**Erythrocyte remodeling by *Plasmodium falciparum* gametocytes in the human host
interplay**

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Keywords: *Plasmodium falciparum* gametocytes; erythrocyte remodeling; cell mechanical
properties; parasite sequestration; malaria transmission.

**The spread of malaria critically relies on the presence of *Plasmodium* transmission
stages - the gametocytes - circulating in the blood of an infected individual, which are
taken up by *Anopheles* mosquitoes. A striking feature of *Plasmodium falciparum*
gametocytes is the long development inside the erythrocytes while sequestered in the
internal organs of the human host. Recent studies on the molecular and cellular
remodeling of the host erythrocyte induced by *P. falciparum* during gametocyte
maturation are shedding light on how these may affect the establishment and
maintenance of sequestration of the immature transmission stages and the
subsequent release and circulation of mature gametocytes in the peripheral
bloodstream.**

Gametocytes in the life cycle of the malaria parasite

The transmission of *Plasmodium* parasites and the spread of malaria in human populations
are intimately linked to the process of sexual differentiation in this protozoan. Only the

31 *Plasmodium* sexual stages (the gametocytes), formed in the bloodstream of infected
32 individuals, are able to survive and transform into male and female gametes once ingested by
33 *Anopheles* mosquitoes in the blood meal. Gamete fertilization and subsequent development
34 in mosquitoes ensure progression of the parasite life cycle, which is enriched by the meiotic
35 reassortment of parasite genotypes in multiclonal infections. The production of gametocytes
36 and their successful maturation in the human host are key phases of the malaria parasite life
37 cycle, and recently are rightly receiving attention in the context of malaria elimination. Five
38 *Plasmodium* species are responsible for malaria in humans: *Plasmodium ovale*, *Plasmodium*
39 *malariae*, *Plasmodium vivax*, *Plasmodium knowlesi* and *Plasmodium falciparum*. The latter
40 stands out not only because it is the most virulent, being responsible for 90% of the malaria
41 related deaths in sub-Saharan Africa [1], where 81% of the worldwide cases of malaria are
42 reported [2], but also because of its unique process of gametocyte maturation.

43 After developing for four to seven days in the liver of its human host, *P. falciparum*
44 establishes a blood-stage infection in which the parasite has two alternative life styles. In
45 asexual multiplication, one mature schizont produces 16–32 merozoites, which burst from the
46 red blood cell, each invading a new erythrocyte and producing a new asexual schizont in 44–
47 48 h. Alternatively, factors as yet undefined trigger a developmental switch in a fraction of
48 maturing schizonts. In these sexually committed parasites, an epigenetic control mechanism
49 [3,4] activating a transcriptional cascade [5,6] results in all merozoites to develop as
50 gametocytes of the same sex [7-9]. While in most *Plasmodium* species the sexual stages
51 mature within two days [10], the gametocytogenesis of *P. falciparum* takes 10–12 days,
52 traditionally classified into five stages of morphological maturation [11]. From 24 h to 30 h
53 after invasion of the host cell, the sexually committed parasite appears as a round cell (stage
54 I gametocyte), morphologically indistinguishable from a mononucleated asexual trophozoite.
55 Profound molecular and cellular rearrangements transform this cell first into a half-moon-
56 shaped stage II gametocyte (day 2), then into a symmetric elongated stage III gametocyte
57 (day 4), which further stretches and acquires pointed edges as a stage IV gametocyte (day
58 6), and finally shows a blunted shape in the stage V gametocyte (day 10). This dynamic
59 morphological differentiation is accompanied by several cellular rearrangements, including (i)
60 the appearance, from late-stage I, of a subpellicular membrane complex subtended by a

microtubule array [12] connected with an F-actin cytoskeleton [13]; (ii) the progressive upregulation of approximately 200 gametocyte-specific genes [14]; (iii) the preferential or specific production of approximately 200 proteins as determined by comparing the proteomes of isolated stage I gametocytes, mature gametocytes, and asexual trophozoites [15]; and (iv) the disassembly of the subpellicular microtubular network at the transition between stages IV and V [16,17].

Plasmodium falciparum's unique sexual development presents many challenging questions as well as several potential targets to be exploited in the fight against malaria and its transmission. Here, we focus on recent evidence on cellular and molecular transformations accompanying the extended period of intraerythrocytic gametocyte maturation in *P. falciparum*. In particular, we consider the mechanisms developed by sexual-stage parasites to remodel the structural and mechanical properties of the host cell and the functional relevance of these modifications for gametocyte maturation, sequestration, subsequent release from internal organs, and ability to circulate in the blood stream of their host.

Sequestration of *P. falciparum* asexual forms and gametocytes

Unlike the asexual and sexual stages of other human malaria parasite species, those of *P. falciparum* do not freely circulate in the peripheral blood for the whole of intraerythrocytic development. Only ring stage parasites and stage V gametocytes are commonly seen in the infected peripheral blood. In the asexual cycle, the maturing trophozoites efficiently adhere to the endothelial cells of the microvasculature 20–24 h post-invasion (p.i.) through parasite ligands exposed on the surface of infected erythrocyte, which bind one or more host endothelial cell receptors [18,19], thus evading splenic clearance. One of the major ligands is the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) family of antigenically variable proteins, encoded by the *var* gene family. PfEMP1 is exposed on structures protruding from the surface of the infected erythrocyte, called knobs, where it binds to a wide range of host endothelial receptors, including thrombospondin receptor (CD36), Intercellular Adhesion Molecule 1 (ICAM-1) and Chondroitin Sulphate A (CSA) [20-22].

In sexual development, the immature stages sequester over approximately 10 days in internal organs, from which they are released only as stage V gametocytes. Little is known about the sites and mechanisms of immature gametocyte sequestration. Early analyses of *postmortem*

specimens and a few recent field and clinical reports suggest bone marrow and spleen as the organs where immature gametocytes (stage II–IV) predominantly sequester [23–29]. A recent analysis conducted with state-of-the-art molecular and cytochemical tools on autopsy specimens from several organs provided the first evidence of a comparative enrichment of immature stages in the bone marrow [30]. Once released into the peripheral circulation, *P. falciparum* gametocytes require another 2–3 days to become infective to mosquitoes [31,32] and further circulate for an estimated 3.4–6.4 days [31,33,34].

Unlike for asexual parasites, the mechanism responsible for gametocyte sequestration is poorly understood. Older studies, generally using stage II–IV gametocytes, produced conflicting data on the adhesion efficiency and possible involvement of PfEMP1 and showed low binding efficiency of immature gametocytes to host endothelial or non-endothelial cells, such as C32 melanoma or bone marrow stromal cell lines, or to purified host ligands, such as ICAM-1 and CD36 [35–38]. One study reported that stage I gametocytes produce knobs on the surface of infected RBCs and bind to CD36-positive C32 melanoma cells with the same avidity as asexual trophozoites [36]. However, by using a protocol to separate stage I gametocytes from asexual trophozoites [15], it was recently shown that early-stage gametocytes show low, if any, binding to CD36/ICAM1 double-positive endothelial cells and confirmed the absence of knobs on early gametocytes [39], the latter previously suggested by ultrastructural studies [40]. Moreover, the Knob Associated Histidine Rich Protein (KAHRP) is undetectable in stage I and II gametocytes, and the expression of the entire *var* gene family is downregulated [39], consistently with the observed poor cytoadhesion of these stages. These results altogether indicate that host cell modifications and cell adhesion properties significantly differ between *P. falciparum* asexual and sexual stages from the onset of parasite differentiation (Figure 1).

Cellular remodeling and changes in mechanical properties of *P. falciparum* gametocyte-infected erythrocytes

The remarkable half-moon shape of *P. falciparum* gametocytes (the name of this species derives from the Latin term for sickle) suggests the existence of major parasite and host cell remodeling. The evidence that more than 10% of the proteins upregulated in stage I gametocytes contain the Host Targeting/*Plasmodium* EXport ELement (HT/PEXEL) signature

that predicts their export to the host cytoplasm, is consistent with this hypothesis. Mass spectrometry identification of HT-PEXEL-processed peptides and experimental evidence for the export of several of such proteins in early gametocytes [15,41,42] confirmed that protein export, originally described during asexual stage development, is also active in early sexual differentiation [43]. At the intracellular level, a role in gametocyte elongation was proposed for a subpellicular membrane complex appearing in late-stage I gametocytes and eventually juxtaposed under the gametocyte membrane throughout maturation. Intriguingly, this structure was recently shown to be molecularly similar to the inner membrane complex (IMC), so far described only in the parasite invasive and motile extracellular stages [17, 44]. It is conceivable that the observed molecular processes have an impact on the infected erythrocyte structure and on the mechanical properties of gametocyte-infected erythrocytes. In asexual stages, the synthesis of novel cellular structures and the export of hundreds of parasite proteins to the erythrocyte cytoplasm enable parasite growth and nutrient acquisition. The concomitant dynamic reorganization of the erythrocyte plasma membrane and of the underlying cytoskeleton is associated with decreased deformability of the host cell membrane [45-47]. These changes, and the presence of the intracellular asexual parasite, impair the ability of infected erythrocytes to deform in response to an applied force with the consequence of not being able to circulate through the splenic sinusoids [48, 49]. The question of whether similar erythrocyte modifications are also induced by the developing gametocyte has been recently addressed through the use of several techniques. An ektacytometry analysis measuring the rheological properties of purified gametocytes of stage III, IV, and V, showed a progressive increase in deformability of the erythrocytes infected by gametocytes during their maturation to stage V [17]. A study combining ektacytometry and microsphiltration showed that immature stage II, III, and IV gametocytes are less deformable compared with stage V gametocytes [50]. Importantly, a distinct switch in deformability was described at the transition from stage IV to V gametocytes, resulting in more deformable gametocyte infected erythrocytes that are significantly less retained when passed through a mesh of microspheres mimicking the physical constraints of the splenic slit [50]. Another study showed that the viscoelasticity of the membrane of the gametocyte infected

erythrocytes progressively decreases during elongation to stage IV gametocytes, but increases significantly in stage V gametocytes [51].

Several factors, besides the intrinsic rigidity of the parasite, determine the mechanical properties of erythrocytes, including membrane viscoelasticity, cytoplasmic viscosity, and cell surface area:volume ratio [52,53]. A decrease in the surface area:volume ratio of the infected erythrocytes is caused by the growth of the parasite while the host erythrocyte volume remains almost constant, leading to a reduction of erythrocyte deformability [54-56]. Despite the striking morphological changes observed during gametocyte maturation, the surface area:volume ratio remains roughly constant except for a transient decrease at stage IV, when the parasite is fully extended [51,56]. During development from stage II to stage IV, 70% of the hemoglobin mass is progressively digested [56], suggesting that the steady progression of this process is unlikely to have an abrupt impact on cytoplasm viscosity at maturation. However, it might be that dissipation of the microtubule network at the onset of stage V leads to a slight increase in the erythrocyte volume with a consequent decrease in hemoglobin concentration [56].

Gametocytes can also directly modify the host erythrocyte membrane and its mechanical properties [51], as supported by evidence that proteins of the STEVOR family show a dynamic association–dissociation with the erythrocyte membrane compartment during gametocytogenesis [50]. Moreover, as most stage V gametocytes passing through the microsphere filter exhibit a strikingly deformed shape compared with the retained immature stages [50], it is clear that an intrinsically higher parasite cell deformability contributes to the overall deformability of stage V infected erythrocytes.

In conclusion, erythrocyte infection with *P. falciparum* immature sexual stages results in increased host cell rigidity, similar to that observed for asexual parasites. This process is reverted at sexual maturation, leading to increased deformability of the stage V male and female gametocyte infected erythrocytes. These are the stages released into the peripheral circulation, where they survive multiple passages through the spleen, for several days, until ingested by the mosquito.

Molecular basis of parasite host cell remodeling

179 Several *P. falciparum* proteins are able to remodel the erythrocyte host cell, altering its
180 mechanical properties. In asexual stages, the Ring-infected Erythrocyte Surface Antigen
181 (RESA), released by invaded merozoites and exported to the erythrocyte membrane,
182 interacts with the spectrin network, leading to the reduced deformability of the infected host
183 cell [47]. Furthermore, KAHRP and *Plasmodium falciparum* erythrocyte membrane protein 3
184 (PfEMP3) have been reported to decrease the erythrocyte deformability [45] and FIKK
185 kinases to induce cell mechanical remodeling through stage-specific phosphorylation of
186 different membrane skeleton proteins [57]. Recently, proteins encoded by the STEVOR
187 multigene family have been shown to contribute to the increased rigidity of asexually infected
188 erythrocytes [49].

189 In gametocyte development, although protein export is active from the onset of differentiation
190 [15,41,42], a direct role in erythrocyte host remodeling for most of the observed proteins
191 awaits experimental confirmation. It is possible, for instance, to speculate that the Mature
192 parasite-infected Erythrocyte Surface Antigen (MESA), which interacts with the host cell
193 cytoskeleton in asexual parasites [58-61], has a similar function in sexual stages. Also, the
194 megadalton protein Pf11-1, localized to the host cell membrane during gametocytogenesis
195 [62] and recently included among the PEXEL-Negative Exported Proteins (PNEPs) [63], or
196 the PEXEL/HT-containing protein MAL7P1.171, whose ablation resulted in failure of stage I
197 gametocytes to mature [64], may interact with host membrane components. Additional
198 candidates are STEVOR and RIFIN proteins, some of which are highly expressed during
199 sexual differentiation and have been shown in some cases to be associated with the
200 erythrocyte membrane in stage III-IV gametocytes [65,66]. A role for STEVOR proteins was
201 directly demonstrated by showing that mature gametocytes overexpressing STEVOR, but not
202 a different transmembrane protein, have a decreased deformability. STEVOR interaction with
203 the erythrocyte membrane is dynamic: a direct association was shown in stage III-IV
204 gametocytes, whereas this was no longer detectable in stage V gametocytes [50]. The
205 combined effects of STEVOR dissociation from the erythrocyte membrane and the
206 disassembly of the microtubule subpellicular network in the transition from stage IV to stage V
207 gametocytes [16,17] are likely to have a major role in the increase in deformability marking
208 the maturation of the *P. falciparum* gametocyte (Figure 2).

Mechanical properties of gametocyte-infected erythrocytes in the interaction with the human host

In asexual development, the parasite stages able to circulate in the blood stream have been shown to be generally more deformable than those, more mature, which sequester in internal organs, both in *P. falciparum* [45,47,57,67] and in *Plasmodium knowlesi* [68,69]. In contrast, the *Plasmodium vivax* infected erythrocytes, which present only a slight tendency to sequester, have been reported to be highly deformable [70]. These observations altogether propose an association between rigidity of infected erythrocytes with sequestration and between ability to circulate with increased deformability.

The recently described changes in parasite and host cell mechanical properties during the long development of *P. falciparum* sexual stages is now triggering hypotheses on their possible functional roles during sequestration of the immature gametocytes and release of stage V gametocytes, and in their ability to circulate in the blood.

Of the few studies investigating the sequestration sites of *P. falciparum* sexual stages, all identified bone marrow and spleen as the organs where immature gametocytes (stage II–IV) are most readily detectable [23-29]. Despite the major differences in physiological functions, general architectures and in the volumes of blood trafficked through these organs, similarities in the blood circulation in the bone marrow and in the spleen might be relevant to the presence of immature gametocytes (see below).

In the bone marrow (Figure 3A), arterial vessels enter the tissue cavity and divide into several arterioles that span toward the endosteum. Thin-walled and low-pressure bone marrow sinusoids are interconnected by intersinusoidal capillaries that are radially distributed around the draining central vein. These vascular channels, comprising a single layer of endothelium in part devoid of a basement membrane, are characterized by a high surface area and a relatively slow flow rate [71,72]. In the spleen (Figure 3B), the splenic artery branches into central arterioles, from where 80–90% of the splenic flow engages in the fast ‘closed’ microcirculation, while the remaining fraction goes through the slow ‘open’ microcirculation. In the fast microcirculation, the erythrocytes pass directly from the perifollicular zone to the venous sinus, whereas in the slow circuit erythrocytes flow to the sinuses through the cords of the red pulp. The red pulp sinuses receive blood from the cords through the sinus wall,

formed by a fenestrated lining of endothelial cells devoid of a basement membrane, positioned in parallel to the vase axis, which are connected by annular fibers running transverse to endothelial cells and by stress fibers oriented along the endothelial cell long axis. The red pulp regulates the passage of the blood cells from the red pulp cords into the sinus and back to the venous system [73]. In sinuses, the blood flows 20 times more slowly than in the fast microcirculation, where blood flow is similar to that in capillaries [74]. Some similarities between bone marrow and spleen may be relevant for gametocyte sequestration: (i) the circulation in the ample vascular structures of the sinusoids in both organs occurs at a reduced flow rate compared with that in capillaries; (ii) mature and immature red blood cells are present, to a different extent, in the extravascular spaces: in the spleen, 10–20% of the blood flow passes through the discontinuous vascular endothelium, whereas in bone marrow reticulocyte maturation occurs extravascularly; and (iii) the lining of the vascular endothelial cells physiologically mediate the passage of immature or mature red blood cells: in the bone marrow, this is the entry in circulation of mature reticulocytes moving from the erythroblastic islands located in the extravascular stroma, whereas in the spleen this is the re-entry in circulation of the erythrocytes that successfully passed the splenic slit checkpoints in the red pulp. How common processes in bone marrow and spleen may have been harnessed by *P. falciparum* to critically ensure maturation and ability to circulate for its transmission stages is discussed in the following sections.

Bone marrow as a possible cradle for gametocytes

The presence of gametocytes in the vascular and in the extravascular spaces of bone marrow has been consistently shown in biopsies from a clinical case report from one subacute *P. falciparum* malaria case [28] and in a systematic survey on 26 bone marrow specimens from fatal cases, the latter relying on molecular, immunological, and ultrastructural identification of sexual stages [30]. Importantly, in both studies, immature gametocytes were observed in significantly higher proportions in the extravascular compartment [28,30]. A related *in vitro* observation is that *P. falciparum* is able to progress from asexual stages to the first steps of gametocytogenesis in the erythrocyte progenitors that naturally inhabit the bone marrow stroma [30,75,76]. Present knowledge is insufficient to answer the fundamental question of whether early gametocytes actively ‘home’ to their sequestration sites or alternatively are

269 formed there by residing asexual parasites [30,77]. This is a difficult question to address
270 because no molecular markers are yet available to identify sexually committed ring stages
271 before the appearance, at 24 h p.i., of the first markers of sexual differentiation [78].
272 Nevertheless, the information gained on host cell remodeling and changes in cell mechanical
273 properties might provide cues and hypotheses for both scenarios.
274 In the 'homing hypothesis', residual PfEMP1 molecules on the surface of stage I gametocyte-
275 infected erythrocytes might mediate low-affinity interactions with endothelial cells in the slow
276 circulation of the marrow sinusoids, as similarly shown for asexual stages in knob-less and
277 KAHRP-less parasites, where these were sufficient to mediate adhesion to CD36 and ICAM1
278 under static, but not 'under-flow', conditions [79]. Once interactions are established, the
279 rigidity of the immature gametocytes might ensure bone marrow retention. Cells with
280 decreased deformability are prone to form aggregates in low shear stress regions, suggesting
281 that increased early gametocyte rigidity promotes a crucial hemorheological behavior in bone
282 marrow sequestration [80]. In a different context, the elongated red blood cells in sickle cell
283 disease were shown to aggregate and accumulate with an efficiency inversely related to the
284 applied shear flow [81] and the onset of vasocclusion by sickle erythrocytes was reported to
285 be associated with their enhanced ability to adhere to endothelial cells [82].
286 In summary, the increasing rigidity of the gametocyte-infected erythrocytes during
287 development might either stabilize the initial low-affinity binding interaction of stage I
288 gametocytes, or even be itself sufficient for an adhesion-independent sequestration solely
289 because of its mechanical properties. However, it cannot be ruled out that as yet unidentified
290 gametocyte adhesins mediate binding of the early sexual parasites to host cells, although
291 probably non endothelial, as suggested by failure of stage I gametocytes to bind to
292 endothelial cells from different organs, including bone marrow [38].
293 The detection of immature gametocytes in the bone marrow extravascular environment
294 requires that the above 'homing hypothesis' accommodates a step of extravasation of the
295 early gametocytes to the bone marrow stroma. Crossing of the bone marrow sinusoid lining
296 by uninfected mature red blood cells has however not been described, to our knowledge, to
297 possibly propose a mechanistic model for this step. Assuming that the physical constraints
298 involved are comparable to those experienced by maturing reticulocytes traversing the

299 endothelium in the opposite direction, it is predicted that only ring-stage parasites are
300 sufficiently deformable [83, 84] to extravasate successfully.

301 As an alternative to the 'homing hypothesis', it was proposed that asexual parasites
302 sequestered in the bone marrow are able (or even are induced by that microenvironment) to
303 produce a sexually committed progeny [77]. This hypothesis has been refined to suggest that
304 such asexual parasites may in fact reside in the bone marrow stroma, where they multiply in
305 erythroid precursors [30] .

306 Although the kaleidoscope of hypotheses on the origin and localization of immature
307 gametocytes awaits experimental assessment, current information on gametocyte
308 deformability provides cues on the mechanisms enabling stage V gametocytes to leave the
309 sequestration sites and circulate in peripheral blood [50]. All models proposing an
310 extravascular sexual development [28,30] imply that stage V gametocytes have to enter the
311 microvasculature through the sinusoid endothelium. In this respect, the switch in gametocyte
312 deformability associated with the transition from stage IV to stage V is fully consistent with the
313 hypothesis that such a change in cell mechanical properties is necessary, and might be
314 sufficient, for extravasation, possibly through similar mechanisms to those used by maturing
315 reticulocytes to cross the sinusoid endothelium [85, 86].

316 **Development, retention, and passage of gametocytes in the spleen**

317 Despite early [23,24,87] and recent [30] observations of immature gametocytes in the infected
318 spleen and of immature gametocytes in the peripheral circulation of a splenectomized malaria
319 patient [27], specific hypotheses on the role of this organ in the development of sexual
320 parasites have not yet been formulated. Given that immature gametocytes of all stages can
321 be observed in the spleen, it is tempting to speculate that they are not cleared as efficiently as
322 asexual stages by the macrophages crowding the red pulp. Whereas the mechanical
323 properties of mature gametocytes can explain their passage through the splenic slits to
324 persist in circulation, the rigidity of immature gametocytes predicts, instead, their retention,
325 and suggest that a successful gametocyte development in the spleen must rely on a
326 mechanism to avoid splenic passage and phagocytosis. Existence of cryptic intrasplenic
327 parasite growth has been hypothesized [74], making it tempting to speculate that this might
328 produce the immature gametocytes observed in this organ.

Wherever their origin is, the ability of mature gametocytes to circulate for 3–6 days [34], surviving passage through an organ that statistically checks the deformability of each erythrocyte every 2–3 h [74], represents a demanding task and an intriguing question in *P. falciparum* biology.

Microsphiltration studies have shown that a high fraction of erythrocytes infected by *P. falciparum* ring stages can pass apertures mimicking the physical constraints experienced through the narrow splenic slits of the sinusoids. This was confirmed in an *ex vivo* spleen perfusion system that consistently showed that a large fraction of ring-stage parasites was able to pass through the spleen tight circulation [84], while an increased sphericity and a decreased surface area was shown to cause the retention of the remaining fraction of ring stages [88]. In the morphologically very different mature sexual stages, achieving a functionally equivalent high deformability is likely to require different specific cellular and molecular modifications, such as the described disassembly of the subpellicular microtubule network and the concomitant STEVOR dissociation from the erythrocyte membrane.

Concluding remarks

It is becoming clear that the molecular mechanisms used by asexual and sexual stages of *P. falciparum* to remodel their host erythrocytes differ from the very onset of the respective developmental programs. This has relevant consequences on the changes in the mechanical properties of the infected erythrocytes along the two pathways of differentiation. The rigidity of the immature gametocyte-infected erythrocytes and the failure of these cells to cytoadhere on endothelial cells through the same ligands used by asexual parasites are proposed to contribute to the specific sequestration of immature gametocytes in internal organs such as the bone marrow. The switch to an increased deformability of gametocytes at the end of their long maturation is possibly involved in their release from the sequestration sites and it is relevant to ensure several days of undisturbed circulation in the blood stream to the mature gametocytes. Although many questions remain, future work should shed light on the molecular basis of host cell remodeling induced by *P. falciparum* gametocytes, which opens new avenues in the identification of targetable processes to interrupt parasite transmission and the spread of malaria.

Acknowledgments

MLT is supported by a fellowship from the EU EVIMalaR Network of Excellence. Research in CL laboratory is supported by CNRS, INSERM, the Labex GrEx and the Bill & Melinda Gates Foundation and in PA laboratory by the Bill & Melinda Gates Foundation and the EU EVIMalaR Network of Excellence. Authors thank Cosimo Marino Curianò, Istituto Superiore di Sanità, Rome, Italy, for the artwork in Figures 1–3.

Glossary:

Ektacytometry – A technique used to quantitatively measure the average elongatibility of populations of cells in suspension under increasing shear stresses. A laser beam directed through the sheared cell population generates a diffraction pattern and the elongation efficiency at increasing shear stresses, or elongation index (EI), is defined as: the ratio between the difference of the 2 axes of the ellipsoid diffraction pattern and the sum of these two axes, $EI = (L - W)/(L + W)$, where L and W are the length and the width of the diffraction pattern, respectively. The elongation index curve is obtained after the calculation of EI at the different applied shear stresses [52,89,90,91].

Gametocytogenesis – The developmental process leading to the formation of parasite sexual blood stages, the gametocytes, from a population of asexual blood stages. Experimental evidence in *Plasmodium falciparum* indicate that the developmental decision to alternatively develop as asexual or sexual stages occurs in the trophozoite to schizont stage, leading to the production of a developmentally committed offspring of merozoites. Experiments in the rodent parasite *Plasmodium berghei* are instead consistent with a model in which the individual merozoite is not committed and developmental cues post erythrocyte invasion influence its subsequent development.

Host Targeting/*Plasmodium* EXport ELe ment– The aminoacid motif found in the N-terminal portion of several Plasmodium proteins, whose consensus sequence is RxLxE/Q/D, computationally identified and functionally shown to mediate trafficking of parasite proteins from the endoplasmic reticulum to the cytoplasm of the infected erythrocyte, across the parasite parasitophorous vacuole [92,93].

388 **Meiotic reassortment** – Generation of novel combinations of parental genetic traits in the
389 offspring obtained by recombination between homologous portions of parental chromosomes
390 paired in the zygote nucleus during the process of meiosis. In malaria parasites the zygote is
391 produced by the fusion of a motile microgamete and a spherical macrogamete in the
392 mosquito gut after the mosquito blood meal.

393 **Micropipette aspiration** – Technique used to provide quantitative information on the
394 rheological properties of individual erythrocytes. Micropipette aspiration enables the
395 measurement of the membrane elastic shear modulus, the overall cell deformability, as well
396 as of parameters such as the time constant for erythrocyte shape recovery, membrane
397 surface viscosity, cell volume and membrane surface area [52].

398 **Microsphiltration** – Technique based on the filtration of erythrocytes through a mixture of
399 calibrated metal microspheres (96.50% tin, 3.00% silver, and 0.50% copper) with two different
400 size distributions (5–15- μm diameter and 15–25- μm diameter) that mimic the geometry of
401 inter-endothelial splenic slits, generating a 1.85- μm -wide aperture. The filterability of a
402 population of cells through this matrix is measured under flow and its readout is the ‘retention
403 rate’, which positively correlates with the rigidity of a cell population [94,95].

404 **Parasitophorous vacuole** - Subcellular compartment of the infected erythrocyte originated
405 by the invagination of the erythrocyte membrane during the process of merozoite invasion,
406 inside which the parasite develops until schizogony in the asexual cycle or until gamete
407 egress in the mosquito gut in sexual development.

408 **Ring stage** – The *P. falciparum* intraerythrocytic stage developing in the first approximately
409 20 hours post merozoite invasion; the parasite morphology resulting from the Giemsa staining
410 of the thin circular cytoplasmic portion and the parasite nuclear dot in thin blood smears is
411 reminiscent of that of a ring (Figure 1).

412 **Schizont** - The stage of *P. falciparum* intraerythrocytic asexual development from the onset
413 of parasite nuclear division to the production of the progeny of individual mature merozoites
414 (Figure 1) ready to disrupt the surrounding parasitophorous vacuole and erythrocyte
415 membrane in the process of schizogony.

Spectrin Network –The cytoskeletal protein network localized at the cytoplasmic surface of the erythrocyte where heterodimers of the spectrin protein associate end-to-end to form tetramers, which are then linked by actin and band 4.1 proteins [96].

Splenic Red Pulp – One of the spleen main compartment, composed of a three dimensional meshwork of splenic cords and venous sinuses associated with phagocytic macrophages actively removing senescent or damaged erythrocytes and blood-borne particulate matter [97].

Trophozoite – The stage of *P. falciparum* asexual intraerythrocytic development in which the parasite is actively degrading haemoglobin and polymerizing the residual protoporphyrin IX as inert haemozoin crystals, the malaria pigment. In this review the term trophozoite is used to distinguish the intraerythrocytic parasite from the younger ring stage, where pigment is still undetectable, and from the subsequent schizont stage, characterized by onset of parasite nuclear division (Figure 1).

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Figure 1. Early divergence in the pathways of host cell remodeling in *Plasmodium falciparum* asexual and sexual development.

Upon invasion of the uninfected erythrocyte, the merozoite committed to asexual growth turns into a ring-stage parasite, which activates expression and export of the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) adhesive ligand, produced from the transcript of one of the 60 *var* genes, and assembles knob structures on the erythrocyte surface. This pattern of erythrocyte remodeling enables the asexual parasite to adhere efficiently to endothelial cells from approximately 24 h p.i., and to sequester from the circulation. In the alternative pathway of sexual differentiation, the expression of the entire *var* gene repertoire is downregulated, and both production of the Knob Associated Histidine Rich Protein (KAHRP) and erythrocyte surface exposure of PfEMP1 are undetectable in stage I gametocytes. The sexual pathway of host cell remodeling results in the failure of stage I gametocytes to adhere to endothelial cells double positive for thrombospondin receptor (CD36) and Intercellular Adhesion Molecule 1 (ICAM-1).

Figure 2. Cellular and molecular rearrangements and modifications in cell mechanical properties in the transition from immature (stage IV) to mature (stage V) gametocytes. (A) Diagram of an immature stage IV gametocyte (left) showing the network of subpellicular microtubules (MTub) and the association of the exported STEVOR proteins with the membrane of the infected erythrocyte. In the subsequent transformation into a stage V gametocyte (right), the subpellicular microtubule network is disassembled, and association of STEVOR proteins to the erythrocyte membrane is no longer detectable. (B) Scanning electron microscopy photographs of a stage IV (left) and a stage V gametocyte (right). Notice the pointed edges of the immature gametocyte that disappear in the transition to stage V.

Scale bar = 1 μ m. (C) Bright-field microscopy photographs of glutaraldehyde-fixed gametocytes isolated in their passage through the microsphere mesh in a microsphere column. Stage IV gametocytes retain a normal shape (left) in the microsphere mesh, where they are efficiently retained. By contrast, mature gametocytes (right) are more deformable, consistently with their poor retention in the microsphere mesh. Notice that the intrinsic high deformability of the parasite cell contributes to the overall mechanical properties of the mature gametocyte-infected erythrocyte. Scale bar = 5 μ m. Abbreviations: IMC: inner membrane complex, the bilamellar membrane structure underlying the gametocyte plasma membrane; OB, osmiophilic bodies, the secretory organelles accumulating in stage IV-V gametocytes. (B) is reproduced, with permission, from Sylvie Perrot, Institut Pasteur.

Figure 3. The structure of blood circulation and schematic representation of the main cell types in human bone marrow and spleen.

(A) Bone marrow. (i) Diagram of a portion of the venous and arterial vasculature in bone. (ii) Diagram of a cross section from (i), showing irregularly shaped sinusoid vessels and their endothelial lining, embedded in the marrow stroma. Typical cell types of the bone marrow microenvironment are shown, such as pericytes juxtaposed to the stromal side of the endothelial cell lining, adipocytes, megakaryocytes, and erythroblasts, the latter associated with erythroblast islands surrounding a centrally located nursery macrophage. Adjacent to these islands, reticulocytes are depicted in the process of crossing the endothelial barrier to enter blood circulation. (B) Spleen. (i) Schematic representation of a portion of the arterial and venous vasculature in the white and the red pulp of the human spleen. (ii) Diagram of a section across (i) showing the sinusoids, hosting the blood 'closed' circulation. Sinusoids are shown embedded either in the white pulp, characterized by the abundant presence of lymphocytes, or in the red pulp, where large numbers of extravascular erythrocytes are present, flowing in the slow, 'open' circulation. Based on images from [72,98,99] in (A) and from [74,100] in (B).