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1 ***Plasmodium falciparum* ring stage plasticity and drug resistance**

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18
19 **Keywords:** *Plasmodium falciparum*, ring stage, temporary growth arrest, antimalarial
20 drug resistance, artemisinin, cell-cell communication.

21 22 **Abstract**

23 Malaria is a life-threatening tropical disease caused by parasites of the genus
24 *Plasmodium*, of which *P. falciparum* is the most lethal. Malaria parasites have a
25 complex life cycle, with stages occurring in both the *Anopheles* mosquito vector and
26 human host. Ring stages are the youngest form of the parasite in the intraerythrocytic
27 developmental cycle and are associated with evasion of spleen clearance, temporary
28 growth arrest, and drug resistance. This formidable ability to survive and develop into
29 mature, sexual, or growth-arrested forms demonstrates the inherent population
30 heterogeneity. This review highlights the role of the ring stage as a crossroads in
31 parasite development and as a reservoir of surviving cells in the human host via
32 temporary growth arrest survival mechanisms.

35 ***Plasmodium falciparum* antimalarial drug resistance**

36 Malaria is a major public health issue in tropical countries and a potentially fatal
37 disease caused by protozoan parasites from the genus *Plasmodium* [1,2]. Among the
38 six *Plasmodium spp.* that infect humans, *P. falciparum* is responsible for most severe
39 cases. Globally, an estimated 247 million malaria cases were recorded in 2021,
40 leading to 619,000 deaths, primarily in sub-Saharan Africa [2].

41 Efforts to eliminate malaria have been strengthened and intensified with the support
42 of the World Health Organization (WHO) [2]. Novel and complementary interventions
43 have been implemented, including the distribution of long-lasting insecticide-treated
44 nets, prompt diagnosis, and effective treatments such as artemisinin combination
45 therapies (ACTs). Currently, ACTs are important for controlling malaria morbidity and
46 mortality in endemic countries. However, progress has been stalled, and the number
47 of cases is estimated to have increased in recent years [2]. A major contributor is the
48 increasing resistance of malaria parasites to most available antimalarial drugs [2].

49

50 This review aims to summarize our current knowledge of the classical mechanisms
51 of drug resistance based on genetic modification and to emphasise alternative
52 survival strategies relying on growth arrest phenotypes, especially in the parasite
53 ring stage.

54

55 ***Antimalarial drugs as weapons against P. falciparum malaria blood-stage parasites***

56 Antimalarial treatments have been used for centuries mainly with natural plant
57 products. As a result of these scientific advances, chemical compounds with
58 antimalarial activity, such as quinine, were first used and then replaced with new
59 chemically synthesised compounds. Commonly used blood-stage antimalarial drugs
60 are shown in **Figure 1**.

61

62 **The 4-aminoquinolines** including chloroquine (CQ), amodiaquine (AQ), piperaquine
63 (PPQ), and pyronaridine (PND), are active against the parasite erythrocytic stages,
64 except for mature gametocytes. These molecules share a similar mode of action by
65 acting exclusively against intraerythrocytic stages, during which the parasite actively
66 degrades haemoglobin. Multiple studies have described these molecules as potent
67 heme-crystallisation inhibitors [3-6]. Inhibition of heme crystallisation induces parasite
68 death via accumulation of toxic heme following haemoglobin proteolysis in the food

69 vacuole (FV). Additionally, chloroquine has been suggested displays the property to
70 interact with the DNA double strand and acts as an intercalant with cytotoxic effects
71 on cells by suppressing DNA replication [7].

72

73 **Aryl-amino-alcohols** (quinine QN, mefloquine MQ, and lumefantrine LUM) have less
74 well understood mechanisms of action. They appear to partially interfere with
75 hemozoin formation and detoxification of haemoglobin degradation by-products, which
76 is a secondary aspect of their mode of action. A recent *in vitro* study suggested that
77 MQ specifically targets the GTPase catalytic site located on the 80S ribosomal subunit
78 of the parasite, thereby inhibiting protein biosynthesis [8]. However, this finding was
79 challenged by Sheridan et al., who reported that MQ had no effect on the 80S
80 ribosomal subunit *in vitro* in *P. falciparum* [9].

81

82 **Sulfadoxine (S) and pyrimethamine (P)** are two antifolate compounds that were
83 used in combination (SP) against CQ-resistant parasites from the 1960s to the 1980s
84 [10]. S and P inhibit the activities of dihydrofolate reductase (DHFR) and
85 dihydropteroate synthetase (DHPS), respectively, two key enzymes involved in the
86 folate pathway responsible for nucleic acid biosynthesis [10,11]. This combination is
87 currently recommended for malaria chemoprevention during pregnancy [10,11].

88

89 **Atovaquone (ATV)** is a ubiquinone analog that acts on parasite mitochondria. ATV
90 selectively inhibits cytochrome *bc1* in the respiratory chain, thereby blocking ATP
91 synthesis [12]. This inhibition causes a collapse of the mitochondrial membrane
92 potential but also inhibits pyrimidine biosynthesis by depriving the parasite enzyme
93 dihydroorotate dehydrogenase of its cofactor ubiquinone. ATV has no activity against
94 female sexual stages but may be active against male gametocytes [13,14]. ATV has
95 also been described as a potent inhibitor of ookinete formation [15].

96

97 **Artemisinin (ART)** and its derivatives (artesunate, dihydroartemisinin, artemether)
98 used in combination with partner drugs (AQ, LUM, MQ, PPQ, SP, or PND) are the
99 current global standards for the treatment of uncomplicated *P. falciparum* malaria
100 [16]. ART is a sesquiterpene lactone with an internal peroxide bridge, extracted from
101 the leaves of *Artemisia annua*. They are highly potent, short-lived, and fast-acting
102 antimalarial drugs capable of reducing *P. falciparum* biomass by up to 10 000-fold

103 per 48-hour asexual blood stage cycle [17]. Dihydroartemisinin (DHA), the major
104 active metabolite of all ARTs, is activated by the cleavage of its endoperoxide bridge
105 by free heme released during haemoglobin digestion and is responsible for
106 antimalarial activity [18]. Once cleaved, ART causes non-specific alkylation of
107 parasite biomolecules such as lipids, proteins, and DNA, leading to parasite death
108 [19,20]. ART is active against all parasite blood stages, including the ring and sexual
109 stages, except stage V gametocytes [21,22].

110 The first cases of ART partial resistance (ART-R) in *P. falciparum* were reported in
111 Southeast Asia in the 2000s [23,24], followed by reports in sub-Saharan Africa,
112 notably Rwanda [25,26], Uganda [27,28], and Eritrea [29]. Clinically, ART-R is
113 defined as an increase in the parasite clearance half-life [1] or the persistence of
114 microscopically detectable parasites on day 3 following the initiation of the 3-day
115 ACT regimen) [2]. Further studies have concluded that ART-R is associated with
116 decreased susceptibility to ring stages [19,30]. The ring-stage survival assay (RSA),
117 which measures the susceptibility of 0–3 h post-invasion ring stages to a
118 pharmacologically relevant concentration (700 nM) of DHA for 6 h, showed a strong
119 association between reduced clearance of clinical ART-R parasites and reduced *in*
120 *vitro* killing of ring stages by DHA [30]. In contrast, *in vitro* RSA showed that the late
121 rings and trophozoites remained susceptible to DHA. Subsequent genomic studies
122 confirmed a direct link between several mutations in the propeller domain of the
123 *Pfkelch13* gene and ART-R, with a limited subset of parasites able to survive short
124 ART exposures [31,32]. The 580C>Y mutation appears to be the most common
125 mutation in Cambodia, increasing parasite survival upon ART exposure by ~5 fold
126 compared to the wild type (WT) [33]. The ART-R parasite exhibits an uncommon
127 phenotype *in vitro*, consisting of a subset of ring stages that undergo transient
128 developmental arrest after DHA exposure and resume growth after drug removal
129 [34]. As the main ART-R biomarker, *Pfkelch13* has been extensively studied for its
130 functional characterization. However, the exact function of *Pfkelch13* remains
131 unclear. Different laboratories have associated ART-R with increased unfolded
132 protein response [35,36], altered mitochondrial physiology [37], PI3K/PI3P/AKT [38],
133 and PK4/eiF2 α [39] pathways, extended ring-stage development, followed by faster
134 progression to trophozoite stages [36,40]. Although not fully understood, [41] it is
135 currently thought that *Pfkelch13*-mediated ART-R consists of reduced activation of
136 ART through lower hemoglobin uptake, and mutations in the *Pfkelch13* gene

137 reduced protein levels and decreased hemoglobin endocytosis, thereby favouring
138 ring stage survival [42,43].

139

140 **Primaquine**, an 8-aminoquinoline derivative, is currently the only drug
141 recommended by the WHO as a gametocytocidal treatment for the prevention of *P.*
142 *falciparum* malaria transmission [93] and is the main treatment for the radical cure of
143 malaria caused by *P. vivax* and *P. ovale* hypnozoite liver stages [16]. Primaquine is a
144 prodrug activated by cytochrome P45, an enzyme that produces hydroxylated active
145 metabolites that generate hydrogen peroxide (H₂O₂). Active metabolites cause
146 significant oxidative stress in parasites and in human cells. Primaquine, when used
147 as a radical cure, is not recommended for glucose-6-phosphate dehydrogenase-
148 deficient individuals, as they may develop acute hemolytic anaemia. The related
149 compound tafenoquine may be a future alternative to primaquine owing to its
150 improved safety profile [44].

151

152 *Antimalarial drug resistance: The parasite's defence for survival*

153 Malaria control and elimination efforts have made significant advances in the recent
154 decades. However, the emergence and spread of antimalarial drug resistance is a
155 major concern. *P. falciparum* has evolved resistance to almost all antimalarial drugs
156 [45]. Several resistance mechanisms have been identified, including active transport
157 of the drug away from its target and alteration of the parasite target, both of which are
158 mediated by nucleotide polymorphisms (SNPs) and copy number variations (CNVs) in
159 the genes encoding these proteins (**Table 1**). In addition, non-specific stress
160 response-based survival mechanisms, such as temporary growth arrest (TGA)
161 phenotypes, have been shown to be linked to drug resistance and survival.

162

163

164 ***P. falciparum* ring stages growth arrest and drug resistance, what do we not 165 know?**

166 *P. falciparum* ring stage: the most resilient and adaptable parasite stage

167 The ring stage is the first stage of the intraerythrocytic developmental cycle (IDC)
168 following merozoite invasion of erythrocytes. It establishes an intracellular niche, the
169 parasitophorous vacuole (PV), which is formed by invagination of the host cell
170 membrane [46] and later modified through lipids addition by the parasite [47]. Over

171 the next 20h, the parasite assumed a typical ring-shape. The ring stage parasite then
172 begins to import haemoglobin by endocytosis with a cellular structure called the
173 cytostome to support its metabolism [48,49]. Nutrient transport channels, including
174 PSAC, EXP1 or hGLUT1 also provide key nutrients (isoleucine, glucose, and ions)
175 from the host serum and serve as possible entrances for drugs. Their expression at
176 a relatively late stage during the ring stage could be a possible explanation for drug
177 resilience as reviewed elsewhere [50].

178 To date, almost all available antimalarial treatments have been unable to specifically
179 target the ring stage [51] (**Figure 1**). Current drugs mainly target FV and are most
180 effective against trophozoites and schizonts due to their high haemoglobin
181 consumption rate [51]. In contrast, metabolite inhibitors, such as SP or ATV, inhibit
182 parasite growth rather than specifically eliminating the ring stage [51]. Currently, ART
183 is the only drug that has direct cytotoxic activity in the ring stage [17] although some
184 candidates such as cipargamin (KAE609) and BCH070 have shown promising
185 results in killing ring stage parasites [52,53]. Among the intraerythrocytic cycles, the
186 ring stage appears to be the most adaptable and resilient: first through splenic
187 clearance evasion [54], second, through its ability to orchestrate a metabolic switch,
188 including the shutdown of core metabolic functions accompanied by high RNA
189 expression in specific pathways, third, through its ability to modulate the immune
190 system through vesicle-mediated intracellular transport [36,55-57] and fourth, by
191 adjusting cell cycle progression [58,59].

192 The cessation of *P. falciparum* parasite progression from the ring to the trophozoite
193 in IDC, defined as ring stage TGA, has been reported in many biological situations.
194 Isoleucine deprivation, an essential amino acid that must be scavenged from the
195 extracellular medium, was directly associated with a reversible ring stage TGA
196 phenotype [60]. Furthermore, experiments with parasites exposed to extreme
197 temperatures ranging from +4°C to +40°C showed that only the ring stage resumed
198 growth and maintained cell viability when the temperature returned to physiological
199 conditions at +37°C [59,61]. TGA phenotypes of malaria parasites have been
200 reported in other *Plasmodium* species (**Table 2**). This suggests that reversible G0
201 cell cycle arrest is a general strategy used by *P. falciparum* ring stage parasites to
202 cope with environmental stress, independent of drug resistance-causing mutations.

203

204 *Drug-induced ring stage TGA: a drug resistance strategy*

205 TGA can be defined as a phenotype based on the cellular mechanisms by which a
206 microorganism can reversibly halt its growth under adverse conditions (**Box 1**). In
207 *Apicomplexa*, this survival strategy has been well described in the bradyzoite stage of
208 *Toxoplasma gondii* [62] , hypnozoites of *Plasmodium vivax* [63], and multiple other
209 *Plasmodium* species (**Table 2**). Research on TGA in *P. falciparum* is still in its infancy.
210 *P. falciparum* blood stage TGA was firstly suggested by Nakazawa et al., who
211 observed parasite recrudescence following SP treatment without detecting any genetic
212 changes in the surviving population [64]. The TGA hypothesis was subsequently
213 validated *in vitro*, which showed delayed progression of ring stages following ART
214 exposure [30,34]. *Pfkelch13* wild type (WT) and mutant ring stages were able to enter
215 growth arrest-mediated processes, but isogenic *Pfkelch13* mutants shifted the
216 subpopulation distribution dramatically, allowing more ring stages to arrest and
217 resume growth after drug removal *in vitro* [30,31]. This has been microscopically
218 documented in ring stages, as evidenced by delayed ring stage progression and
219 unaltered morphology at 24h post-invasion, using a 24-hour DHA exposure at 700 nM
220 [30]. Nevertheless, the potential dose-response of the ring stage and TGA induction
221 when exposed to DHA should be tested, which could provide useful insight into the
222 biological mechanisms that govern TGA.

223 Other studies have also reported the recovery of TGA ring stages for up to several
224 weeks after a single pulse of ART [34,37,65-71]. These results differ significantly
225 from the TGA phenotype initially observed, with an immediate resumption of growth
226 after drug removal [30]. These conflicting results can be explained by the fact that
227 different experimental parameters were used to induce the TGA. However, it remains
228 unclear whether these phenotypes are biologically similar or distinct. From this
229 perspective, the use of a more general term such as TGA may better reflect the
230 observed phenotype and avoid confusion until the different biological mechanisms
231 behind TGA are unveiled (**Box 1**). Nevertheless, one of the most concerning
232 conclusions drawn from these studies is that TGA could be a mechanism that
233 maintains a reservoir of recrudescence parasites in the human host, which lead to
234 therapeutic failure [71].

235

236 Consequently, many questions remain regarding the TGA. Why does only one
237 subpopulation undergo TGA in an isogenic population? What is the duration of the
238 TGA? However, a theoretical framework of three key steps can be suggested: i) the

239 initial stress inducing TGA, ii) the maintenance in a growth-arrested state for a given
240 time, and iii) the resumption phase when the stress is removed.

241 Metabolic and transcriptomic studies of TGA rings at different times following stress
242 could provide useful data to understand the duration of this phenotype. The link
243 between TGA and *Pfkelch13*-mediated ART-R is unclear, but it could be hypothesised
244 that TGA is mediated by the haemoglobin consumption rate, which is altered in
245 *Pfkelch13* mutant strains. Indeed, ART-R *Pfkelch13* C580Y ring stage have lower
246 haemoglobin uptake than the *Pfkelch13* WT strain [42,43], which limits the chemical
247 activation of ART by free heme, thus promoting ring stage survival. To overcome the
248 reduced nutrient uptake through haemoglobin endocytosis, *Pfkelch13* C580Y showed
249 nutritional compensatory mechanisms through the expression of EXP1, a parasite FV
250 transmembrane protein that imports amino acids from host serum [72]. Such a
251 mechanism may provide sustenance during the TGA. Lastly, *Pfkelch13* mutation
252 appears to be particularly important for the ring stage development as an impaired
253 K13 protein results in a slower progression of the ring stage *in vitro* [42].

254 A recent metabolic study of the ART-sensitive and ART-R ring stages induced by a
255 single pulse of DHA suggested that arrested parasites rely on a different energy
256 metabolism than parasites growing under normal conditions [55]. In particular, the
257 authors showed that the TGA ring stage is in a metabolically active state that relies on
258 alternative nutrient sources such as haemoglobin and glycolysis, and TGA and
259 *Pfkelch13*-mediated ART-R may be distinct but complementary mechanisms that
260 promote survival in the population by reducing ART sensitivity. Additionally, another
261 key work provides new insights into the transcriptome of the TGA ring induced by ART,
262 with the parasite requiring 4-5 days to differentiate into the TGA ring, expressing
263 signatures associated with DNA repair and repressed glycolysis [73]. More recently,
264 a study identified a novel gene (PF3D7_1136600, named MRST for Modulator of Ring
265 Stage Translation) associated with changes in parasite translational metabolism
266 during ART drug response, with a possible link to parasite stress response
267 mechanisms, supporting its role as an essential modulator of ring stage translation
268 [74]. From this perspective, it could be relevant to study the involvement of MRST in
269 the TGA molecular pathways. Strikingly, an *in vitro* study showed that pre-exposure to
270 low temperature (+4°C) prior to DHA exposure at a clinical dose of DHA (700 nM)
271 resulted in a significant increase in the parasite survival expressed by the RSA in
272 *Pfkelch13* WT (ART-S) and *Pfkelch13* mutant ART-R ring stages [75], suggesting that

273 TGA can be induced by low temperature and may be a potential *Pfkelch13*
274 independent mechanism.

275 Collectively, these data suggest that TGA may be a generalist strategy not necessarily
276 based on genetic changes but rather an environmentally induced mechanism. This
277 hypothesis may explain why TGA was observed in a fraction of the ring stage
278 population under adverse conditions in a clonal population. As TGA has been reported
279 in *P. falciparum* ring stages under various stresses, it could be speculated that the
280 parasite is able to actively sense its environment (**Figure 2**), suggesting the possible
281 induction from the extracellular compartment.

282

283 *Is TGA triggered externally? Individuals die, and the population survives*

284 Is the TGA phenotype observed after ART exposure caused by the drug itself or by a
285 signal emitted by a stressed parasite population? Such a question is currently
286 unanswered, but it highlights the internal heterogeneity within a clonal parasite
287 population, as it implies that healthy, stressed, and dying parasites share the same
288 environment. This heterogeneity between growing and arrested parasites could be
289 explained by environmental signals. A similar model has already been described for
290 *Trypanosoma brucei*, in which small oligopeptides from the extracellular medium are
291 used as communication signals to induce parasite differentiation from slender to
292 stumpy forms [76]. Despite the lack of direct evidence between TGA and
293 extracellular signalling, several *in vitro* studies have described the innate ability of *P.*
294 *falciparum* to sense and respond to its environment in different biological contexts.
295 The sexual maturation of *P. falciparum* has been linked to extracellular factors.
296 Xanthurenic acid (XA) is present in human serum and in mosquitoes, and XA
297 concentration has been linked to pH changes and temperature drops from the
298 human host to the mosquito, which is responsible for the maturation of the parasite
299 sexual stage [77]. Another example was found *in vitro* using the human host
300 compound lysophosphatidylcholine (LPC) as an environmental factor that drives
301 sexual commitment in *P. falciparum* parasites during the blood stage [78]. A direct
302 relationship between LPC levels and parasite response was demonstrated *in vitro*,
303 with the parasite maintaining its asexual growth when LPC is not limiting and
304 undergoing gametocyte differentiation upon LPC shortage, particularly in the host
305 bone marrow where LPC levels are constitutively low. Following isoleucine
306 starvation, the parasite can carry out TGA until the environment becomes more

307 favourable [60], meaning that parasites are somehow able to sense when the
308 extracellular medium becomes limiting for isoleucine *in vitro*. In response, the
309 parasite actively expresses the RNA polymerase III repressor *Maf1* during amino
310 acid starvation induced TGA to stop RNA transcription, thereby limiting cellular
311 biological activity and promoting survival [79,80]. Quorum sensing (QS) is another
312 type of extracellular sensing. When cultured under optimal conditions, the parasite
313 population cannot reach 100% parasitemia before collapse. This collapse phenotype
314 was shown to be due to the specific expression of apoptotic genes in the population
315 [81,82], ruling out the involvement of nutrient deprivation, as the parasite was
316 cultured under non-limiting nutrient conditions. It has been suggested that QS
317 between parasites that keeps the population under control is mediated by molecules
318 of the homoserine lactone family.

319 Interestingly, TGA appears to be modulated by *ex vivo* exposure to phytohormones
320 such as abscisic acid (ABA) and gibberellic acid (GA), as shown in a recent *in vitro*
321 study [83]. The addition of either ABA or GA accelerated the recovery time of ART-
322 induced ring stage TGA compared to ART alone, whereas the plastid-targeting
323 herbicide fluridone had the opposite effect. This supports the idea that growth-
324 arrested ring stages are still able to sense their environment, despite reduced
325 metabolic activity. It also identifies the apicoplast as an important player in TGA
326 regulation. ABA and GA have been described as stress hormones in plants,
327 particularly during abiotic stress [84]. However, their role in malaria infection,
328 particularly in TGA regulation, is unknown. Additional studies are needed to confirm
329 whether malaria parasites can produce and disseminate phytohormones in their
330 environment as a signal that promotes TGA. This hypothesis is supported by the
331 relatively close relationship between algae and the apicoplast, an organelle obtained
332 by primary endocytosis in Apicomplexa [85]. Another recent study showed that
333 cripowellins, a pesticide extracted from *Crinum erubescens*, induced reversible
334 inhibition of ring stage growth within 24 h of treatment while being cytotoxic in the
335 trophozoite and schizont stages [86]. Whole transcriptome analysis confirmed that
336 cripowellin B could reversibly halt the ring stage transcriptional program required for
337 trophozoite progression.

338 Extracellular vesicles (EVs) containing both host and parasite materials (proteins,
339 lipids and nucleic acids) have been found in the extracellular medium of malaria-
340 infected cells and have been shown to act on immune cells [56,57,87,88], suggesting

341 the existence of an intercellular communication network between the parasite and
342 human host [88]. Additional studies have demonstrated the ability of *P. falciparum*
343 ring stage parasites to exchange genes via EVs. PfPTP2, which is involved in
344 molecular trafficking, is an important molecular factor that controls these processes
345 [57]. Finally, the parasite protein PfAB4 has been described as a potential
346 environmental sensor because of its ability to sense serum depletion, osmotic
347 changes, and cytokine concentrations in extracellular medium *in vitro* [89]. This
348 sensing ability is mediated by multiple phosphorylation events that occur in the
349 proteins localised in the cytoplasm of infected erythrocytes. However, the association
350 between PfAB4 expression and TGA remains unclear.

351

352 Taken together, these data suggested the existence of an extracellular communication
353 network and environmental sensing in *P. falciparum* blood-stage parasites. The
354 molecules, their signalling pathways, and their potential involvement in TGA remain
355 elusive. However, the existence of such extracellular communication implies
356 heterogeneity within the parasite population; only parasites that receive signals
357 respond accordingly. In the case of TGA, this implies that such a signal is produced
358 shortly after stress exposure to ensure that as many parasites as possible receive the
359 signal and enter TGA before they die, thereby making the signal concentration and
360 diffusion key parameters. As shown previously, the ring stage is exposed to many
361 extracellular factors (**Figure 2**) and can respond to TGA, sexual commitment, or
362 asexual growth. This makes the ring stage a true crossroad in the parasite's asexual
363 blood cycle (**Figure 3**).

364

365

366 **Concluding remarks**

367 The ring stage exhibits extreme plasticity under adverse conditions. CNVs and SNPs
368 associated with antimalarial drug resistance are heritable and confer survival to almost
369 all individuals exposed to the drug, whereas TGA, an innate and passive resistance to
370 many stressors, ensures that a small proportion of individuals in the population can
371 survive in the host under a wide range of sudden adverse conditions.

372 This suggests that TGA relies on environmental sensing (**Figure 2**), including
373 temperature, small diffusible molecules (hormones or cytokines), nutrient availability,
374 population density, drug exposure, and cell fate at the ring stage, which can be

375 controlled by extracellular signals. The possible involvement of EVs in TGA regulation
376 requires further investigation (**see Outstanding Questions**). This makes the ring
377 stage a target of choice, as it is at the crossroads of the three known cell fates (**Figure**
378 **3**).

379

380 Targeting the ring stage in TGA is critical and specific biomarkers of TGA need to be
381 identified. Metabolomic and single-cell RNA-seq approaches could be important tools
382 to unveil the biological mechanism(s) behind TGA phenotypes and shed new light on
383 this survival strategy by providing a much-needed molecular signature. Such
384 biomarkers could provide new insights into the molecular regulatory pathways and
385 duration of TGA, as well as a better overview of the global parasite population in the
386 human host, especially in asymptomatic patients. Therefore, new therapeutic tools that
387 specifically target the ring stage in TGA are required.

388

389

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399 **Declaration of interests**

400 The authors declare no competing interests.

401

402

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733 dormancy in the rodent malaria model *Plasmodium vinckei*. *PLoS One* 6, e26689.
734 10.1371/journal.pone.0026689
735

736

737 **Glossary**

738

739 **ACTs: Artemisinin-combined therapies.** Three daily doses of a combination of
740 artemisinin and partner drugs is the therapeutic approach. The goals of antimalarial
741 treatment are to prevent drug resistance and to ensure parasite elimination. There
742 are currently six ACTs in use: ASAQ (artesunate-amodiaquine), AL (artemether-
743 lumefantrine), ASMQ (artesunate-mefloquine), DHA-PPQ (dihydroartemisinin-
744 piperazine), AS-SP (artesunate-sulfadoxine-pyrimethamine), AS-PND (artesunate-
745 pyronaridine).

746

747 **ART: Artemisinin.** ART is the main antimalarial compound in ACTs. When the
748 parasite digests haemoglobin, it releases haem, which activates ART and causes
749 indiscriminate and lethal alkylation of cell biomolecules. One of the most important
750 areas of malaria research with immediate impact is the mechanism of drug
751 resistance to ART.

752

753 **EVs: Extracellular vesicles.** The infected red blood cell releases vesicles known as
754 EVs, which are composed of protein, lipids and nucleic acids in addition to parasite
755 material. In recent years, there has been growing interest in their roles, which include
756 modulating the host immune system and demonstrating the parasite's environmental
757 adaptability.

758

759 **Pfkelch13: *Plasmodium falciparum* kelch gene (PF3D7_1343700).** The parasite
760 K13 protein, which is involved in haemoglobin endocytosis, is encoded by a gene
761 located on chromosome 13. Over the past decade, scientific interest has increased
762 significantly due to the direct correlation between ART resistance and mutations in
763 the propeller domain of K13.

764

765 **RSA: Ring stage Survival Assay.** The RSA is an in vitro phenotypic test that can
766 be used to determine whether a particular strain of *P. falciparum* is resistant to ART.
767 The test is based on a synchronous 0-3 ring stage exposed to 700 nM DHA (an
768 active metabolite of all ARTs) or DMSO (vehicle) for 6 hours, followed by a 66 hour
769 recovery period. ART resistance is validated by a parasite survival rate (RSA)
770 greater than 1%.

771

772 **TGA: Temporary Growth Arrest.** TGA is any phenotype of cell growth arrest that
773 occurs after exposure to unfavourable conditions. As a result, a wide range of
774 phenotypes that may be dependent on many biological processes are covered by

775 this broad description. The aim of such a definition is to bring clarity to a field where
776 there are multiple names for the same observed phenotype. The TGA is intended to
777 serve as a temporary definition until each growth arrest phenotype is fully
778 understood and can be described more precisely with experimental data.

779

780

781 **Box 1. Cell growth arrest: Several words for the same phenotype**

782 The TGA phenotype has been described in many models as a reversible metabolic
783 growth arrest that occurs under adverse growth conditions. Various terms, such as
784 persister cells [122], quiescence [123], diapause [124], latency, and dormancy [125],
785 have been used to describe this phenotype in a wide variety of organisms, including
786 plants, bacteria, and cancer cells, including *P. falciparum* (**Table 2**). Several
787 phenotypes of growth arrest have been reported in *P. falciparum* in different biological
788 contexts. 'dormancy', 'persister', 'latency' and 'quiescent ring' are being used to
789 describe the subpopulation of ring stage that survive ART exposure [37,90] as well as
790 amino acid starvation [79]. In this context, the term 'hibernatory state' is used to define
791 growth-arrested parasites following isoleucine deprivation [60]. It is currently unclear
792 whether these growth arrest phenotypes are based on similar or different biological
793 mechanisms. Therefore, the use of the term 'temporary growth arrest' may better
794 define these phenotypes and may be used as a consistent terminology for this family
795 of phenotypes, pending further data that may reveal the possible underlying biological
796 mechanism(s). Thus, TGA can be defined as a phenotype of reversible growth arrest
797 following stress exposure. This flexible definition allows the various growth arrest
798 phenotypes described above to be included until they can be properly characterized
799 and therefore given their own definition based on experimental data.

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810 **Table 1. List of the molecular signatures associated with antimalarial drug resistance.**

Gene	Molecular signatures of resistance	Mechanisms	Reference
<i>Plasmodium falciparum</i> chloroquine resistance transporter (<i>Pfcr</i> t)	Chloroquine resistance (CQ-R) is associated with mutations in codon 76 (K>T), combined with other mutations (74 M>I, 75 N>E, and 72 C>S)	by actively effluxing CQ from FV into the cytoplasm, maintaining the cellular process of heme crystallisation	[91,92]
	Piperaquine resistance (PPQ-R) has been linked with mutations in codons 97H>Y, 145F>I, 343M>L, and 353G>V in Southeast Asia and mutation in 350C>R in South America.		[93-97]
<i>P. falciparum</i> multidrug resistance protein 1 (<i>Pfmdr</i> 1)	Mutations in codons 86N>Y, 1034S>C, 1042N>D, and 1246D>Y	<i>Pfmdr</i> 1 gene encodes an ABC transporter (ATP-binding cassette) located in the FV membrane. Mutations in codons 86N>Y, 1034S>C, 1042N>D, and 1246D>Y have been found to decrease susceptibility to CQ and AQ but increase susceptibility to LUM, MQ, and ARTs	[98-100]
		The opposite effects of CQ and MQ have been reported; the 86N>Y mutation decreases parasite susceptibility to CQ, but increases susceptibility to MQ	[101]
	CNV	<i>Pfmdr</i> 1 gene amplification increases protein expression and has been implicated in resistance to MQ, LUM, QN, and ART, but conversely increases sensitivity to CQ and PPQ	[95,102-107]
<i>plasmepsin II and III</i>	CNV	<i>Pfpm</i> II and <i>Pfpm</i> III genes amplification are associated with resistance to PPQ (PPQ-R)	[45,108]
<i>dihydrofolate reductase (dhfr)</i> & <i>dihydropteroate synthetase (dhps)</i>	Double mutations 437A>G and 540K>E in <i>dhps</i> , associated with triple mutations in <i>dhfr</i> at residues 51N>I, 59C>R, and 108S>N, suggest that the protective efficacy of SP may be reduced. <i>dhfr/dhps</i> sextuple mutants (quintuple mutants with an additional mutation, <i>dhps</i> 581A>G) have been associated with clinical failure and loss of SP protection	The resulting structural changes in the DHFR and DHPS enzymes are located at SP-binding sites, reducing the affinity of the drugs for their targets	[109-112]
<i>cytochrome bc1</i>	Mutation in codon 268Y>N/S/C	ATV resistance (ATV-R) is mediated by structural changes in mitochondrial cytochrome bc1. Mutations have been shown to reduce both the stability of cytochrome bc1 and its affinity for ATV, leading to a drug-resistant phenotype in mutant parasites	[113-115]

811

812 **Table 2. TGA phenotypes previously described among *Plasmodium* species**

Plasmodium species	TGA phenotype	Mammalian host	Parasite stage	References
<i>P. falciparum</i>	Yes	Human	Oocyst, sporozoites (vector), Ring (blood stage)	[19,30,34,116],
<i>P. vivax</i>	Yes	Human	Hypnozoite (liver stage)	[63]
<i>P. malariae</i>	Not described	Human	N/A	N/A
<i>P. ovale</i>	Suspected	Human	Possibly hypnozoite (liver stage)	[117]
<i>P. berghei</i>	Yes	Rodent	Oocyst (vector)	[116]
<i>P. knowlesi</i>	Not described	Monkey, Human	N/A	N/A
<i>P. cynomolgi</i>	Yes	Monkey	Hypnozoite (liver stage)	[118,119]
<i>P. chabaudi</i>	Not described	Rodent	N/A	N/A
<i>P. yoelii</i>	Not described	Rodent	N/A	N/A
<i>P. vinckei</i>	Yes	Rodent	Ring (blood stage)	[120]

813 *N/A: Not available.*

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828 **Figure 1. Cellular targets and timing of antimalarial drug action.** Several
829 compounds have been used against *P. falciparum* blood stages to target a wide range
830 of cellular processes. Our current arsenal can effectively inhibit the haem
831 neutralization process, block the pyrimidine biosynthetic pathway, disrupt the
832 mitochondrial membrane potential or induce massive non-specific alkylation. The
833 exact mechanisms of action of several drugs are not fully understood and require
834 further research. Most currently used molecules target mature blood stages, while only
835 ART and its derivatives have a spectrum of action that extends to the ring stage.

836

837 **Figure 2. The ring stage develops and adapts to different signals in the**
838 **extracellular medium.** The extracellular medium is a complex and diverse
839 environment to which the ring stage parasite must adapt. Some of the known
840 components of the parasite extracellular medium are nutrients (glucose, hypoxanthine
841 and amino acids), secreted vesicles, host immune proteins (such as antibodies and
842 complements) and small soluble molecules. The extracellular medium is altered,
843 particularly by the host's diet, which directly affects the availability of nutrients in the
844 peripheral circulation, which in turn affects the parasite's ability to grow. In addition,
845 acute malaria infection leads to increased production of immune proteins,
846 hyperglycaemia, temperature elevation (fever) and possibly drug uptake by the host.
847 The parasite thus evolves in a rapidly changing environment that it must decipher to
848 survive.

849

850 **Figure 3. Plasticity of the ring stage during the intraerythrocytic developmental**
851 **cycle.** The three fates of the ring stage are described. The ring stage can develop into
852 asexual growth and proliferation (blue), undergo sexual conversion to produce
853 gametocytes (green) or initiate TGA (purple). Our model suggests that such decisions
854 depend on environmental sensing and extracellular communication involving biotic
855 and abiotic factors (coloured question marks).

856

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858

859

Figure 1

Antimalarial compounds used against malaria blood stage

Endoperoxide
ART Artemisinin and derivatives

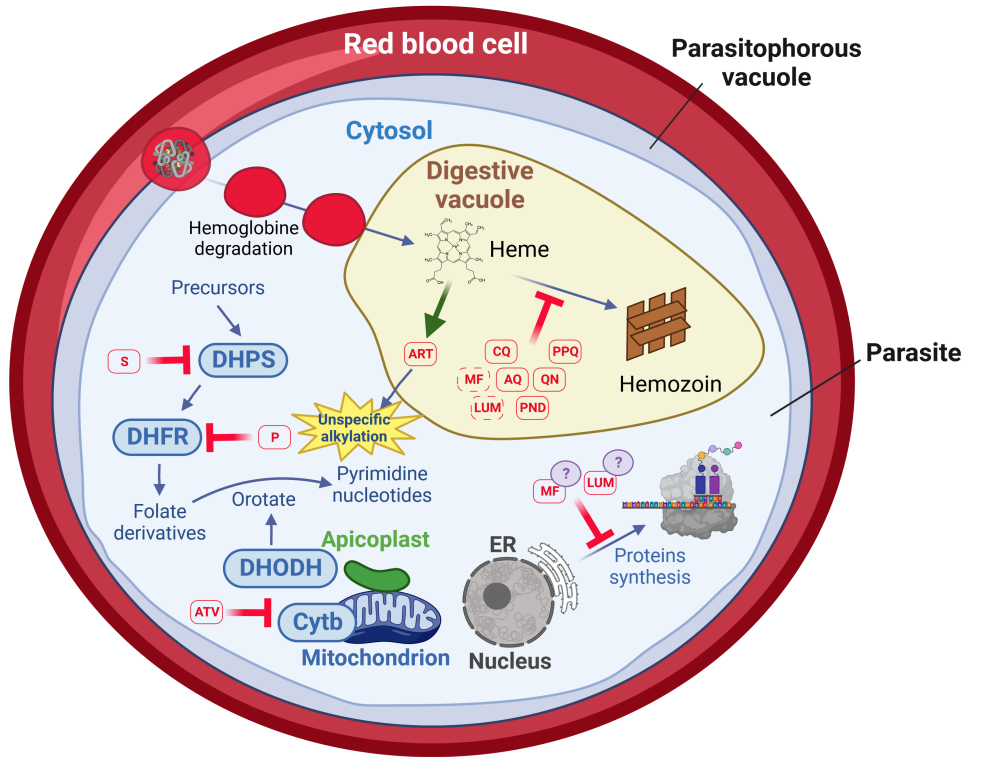
4-Aminoquinoline
CQ Chloroquine
PPQ Piperaquine
AQ Amodiaquine
PND Pyronaridine

Aryl-amino-alcohol
QN Quinine
MF Mefloquine
LUM Lumefantrine

Antifolates
S Sulfadoxine
P Pyrimethamine

Naphthoquinones
ATV Atovaquone

Activation
 Inhibition
 Parasite enzyme
 Unclear mechanism



Window of action of blood-cycle specific antimalarials

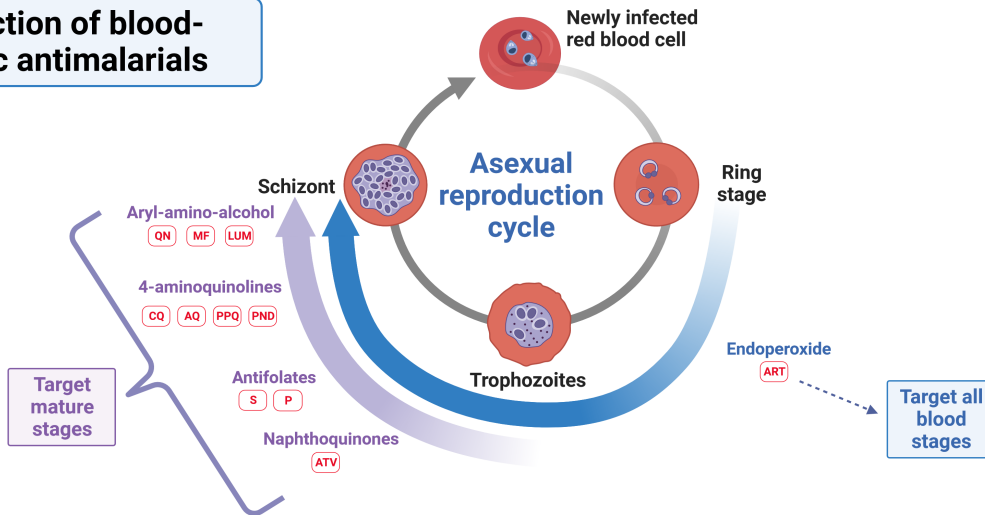
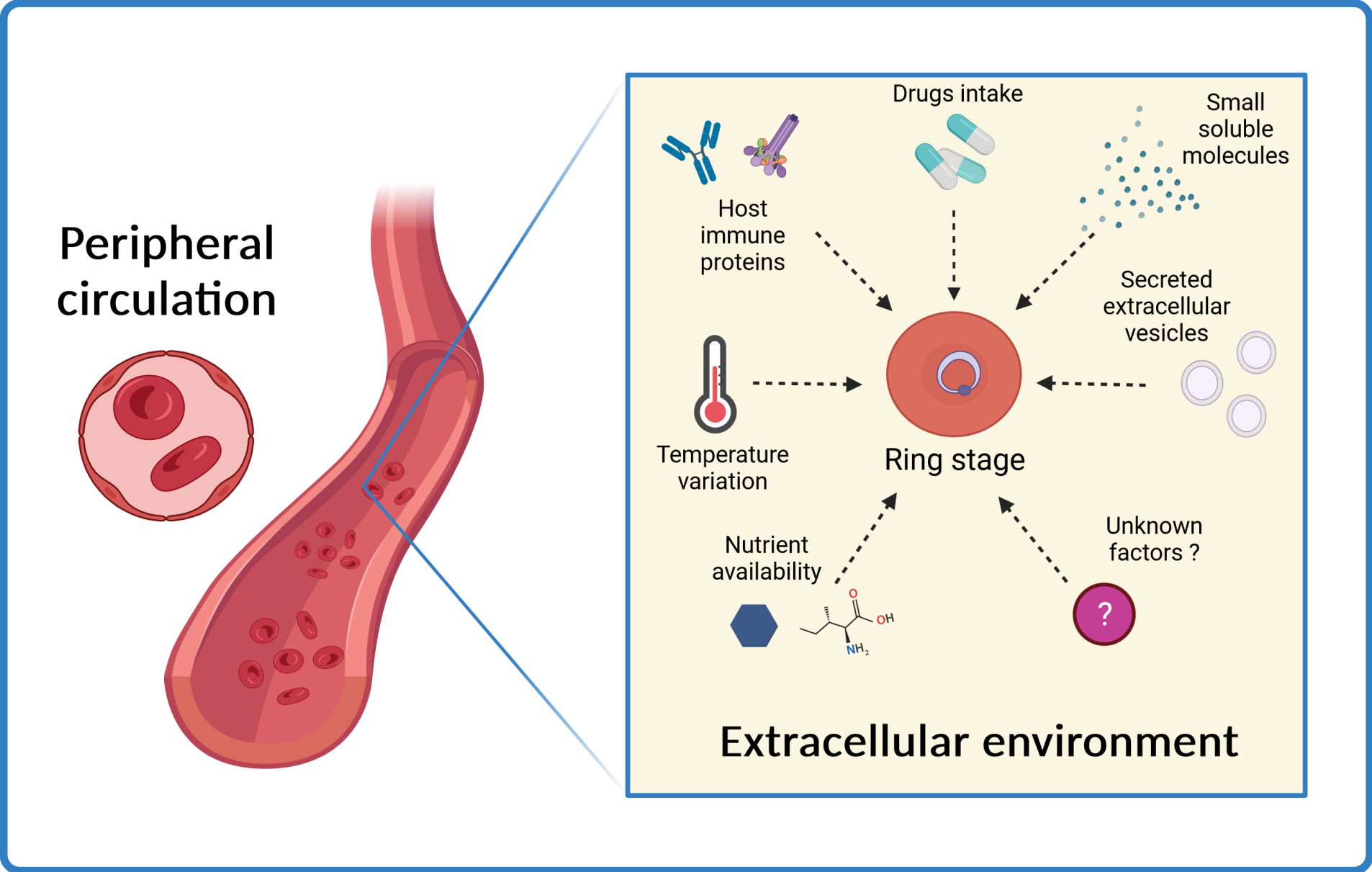


Figure 2



- Lysophosphatidylcholine
- CXCL10
- Extracellular vesicles ?
- Unknown(s) factor(s) ?

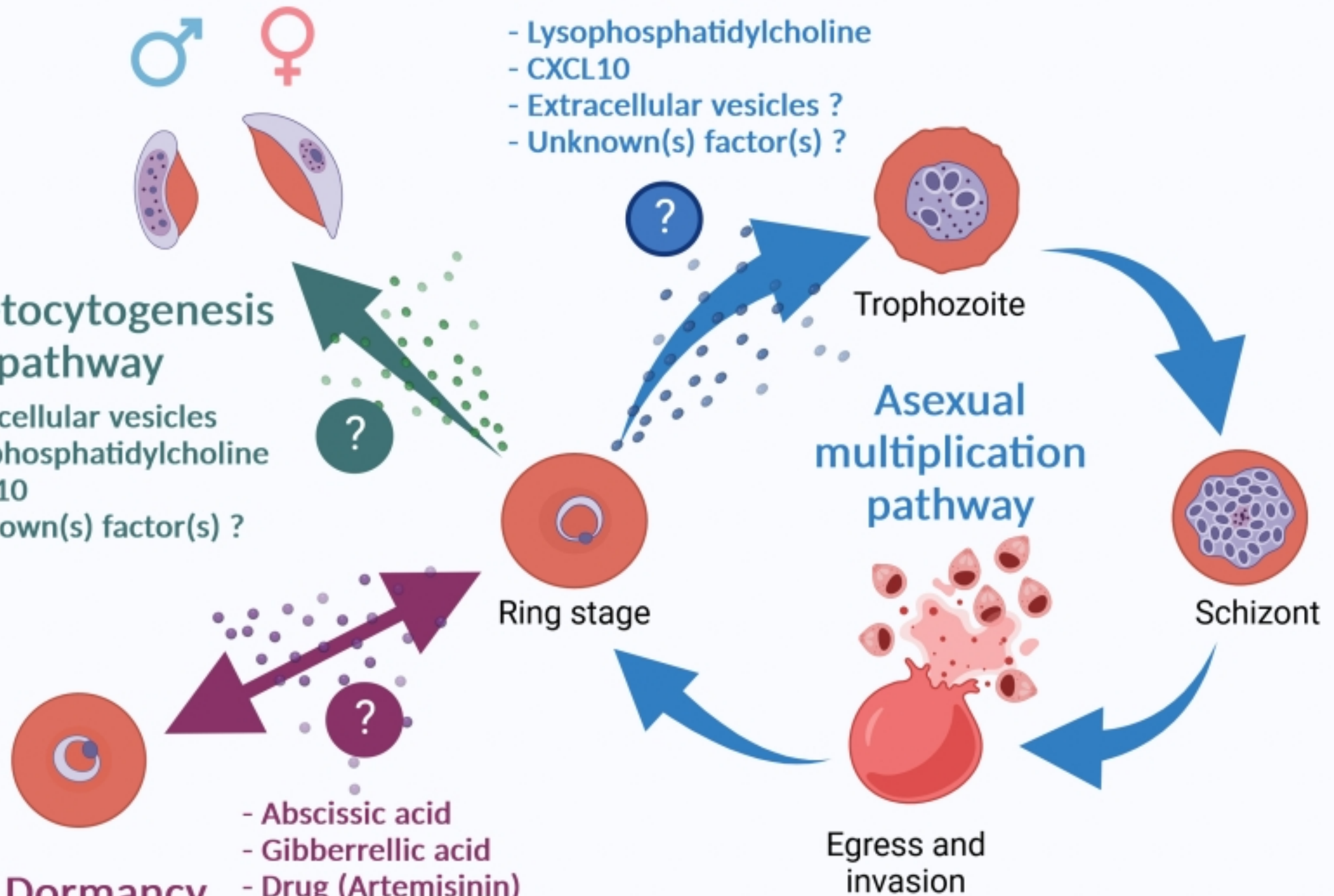
Gametocytogenesis pathway

- Extracellular vesicles
- Lysophosphatidylcholine
- CXCL10
- Unknown(s) factor(s) ?

Asexual multiplication pathway

Dormancy pathway

- Abscissic acid
- Gibberellic acid
- Drug (Artemisinin)
- Starvation
- Extracellular vesicles ?
- Unknown(s) factor(s) ?



1 **Highlights**

- 2 - *P. falciparum* has evolved resistance to almost all antimalarial drugs through
3 genomic changes (SNPs and CNVs) and a non-specific stress response-based
4 survival such as the ring-stage temporary growth arrest (TGA).
- 5 - The ring-stage can develop into asexual or sexual stages or stop growing when
6 conditions are unfavourable.
- 7 - The ring-stage shows extreme plasticity under adverse conditions, such as
8 artemisinin exposure. The ring-stage TGA stage is an innate and passive
9 resistance to many stressors, which ensures that some individuals in the population
10 can survive in the host.
- 11 - *P. falciparum* ring-stage TGA may rely on environmental sensing and extracellular
12 communication between the parasites.
- 13 - Ring-stages TGA may be an underestimated parasite reservoir, especially in
14 asymptomatic individuals.
- 15 - No treatment that specifically targets growth-arrested ring stages is currently
16 available.
- 17

1 **Outstanding questions**

- 2 - What proportion of *P. falciparum* ring-stages can lead to a temporary growth arrest
3 (TGA)? How long can the ring stage be arrested?
- 4 - How can growth-arrested ring-stage parasites be detected? What are the specific
5 metabolic and/or transcriptomic biomarkers of growth-arrested ring-stage
6 parasites?
- 7 - Are the growth-arrested *P. falciparum* ring-stages responsible for asymptomatic
8 carriage and chronic infections *in vivo*?
- 9 - Is the TGA ring-stage population stable over time or is it specifically induced during
10 a stress episode?
- 11 - Can the ring stage of other clinically relevant *Plasmodium* species also use the
12 TGA survival mechanism?
- 13 - Which molecules mediate the growth arrest phenotype? Is the growth arrest
14 phenotype mediated by extracellular vesicles or small molecules released by
15 stressed parasites or both?
- 16 - Can we target growth-arrested ring stages and develop a 'wake and kill' therapeutic
17 strategy?
- 18