution of life cycles across haemosporidians suggests that establishment of a bone marrow reservoir emerged with blood schizogony, either in parasites infecting birds and/or once they infected mammals (with bats as the most likely origin).

Figure 1: Life cycle of *Plasmodium falciparum* in humans and mosquitos. a. *P. falciparum* sporozoites are injected into the skin during the blood meal of an infected mosquito. They will migrate to and enter a blood capillary. **b**. Through the blood stream the sporozoites reach the liver sinusoids and there they leave the blood circulation to invade a hepatocyte, after multiple transmigration events. In the hepatocyte, they undergo one asexual replication cycle that results in a liver schizont containing thousands of merozoites. The merozoites enter the blood stream in membrane-bound structures termed merosomes. Once released, merozoites infect red blood cells to initiate the intra-erythrocytic parasite cycle. **c**. In the blood, *P. falciparum* parasites

undergo cycles of asexual replication. After invasion of a red blood cell, they develop from ring stages to trophozoites and then to schizonts. Mature schizonts burst to release merozoites that initiate another replication cycle. A subpopulation of parasites commits to produce male and female sexual progeny or gametocytes (green). **d.** A female Anopheles mosquito picks up gametocytes while feeding on an infected human. Male and female gametocytes undergo gametogenesis within the midgut of the mosquito. The gametes then fertilise to form a zygote, which further develops into motile ookinetes. Ookinetes cross the midgut epithelium to form an oocyst beneath the basal lamina. In the oocyst, thousands of sporozoites form, which upon bursting of the oocyst wall, enter the haemolymph to invade the salivary gland. From there, sporozoites are transmitted to the next human during the subsequent mosquito bite, closing the complex life cycle of the parasite.

Figure 2. Sexual development of *Plasmodium falciparum*. A subset of schizonts commit to the sexual cycle, producing sexual merozoites. Merozoites and young gametocytes home to the bone marrow, leave the sinusoids and enter the parenchyma. Alternatively, the gametocytes form in the parenchyma from committed schizonts. In the bone marrow parenchyma, gametocytes develop from stage I to stage IV. Remodelling of the membrane of the host red blood cell results in transient deposition of surface antigens (orange) and reversible increase in cellular rigidity (purple). Restored deformability during maturation to stage V gametocytes triggers their release back into the blood stream, where they can be taken up during another mosquito bite. Asexual replication in the bone marrow parenchyma most likely contributes to the accumulation of asexual parasites and sexual commitment in this compartment.

Figure 3: Intravascular sequestration of *Plasmodium falciparum*. Trophozoite and schizont stages of asexual *P. falciparum* parasites sequester in the capillaries of several organs including

947 brain, lung, spleen and bone marrow. Cytoadherence of infected red blood cells to endothelial
948 cells and to uninfected red blood cells (rosetting) facilitates sequestration. The main parasite
949 ligand is PfEMP1, which is exposed on the surface of infected red blood cells by knob-like
950 structures. The different variants of PfEMP1 interact with diverse endothelial cell receptors
951 such as EPCR, ICAM1, PECAM1 and CD36. In pregnant women, *P. falciparum* also
952 sequesters in the placenta through the interaction of the PfEMP1 variant var2CSA and the
953 placental receptor CSA. Ligand receptor interactions involved in rosetting are not clearly
954 defined, but likely involve RIFIN and STEVOR as well as PfEMP1.
955
956 **Figure 4: *Plasmodium falciparum* development in the hematopoietic niche of the bone**
957 **marrow.** In the hematopoietic niche of the bone marrow, erythropoiesis occurs in
958 erythroblastic islands, consisting of a central macrophage surrounded by erythroid cells.
959 Coinciding with maturation of the erythroid cells from polychromatic to orthochromatic
960 nucleated red blood cell precursors and then to reticulocytes, erythroblastic islands move closer
961 to the sinusoids. When the reticulocytes have lost their cell nucleus, they are eventually
962 released and enter (either through or between endothelial cells) into the sinusoidal lumen. The
963 asexual parasite cycle in the bone marrow parenchyma is likely established both by influx of
964 asexual merozoites or ring stages from the sinusoids, and by a genuine asexual cycle in the
965 bone marrow. In the parenchyma, asexual parasites may invade and develop in association with
966 erythroblastic islands, or with other cell types. The exported proteins GEXP07 and GEXP10
967 on the surface of infected red blood cells interact with the host receptor CX3CL1, which is
968 expressed on different host cells including BM-MSCs, providing a potential mechanism by
969 which parasites are retained in the bone marrow parenchyma. Gametocytes mature in the bone
970 marrow and are derived either from extravasated sexual merozoites or rings, or from
971 extravascular schizonts that commit to produce sexual progeny in the bone marrow

environment. Most gametocytes associate with erythroblastic islands. Stage I and II gametocytes express GEXP07 and GEXP10, which might contribute to bone marrow retention by interacting with other cell types, such as the nursing macrophages and BM-MSCs. Stages III and IV are retained in the bone marrow by their high rigidity, preventing passage through the endothelium. Ultimately, mature stage V gametocytes enter the sinusoids.

Figure 5. Revisiting interventions to block *Plasmodium falciparum* transmission. a. Current transmission blocking vaccines target parasite processes in the mosquito, posing formidable technical challenges. Antibodies are taken up with the few microliters of a mosquito blood meal and require high titres in human blood. Many of the target proteins are not expressed during human infection, and hence there is no natural boosting of the immune response. Finally, efficacy testing requires mosquito feeding assays, which are cumbersome. **b.** Alternatively, transmission can be blocked by targeting gametocytes with stage V density in the circulating blood as a readout. Many antimalarials that are active against asexual blood-stage parasites are also active against immature gametocytes, including artemisinin and its derivatives. A few antimalarials are mostly active against stage V gametocytes, in particular primaquine. Identification of the bone marrow as a reservoir for asexual parasites and gametocytes opens new opportunities for interventions targeting both asexual parasite burden and transmission. i) Vaccines or human monoclonal antibodies could target receptor-ligand interactions required for parasite homing and extravasation, thereby blocking establishment of bone marrow infection and gametocyte development. ii) They could also inhibit interactions at the erythroblast island or with mesenchymal stem cells, possibly triggering premature parasite release into circulation and subsequent clearance in the spleen. iii) Drugs that inhibit the deformability switch may lead to the accumulation of mature gametocytes in the bone marrow parenchyma, and hence prevent their release into circulation and transmission.

Glossary

Gametogenesis: Maturation of male and female gametes.

Meiosis: Cell division involving chromosome duplication and genetic exchange.

Sporozoite: Only parasite stage that can invade the vertebrate host upon insect bite.

Sinusoid: Special capillaries lacking a basal lamina and present in bone marrow, liver, spleen and adrenal glands.

Parasitophorous vacuole: Membrane compartment surrounding the parasite and separating it from the host cell.

Schizont (meront): Replicative parasite stage in the vertebrate host producing daughter merozoites.

Merozoite: Only parasite stage that can invade red blood cells.

Hypnozoite: Non-replicative dormant parasite stage in the vertebrate host liver that can reactivate and lead to relapses.

Reticulocyte: Immature RBC developing in the bone marrow before final maturation in blood circulation.

Microgamete: Male gamete.

Macrogamete: Female gamete.

Zygote: Union of male and female gamete where meiosis takes place.

Ookinete: Motile zygote that forms the oocyst upon crossing the basal lamina of the mosquito midgut.

Oocyst: Replicative stage in the mosquito host producing daughter sporozoites.

Haemolymph: Equivalent to blood in arthropods and other invertebrates.

Haematopoietic stem cell: Cell type that gives rise to all blood cells in the process of haematopoiesis.

Parenchyma: Extravascular compartment of the bone marrow where haematopoiesis takes place.

Central tolerance: Process of eliminating developing T and B cells that are reactive to self.

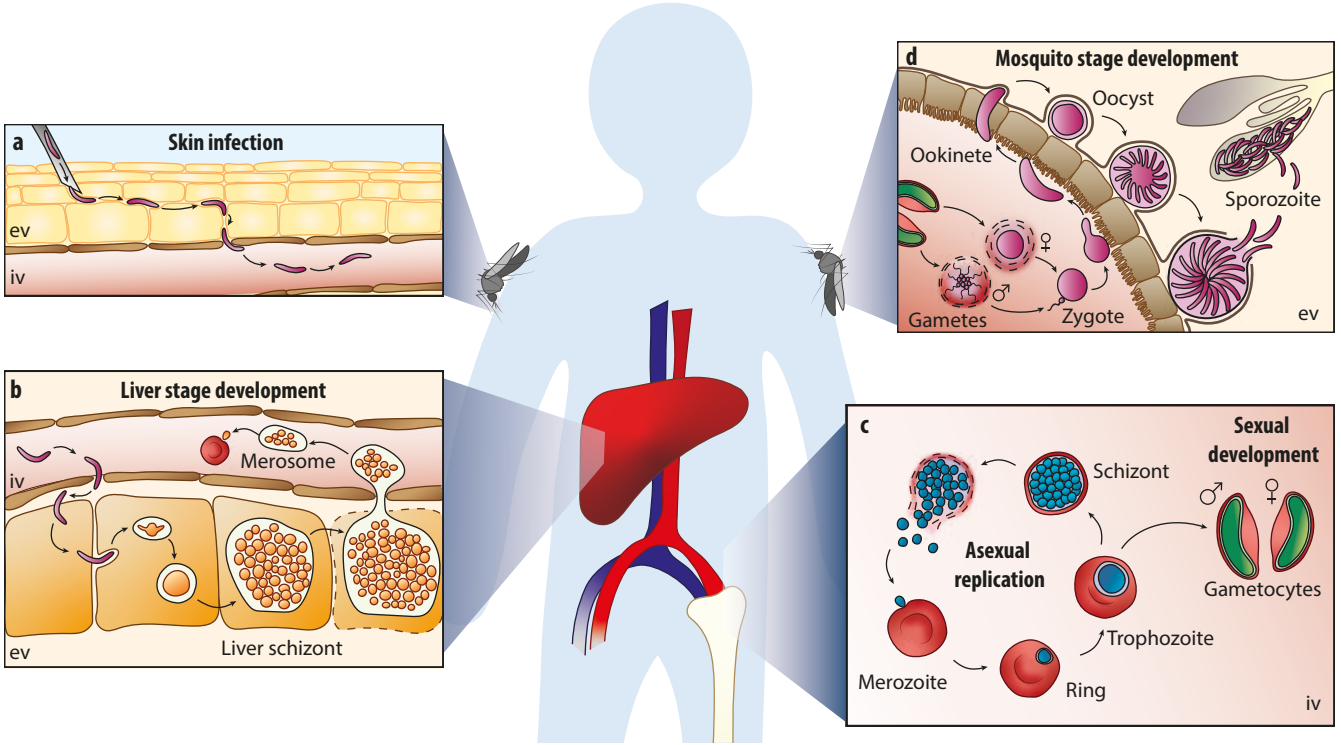
Recrudescence: Recurrence of detectable parasitaemia upon clearance to submicroscopic levels.

Phanerozoite: Secondary exo-erythrocytic schizont in avian and reptile malaria parasites.

Dyserythropoiesis: Defective development of red blood cells, or erythropoiesis.

ToC blurb

Plasmodium falciparum and other malaria parasites have complex life cycles, inhabiting different host cells and tissues during their multi-stage development. In this Review, Marti and colleagues discuss parasite infection in the haematopoietic niche of the bone marrow, a newly discovered reservoir for parasite growth and transmission.


a
Skin infection
ev
iv
b
Liver stage development
Merosome
iv
ev
Liver schizont
c
Sexual development
Schizont
Asexual replication
Gametocytes
Trophozoite
Merozoite
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iv
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Mosquito stage development
Oocyst
Ookinete
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Gametes
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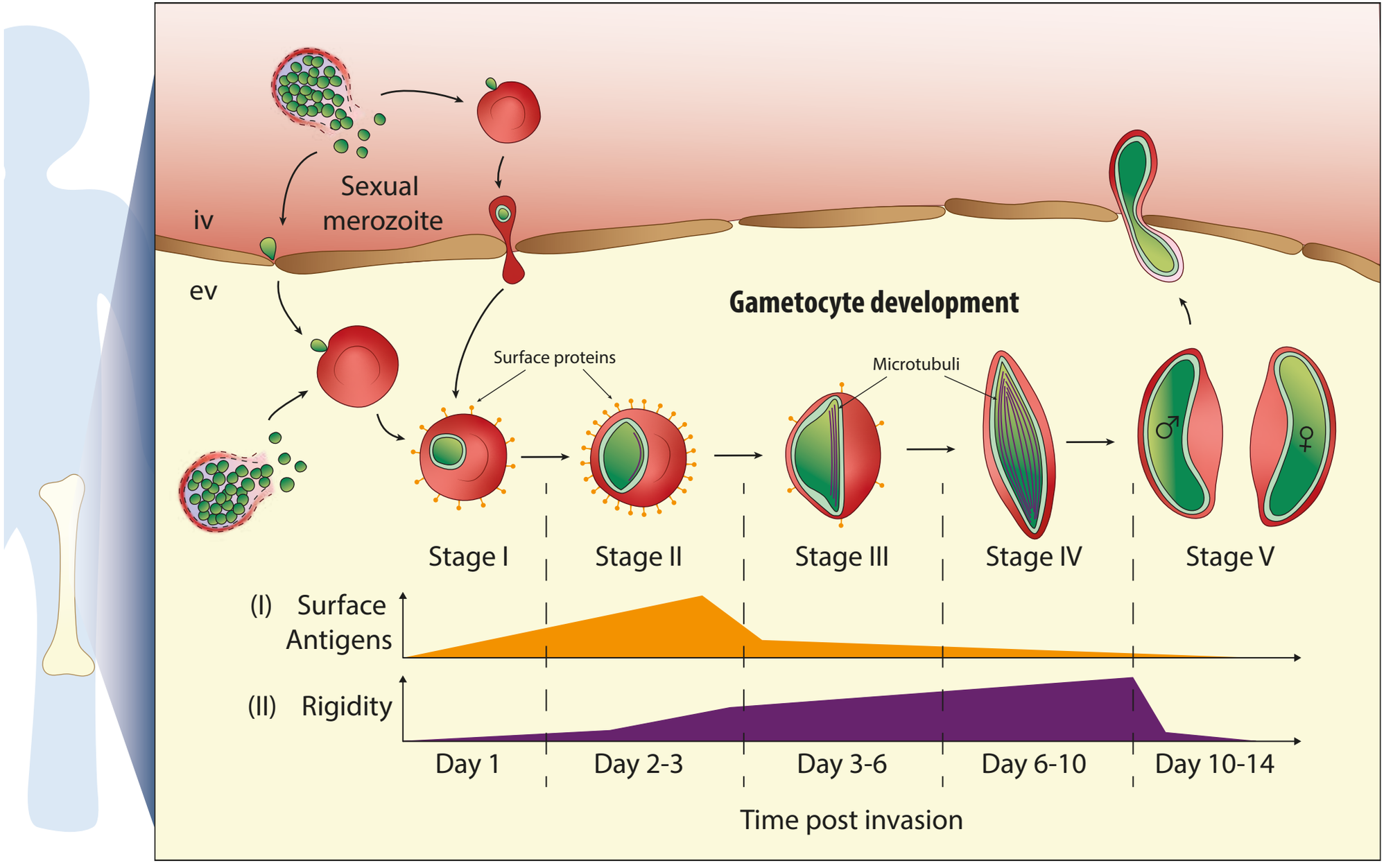
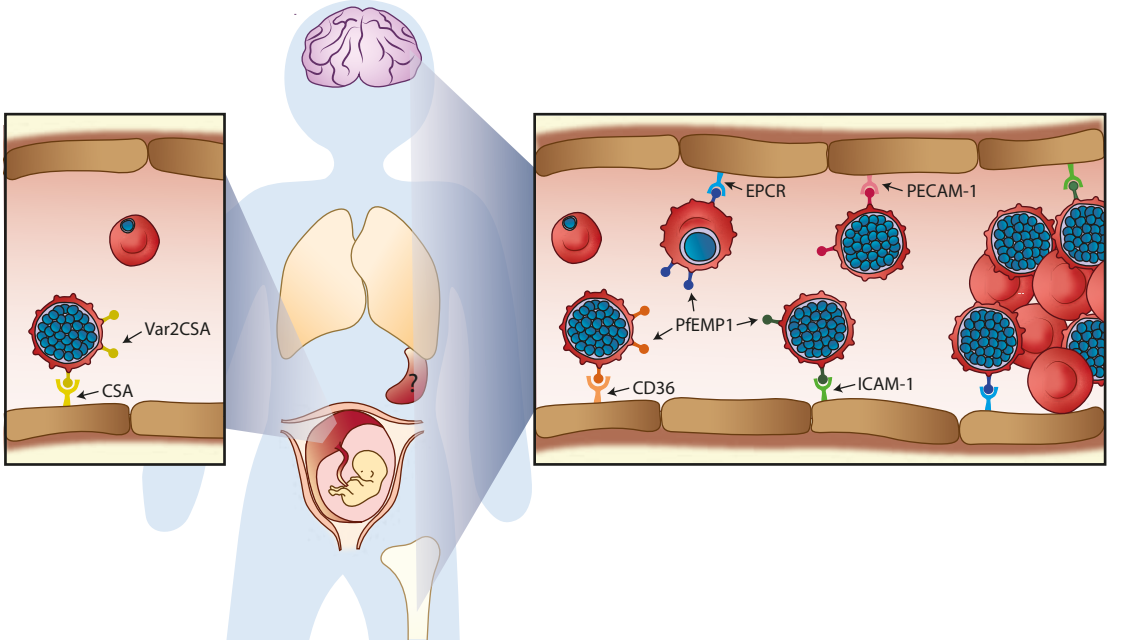
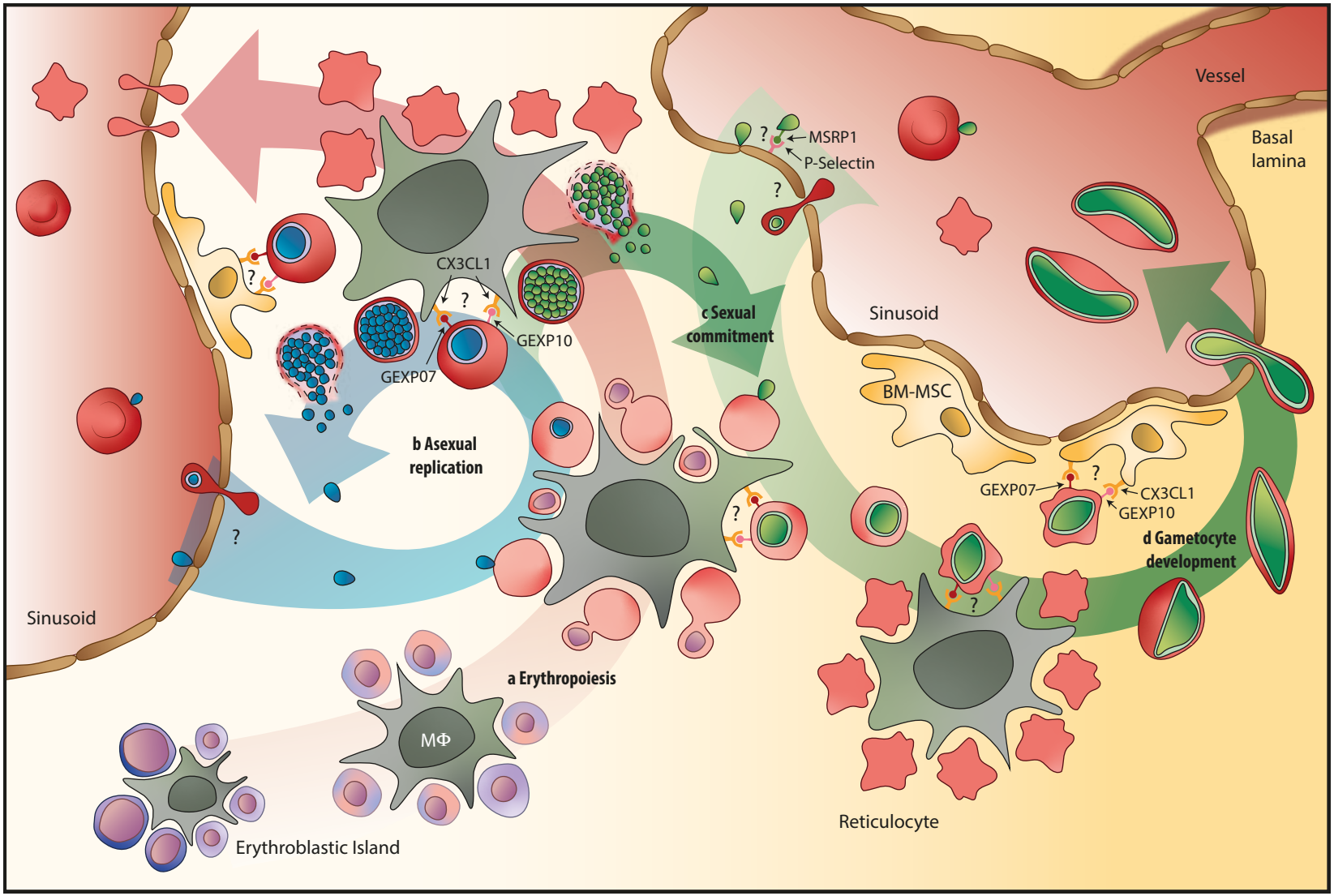
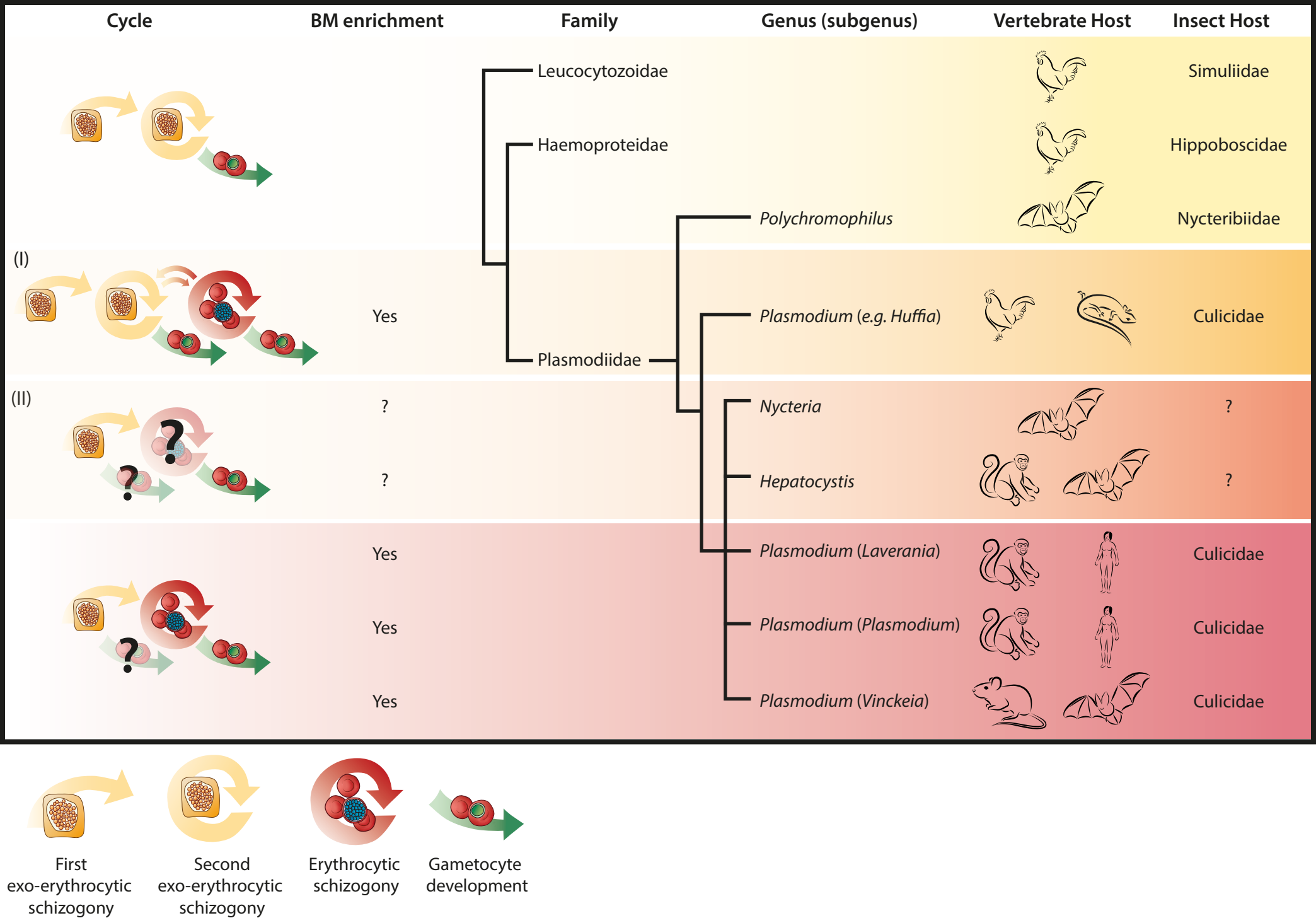
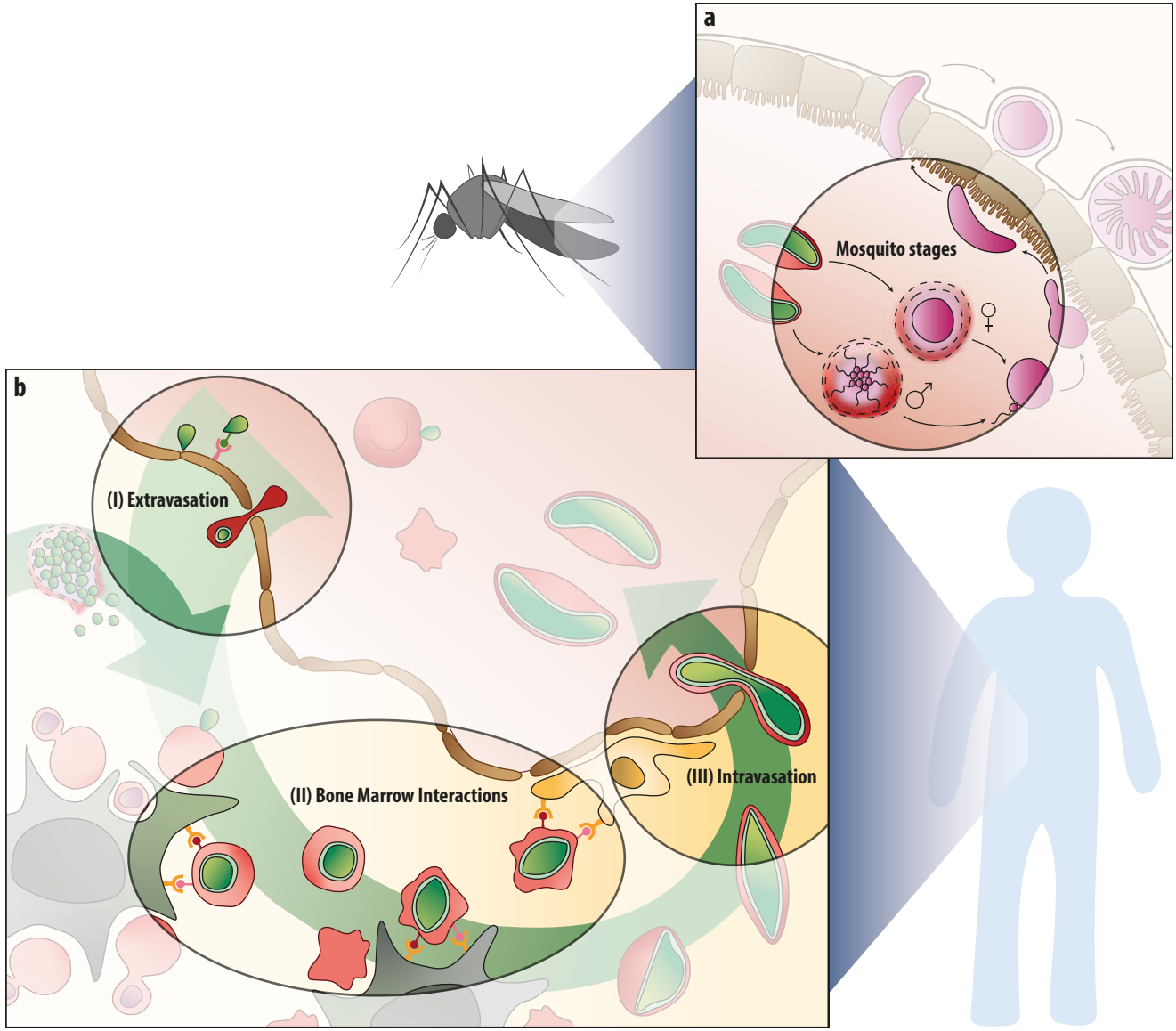

Sexual merozoite
iv
ev
Gametocyte development
Surface proteins
Microtubuli
♂
♀
Stage I
Stage II
Stage III
Stage IV
Stage V
(I) Surface Antigens
(II) Rigidity
Day 1
Day 2-3
Day 3-6
Day 6-10
Day 10-14
Time post invasion


Var2CSA
CSA
?
EPCR
PECAM-1
PfEMP1
CD36
ICAM-1


Vessel
Basal lamina
MSRP1
P-Selectin
?
CX3CL1
GEXP10
GEXP07
c Sexual commitment
Sinusoid
BM-MSC
b Asexual replication
d Gametocyte development
a Erythropoiesis
MΦ
Sinusoid
Erythroblastic Island
Reticulocyte


Cycle
BM enrichment
Family
Genus (subgenus)
Vertebrate Host
Insect Host
Leucocytozoidae
Haemoproteidae
Plasmodiidae
Polychromophilus
Simuliidae
Hippoboscidae
Nycteribiidae
(I)
Yes
Plasmodium (e.g. Huffia)
Culicidae
(II)
?
?
Nycteria
Hepatocystis
?
?
Yes
Yes
Yes
Plasmodium (Laverania)
Plasmodium (Plasmodium)
Plasmodium (Vinckeia)
Culicidae
Culicidae
Culicidae
First exo-erythrocytic schizogony
Second exo-erythrocytic schizogony
Erythrocytic schizogony
Gametocyte development


a
Mosquito stages
♀
♂
b
(I) Extravasation
(II) Bone Marrow Interactions
(III) Intravasation