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► **To cite this version:**

Benoît Henry, Camille Roussel, Mario Carucci, Valentine Brousse, Papa Alioune Ndour, et al.. The Human Spleen in Malaria: Filter or Shelter?. Trends in Parasitology, 2020, 36, pp.435 - 446. 10.1016/j.pt.2020.03.001 . hal-03490391

HAL Id: hal-03490391

<https://hal.science/hal-03490391>

Submitted on 22 Aug 2022

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1 **The human spleen in malaria: filter or shelter?**

2

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12

13 **Keywords:** malaria; spleen; *Plasmodium*; gametocytes; hyper-reactive malarial
14 splenomegaly; artemisinin.

15

16 **Abstract**

17 The human spleen is an immune sentinel and controls red blood cell (RBC) quality. By
18 mechanically retaining subsets of infected RBC, the spleen may reduce the pace at which the
19 parasite biomass increases before the adaptive immune response operates. Conversely, the
20 spleen may contribute to malaria pathogenesis, particularly anemia that is associated with
21 splenomegaly. Large spleens may also shelter parasites in chronic carriers. Upon treatment
22 with artemisinins, the spleen clears circulating parasites by pitting and releases “once-
23 infected” RBC in circulation. This triggers post-artesunate delayed hemolysis and explains the
24 long post-treatment positivity of HRP2-based dipsticks. Importantly, splenic retention of RBC
25 also applies to gametocytes, the clearance of which may be enhanced by stiffening them with
26 drugs, a potential way to block malaria transmission.

27 **Major importance of red blood cells-spleen interactions in the pathogenesis of human**
28 **malaria**

29 Malaria is the most prevalent severe parasitic disease affecting humans. The causal
30 microorganism, *Plasmodium* spp., develops in hepatocytes then in red blood cells (RBC), but
31 only the asexual multiplication in RBC causes clinical manifestations. Invasion of RBC by
32 *Plasmodium* triggers major alterations in this host cell. *P. falciparum*, the human-infecting
33 *Plasmodium* specie that has been studied in greatest details, expresses parasitic proteins at the
34 RBC surface, and modifies its deformability and adhesiveness. Adhesion of parasitized RBC
35 to endothelial cells (and to other infected or uninfected RBC) is central in malaria
36 pathogenesis but parasite-induced alterations of RBC deformability have also been associated
37 with the severity of malaria [1–3].

38 **White and red pulp** structures (See Glossary) have specific functions in the human spleen.
39 The white pulp is a major operator of the humoral immune response, especially to circulating
40 antigens. The red pulp exerts a unique and subtle control on the surface integrity and
41 biomechanical properties of RBC. To be left in circulation, RBC must be fit enough to cross a
42 very specific structure of red pulp sinuses, the **inter-endothelial slit** (IES; Figure 1). Older
43 RBC, or RBC modified by innate or acquired conditions are eventually retained in the splenic
44 red pulp and processed by red pulp macrophages (RPM) [4].

45 The major modifications of RBC biomechanical properties induced by *Plasmodium* infection,
46 point to the spleen as an important player in the pathogenesis of human malaria. The
47 proportion of subjects with splenomegaly (the splenic index) has long been a defining marker
48 of malaria endemicity. Although knowledge has progressed slowly due to the risk associated
49 with splenic puncture or biopsy and limited access to imaging in endemic areas, significant
50 advances have been made on the role of the spleen in malaria over the last few decades,

51 especially regarding the innate control of infection and transmission [4,5] (Box 1). *Ex vivo*
52 perfusion of human spleens [6] and the development of biomimetic tools [7–9] have generated
53 new insights. The role of cell-mediated immunity has been extensively studied in murine
54 models of malaria, and in malaria-infected or exposed humans through the analysis of
55 circulating cell populations. However, the aforementioned constraints have hampered
56 progress in the understanding of intra-splenic immune responses to malaria in humans. If the
57 important role of platelets in malaria pathogenesis is likely [10], contribution of intra-splenic
58 platelets, which represent a third of the total human pool, remains largely unexplored. In
59 addition, substantial anatomic differences exist between human and murine spleens,
60 especially regarding the red pulp which is devoid of bona fide IES in mice [11] (although
61 mechanical filtration exists), and between human and murine *Plasmodium* species. Whether
62 mouse models of malaria are relevant to explore the pathogenesis of human malaria is still a
63 matter of controversy [12]. Unlike the spleen of humans and rats, the mouse spleen is devoid
64 of sinuses. The mechanical sensing of RBC thus depends on holes in venules rather than on
65 inter-endothelial slits in sinus walls [13]. It is unclear whether these markedly different
66 microanatomic structures display a close or loose functional convergence, and whether the
67 mouse spleen can pit RBC containing undeformable bodies, a major parasite clearance
68 process in humans (please see section “**Role of the spleen in parasite clearance following**
69 **antimalarial therapy**”). Whether the splenic function is preserved in humanized mice
70 infected with *P. falciparum* is also questionable as stiff immature gametocyte and mature
71 asexual stages – which should be filtered out by the spleen – are observed in the circulation of
72 these mice [14]. We have therefore limited this review to observations in humans.

73

74 **Functional anatomy of the human spleen**

75 *General features*

76 The spleen is an oval retroperitoneal organ of 10 to 12 cm in its major axis, weighing 100 to
77 200 grams in adults, surrounded by a collagenous, poorly extensible capsule. It is considered
78 poorly contractile in humans compared to diving or fast-running other animal species (seals,
79 whales, dogs, horses) which harbor large “storage spleens”. Splenic parenchyma is composed
80 of 2 specific tissues, the red pulp and the white pulp. The **white pulp** is made of immune
81 cells, organized in periarterial lymphoid sheaths containing mostly T cells, and lymphoid
82 follicles containing mostly B cells. Follicles are surrounded by the marginal and perifollicular
83 zones. The red pulp accounts for 75% of splenic volume and is composed of a unique
84 association of **cords** and **venous sinuses**. Cords are circulatory structures that contain
85 fibroblasts and, very predominantly, specific macrophages (RPM). Venous sinuses which
86 collect blood from the cords, are made of endothelial cells, which are parallel to the blood
87 flow, and are surrounded by a helicoid basal fiber (i.e., highly discontinuous) which leaves
88 room for narrow spaces between endothelial cells (0.2 to 2 μm), named inter-endothelial slits
89 [13]. Rare plasma cells, mastocytes and other white blood cells are also observed in the red
90 pulp.

91

92 *Microcirculation in the human spleen*

93 Overall, about 5% of arterial flow enters the spleen through the splenic artery, and is divided
94 into 2 paths: approximately 85% of splenic flow enters a fast, closed circulation, which
95 navigates around white pulp structures and follows a specific microcirculation from arterioles
96 to venules delimited by specific MAdCAM1-positive cells in the perifollicular zone; 15%
97 engage into the open and slow circulation in the red pulp, through the cords, then cross the
98 unique structure of IES, to join venous sinuses upstream from venules. Crossing the IES is the

99 most stringent biomechanical challenge for circulating RBC. RBC altered by heat [15],
100 stiffening chemicals [16], genetic defects [17], possibly pretransfusion storage [18], and *P.*
101 *falciparum* [19,20] are retained in the cords, where RPM can process them.

102

103 *Variable definitions of the human marginal zone*

104 In rodents, a marginal zone (MZ), containing fibroblasts, dendritic cells, lymphocytes and
105 specific macrophages (“metallophilic macrophages” and “MZ macrophages”) has been
106 described in the surroundings of lymphoid follicles, and is separated from the white pulp by
107 the marginal sinus [13]. Such a structure is not present in humans, although an important
108 population of B cells is found in this area, the MZ B cells, which have a major role in the
109 early, T-independent antibody response to circulating antigens. Hence, the “human equivalent
110 of MZ” is sometimes renamed peri-follicular zone, or as recently proposed, superficial zone
111 [11].

112

113 **Malaria in splenectomized subjects: a more severe disease?**

114 From 1931 through 2019, 133 malaria attacks were reported in splenectomized patients,
115 among which 69 were due to *P. falciparum*, 26 to *P. vivax*, 6 to *P. knowlesi*, and 3 to *P.*
116 *malariae*. Infection was mixed in 1 case and in 28 cases, *Plasmodium* species was not
117 reported [4] (supplemental Table S1). Only 2 cohorts assessing the risk of malaria in
118 splenectomized subjects (33 in Malawi, 11 in Indonesia) have been prospectively followed
119 and compared to controls. In both cohorts, acute malaria attacks were significantly more
120 frequent in splenectomized patients [21,22]. In this population, severe attacks are also more
121 frequent, mature forms of *P. falciparum* - which are normally sequestered by adherence to the

122 endothelium - are observed in circulation, and post-treatment parasite clearance is delayed
123 [23,24]. However, the over-incidence of severe forms and mortality has not been confirmed
124 by prospective controlled studies and its high frequency in published case reports or series
125 may either reflect a genuine over-susceptibility or result from a publication bias. An increased
126 risk of acute *P. vivax* attacks after splenectomy has been recently reported, and outweighs the
127 risk of *P. falciparum* attacks (hazard ratio 7.7 vs 2.3) [22], without evidence for an increased
128 risk of severe forms in this immune population. One case of acute malaria due to *P. knowlesi*
129 has been described in an asplenic patient [25], and in another case a prolonged parasite
130 clearance has been reported [26]. In summary, the increased risk of malaria has been robustly
131 described in immune or semi-immune subjects, and is strongly suspected in non-immune
132 travelers [27]. Non-immune splenectomized hosts should be very cautious when travelling to
133 malaria-endemic areas. If these patients must travel to endemic areas, strict compliance to
134 antimalarial prophylactic measures is warranted.

135

136 **The spleen in acute malaria**

137 *Protective role*

138 The human spleen senses subtle alterations in RBC deformability. This decreased
139 deformability of *Plasmodium*-infected RBC (asexual stages), first observed with *P. knowlesi*
140 [3], then with *P. falciparum* [1] is intense in RBC infected by mature forms, but already
141 present, albeit moderately, at the ring stage [1,7,19,20,28]. Mechanisms include alterations of
142 surface-to-volume ratio [20], and interactions between parasite proteins and the RBC
143 cytoskeleton [29,30] which induce membrane rigidification. Importantly, **ektacytometry** - a
144 reference technique to quantify RBC deformability [31] - and microfluidics have also

145 demonstrated that during acute malaria attacks, even non-infected RBC are less deformable
146 than normal control RBC [2,28,32].

147 As evidenced by *ex vivo* perfusions of human spleens with *P. falciparum*-infected RBC, the
148 spleen retains a proportion (approximately half) of ring-infected RBC, in addition to the
149 expected, almost complete, retention of schizont-infected RBC [19]. The mechanical retention
150 of schizont-infected RBC is expected to display a small pathogenic impact *in vivo*, as the
151 majority of mature forms cytoadhere in other organs, which prevents them from being filtered
152 by the spleen. Retention of rings which affects a predominantly circulating component of the
153 parasite biomass, takes place in the red pulp. This mechanism, which is difficult to explore
154 directly in human subjects, is expected to reduce the circulating parasitic load, thereby also
155 reducing the parasite biomass amenable to sequester in other organs [19].

156 The increased clearance of all RBC (infected and uninfected) by the spleen after acute malaria
157 has been demonstrated through the autotransfusion of radiolabeled RBC. Thai patients with
158 acute malaria had indeed an enhanced clearance of heat-stiffened RBC when splenomegaly
159 was present [15] that also affected labeled normal RBC in a similar post-malarial context [33].
160 This enhanced splenic clearance of abnormal and normal RBC, which last several weeks, may
161 contribute to the partial control of parasite loads. When comparing cerebral malaria with other
162 severe forms (anemia, respiratory distress, acute renal and/or liver failure, shock), post-
163 mortem studies also showed that intra-splenic **erythrophagocytosis** was more frequent in the
164 latter [34]. Splenomegaly is also less frequent in cerebral malaria than in severe malarial
165 anemia, as demonstrated by field studies performed in Uganda and Sudan, and including a
166 total of 1108 children [35–37]. In one of these studies, splenomegaly was also associated with
167 a survival advantage [37]. The human spleen can thus retain part of the parasite biomass,
168 which may partially control the course of infection, potentially reducing the risk of cerebral
169 malaria. This would, however, come at the expense of an increased retention and clearance of

170 non-infected RBC, leading to splenomegaly and anemia (Figure 1). Exploration of the innate
171 retention of (dead or live) rings in the controlled human infection model [38] would be the
172 most direct and relevant way to assess whether this potentially powerful protective process
173 indeed exists, in a context of limited splenic activation at early steps of human infection, when
174 splenomegaly is not yet present [15].

175

176 *Detrimental role*

177 The pathogenesis of acute malarial anemia is complex and only partially understood. It is
178 associated with intra- and extra-vascular hemolysis, dyserythropoiesis, and the clearance of
179 larger proportions of uninfected than infected RBC [39]. Several observations point to a
180 splenic contribution to the pathogenesis of acute malarial anemia. Congestion of the red pulp
181 is a key post-mortem feature in fatal malaria [40,41]. Decreased RBC deformability, which
182 triggers intrasplenic retention *ex-vivo* (see above), correlates with hemoglobin nadir [32]. As
183 stated above, splenomegaly is more prevalent in patients with severe malarial anemia than in
184 other severe forms, and splenic clearance of RBC is increased after malarial attacks. Not least,
185 erythrophagocytosis is enhanced in acute malaria and alterations of infected RBC render them
186 prone to phagocytosis [42]. The phagocytic function of monocytes is also activated in malaria
187 [43]. That erythrophagocytosis in malaria predominantly takes place in the macrophage-rich
188 spleen remains to be directly demonstrated in humans.

189 Very rarely, splenic-specific complications of malarial infection occur: pathologic splenic
190 rupture, intra-splenic hematoma and splenic infarction. These complications have received
191 little attention so far, and their precise mechanisms remain elusive [44]. In summary, multiple
192 observations suggest a protective role for the spleen in acute malaria, even if definitive
193 evidence from controlled studies is lacking. The innate retention of rings can prevent the rise

194 of peripheral parasite load thereby decreasing the risk of cytoadherence and sequestration of
195 parasitized RBC in vital organs [45]. The potential down-side of this phenomenon is a splenic
196 contribution to malarial anemia.

197

198 **Role of the spleen in parasite clearance following antimalarial therapy**

199 *The pitting process*

200 In patients recently cured from malaria by artemisinin derivatives, a proportion of circulating
201 RBC display parasite proteins at the internal layer of their plasma membrane [46]. These
202 “once-infected” RBC do not contain parasites [23,46,47] and their presence results from a
203 spleen-specific physiological process called **pitting**, whereby an intra-erythrocytic body is
204 extracted from the RBC without cell lysis [48]. Pitting clears physiological intra-erythrocytic
205 items such as Howell-Jolly or Pappenheimer bodies. Malaria-related pitting can be quantified
206 in the peripheral blood of patients through the double-staining of RBC with a nucleic acid
207 marker and an antibody against parasite proteins like RESA (Ring-infected Erythrocyte
208 Surface Antigen). Pitted RBC are parasite-negative and parasite membrane protein-positive.
209 Pitting is not observed in splenectomized subjects [23], and has been replicated in human
210 spleens perfused *ex vivo* with parasitized RBC pre-exposed to artemisinin derivatives [6].
211 Pitting does not occur (or to a very low level) with RBC containing live parasites, and has
212 been so far explored only in *P. falciparum* infections.

213

214 *Contribution of pitting to parasite clearance*

215 In non-immune travelers treated with artemisinins, pitting is a major mechanism of parasite
216 clearance [49]. In artemisinin-treated children living in malaria-endemic Mali, clearance

217 mechanisms may be more complex and influenced by the immune status of the host. Pitting
218 appears to be an important contributor to clearance in infants, who are expected to have low
219 anti-parasite humoral immunity; however in older children, immunity correlates with an even
220 faster parasite clearance, likely through immune-mediated phagocytosis, and possibly through
221 splenic retention without pitting [50]. Pitting is marked in patients treated with intravenous
222 artesunate or oral artemisinin derivatives, and much lower after treatment with quinine,
223 mefloquine or atovaquone-proguanil [51].

224

225 *Contribution of pitting to post-artesunate hemolysis*

226 In Japanese then in German travelers treated with artesunate for hyperparasitemic severe
227 malaria [52,53], acute hemolysis was observed 1 to 2 weeks after therapy. These hemolytic
228 episodes were moderately intense and affected 5-27% of travelers [54,55]. Transfusion was
229 required in 5 to 50% of cases [56] and no conventional cause of hemolysis was found. Initial
230 observations in Southeast Asia had shown that once-infected RBC have a shorter lifespan than
231 normal RBC [47]. In travelers, these hemolytic episodes (named **Post-Artesunate Delayed**
232 **Hemolysis**, PADH) and the disappearance of once-infected RBC from the circulation were
233 simultaneous. PADH is thus likely triggered by the clearance of once-infected RBC [57]. The
234 peak concentration of once-infected RBC quantified by flow cytometry, 2-7 days after
235 treatment initiation, is predictive of PADH [57,58]. Predicting PADH in resource-limited
236 settings is important, as weekly follow-up of all patients recovering from severe malaria and
237 preparation of transfusion are logistically demanding. Like RESA, the parasitic Histidin-Rich
238 Protein 2 (HRP-2) is left as an imprint at the membrane of pitted RBC [58]. HRP-2 is the
239 antigen used in many immunochromatographic malaria **rapid diagnostic tests**. The persistent
240 positivity of the diagnostic dipstick, performed on diluted whole blood 3 to 7 days after

241 initiation of artesunate accurately predicted PADH [58]. Whether PADH is a significant
242 problem in endemic areas has been a subject of debate; a prospective study following 217
243 Congolese children with acute malaria, treated with quinine or IV artesunate, and followed
244 until 42 days after treatment only showed PADH in less than 1% of cases [59]. These patients,
245 however, had parasitemia below the conventional 10% threshold for hyperparasitemia and
246 had uncomplicated malaria. Loosely defined delayed hemolysis has been observed in 7% of
247 African children treated with artesunate for severe malaria [60]. Further prospective studies in
248 African children are ongoing. Interestingly, this approach also explains why HRP2-based
249 dipsticks remain positive days to weeks after treatment of a malaria-attack with artemisinins.
250 HRP2 persists in circulation not as soluble protein in plasma but as a cytoskeleton-associated
251 protein in once-infected RBC.

252

253 **The spleen in chronic parasite carriage.**

254 *A splenic contribution to chronic malarial anemia?*

255 The mechanisms of malarial anemia are influenced by the infecting *Plasmodium* species, age
256 and transmission intensity, amongst others factors. Excellent reviews have dissected these
257 complex parameters [39,61]. Briefly, most important contributors are intra- and extra-vascular
258 hemolysis, erythrophagocytosis, dyserythropoiesis (favored by frequent comorbidities such as
259 iron or vitamin B12 deficiency, or malnutrition), some degree of bone marrow insufficiency,
260 and possibly splenic clearance. The role of the spleen in chronic parasite carriage is complex
261 and very difficult to explore. One study carried in Ghana [62] found a relationship between
262 chronic parasite carriage, anemia and splenomegaly, as observed in multiple other populations
263 and settings. This may reflect any combination of long-lasting RBC trapping in the spleen,
264 enhanced erythrophagocytosis or increased immune reactivity.

265

266 *Parasite biomass in the spleen*

267 Recently, comprehensive pathological and molecular examinations of spleens (and
268 concomitantly of peripheral blood) in mostly untreated patients undergoing splenectomy in a
269 highly *P. falciparum* and *P. vivax*-endemic area of Indonesia have shown that, compared to
270 peripheral blood, non-phagocytosed parasitized RBC concentrate into the spleen by several
271 orders of magnitude [63]. Concentration of parasitized RBC was greatest in the splenic red
272 pulp of *P. vivax*-infected patients. Red pulp congestion by infected and uninfected RBC was
273 proportional to spleen weight. These major observations not only bring some substance to the
274 protective role of the spleen filter in parasite clearance but also suggest that the spleen may be
275 a parasite shelter, possibly supporting a cryptic intrasplenic parasitic cycle [4,64].

276

277 *Hyper-reactive malarial splenomegaly*

278 This condition, initially named tropical splenomegaly syndrome, then more precisely
279 described in 1957, has been progressively deciphered over the last decades, the name **hyper-**
280 **reactive malarial splenomegaly (HMS)** being proposed in 1983 [65]. The currently accepted
281 diagnostic criteria for HMS associate marked splenomegaly, total plasma IgM above 2 times
282 the local standard deviation, clinical response after antimalarials, and polyclonal lymphocytic
283 response [66]. HMS is believed to account for an important proportion (40%) of massive
284 splenomegaly in malaria-endemic areas [67], but recent and precise epidemiological data
285 regarding its prevalence in general population are scarce. In the Gambia, 3 decades ago, the
286 prevalence of HMS was estimated at 0.16% [68]. HMS has essentially been described in
287 subjects chronically exposed to the parasite and is very rare in travelers. Typically, patients
288 present with a gross and constant splenomegaly, sometimes clinically patent (abdominal

289 discomfort) and inconstantly associated with hepatomegaly. Total IgM are markedly elevated,
290 with a polyclonal pattern. This elevation usually precedes splenomegaly. Cytopenia of
291 variable importance can be present, usually attributed to hypersplenism. Positive diagnosis of
292 malaria is difficult in HMS, as parasite load is usually very low, and only detected by
293 molecular methods. Biological features of autoimmunity (cryoglobulinemia, rheumatoid
294 factor, anti-nuclear antibodies) have been associated with HMS. Recently, a relatively specific
295 pattern of anti-nuclear antibodies has been associated with chronic malaria [69].

296 A genetic component of HMS is suspected, based on familial studies [70], but its precise basis
297 remains unknown (although certainly not Mendelian). Pathologic examination of the spleen in
298 HMS has been rarely reported; published cases described essentially red pulp congestion and
299 erythrophagocytosis, with only brief descriptions of the white pulp [71–73]. The pathogenesis
300 of HMS is poorly understood, the most commonly accepted hypothesis involving a defect in
301 regulatory T cells, leading to a B cell expansion after malaria infection, production of immune
302 complexes which would be eventually phagocytosed by splenic macrophages. If this
303 hypothesis is in line with splenomegaly and the enhanced immune response observed in
304 HMS, it however falls short in providing an explanation for anemia and lower parasitic load.
305 The evolution of HMS in endemic areas remains largely unknown. Historical data pointing a
306 very important mortality (>50%) might be biased by the imprecision regarding the causes of
307 death [74]. Scarce data have been collected in travelers, showing that regression of hyper IgM
308 and splenomegaly are slow, over several months [75]. Travelers or expatriates affected by
309 HMS exhibit a tendency to suffer from similar episodes of HMS upon re-exposure to the
310 parasite, consistent with an innate pathogenic component and genetic susceptibility [76]. A
311 major issue is the risk of evolution towards a low-grade marginal-zone splenic lymphoma
312 (MZSL). This association, suspected 50 years ago has been essentially observed in West
313 Africa. MZSL shares many features with HMS. In one study, B cell receptor clonality was

314 assessed in Ghanaian patients with HMS separated according to their response to
315 antimalarials: clonality was absent in responders, constant in non-responders and observed in
316 2 out of 13 partial responders [77]. These findings, along with others, led to the hypothesis
317 that splenic lymphoma represents an ultimate evolution of untreated HMS [78], but this risk
318 has not been precisely quantified.

319 Antimalarial treatment of HMS relied on prolonged courses of primaquine, proguanil,
320 mefloquine or chloroquine. In travelers not returning to malaria-endemic areas, a short
321 regimen is as effective as prolonged treatments [76]. The value of such short courses remains
322 unknown in endemic areas and the usual policy is to administer an intermittent prophylaxis to
323 HMS patients persistently exposed to the parasite [79]. Splenectomy may prove dangerous in
324 HMS patients, as it carries a risk of post-operative acute malaria [64,80,81] and exposes to the
325 long-term risks associated with splenectomy [82].

326

327 *The Fulani enigma*

328 Fulani subjects, present across Sahelian Africa (from Southern Sudan to Eastern Senegal)
329 used to be nomad pastoralists. In Mali and Burkina Faso, compared to other sympatric
330 subjects, Fulani exposed to *Plasmodium* display a distinguishable phenotype, with frequent
331 splenomegaly, enhanced anti-plasmodial humoral response, lower body temperature, lower
332 hemoglobin and lower parasitic load. For these reasons, Fulani have been considered as
333 protected from malaria. This phenotype is however neither complete nor constant
334 (Supplemental Table S2), the most constant feature being palpable splenomegaly. The
335 determinants of the “Fulani phenotype” have been mostly studied from an immunological
336 standpoint, showing, in Fulani, an activated state of monocytes [83], enhanced
337 proinflammatory cytokines production [84], strong response of dendritic cells to toll-like

338 receptors agonists [85], and a defect in regulatory T cells [86]. The genetic basis of this
339 phenotype remains unclear, as the conventional RBC polymorphisms associated with malaria
340 protection have not been found to be more frequent in Fulani [87]. The Fulani phenotype is in
341 many aspects reminiscent of HMS, and HMS is over-prevalent in Fulani [68].
342 Comprehensive, multidisciplinary field studies are underway to decipher the determinants of
343 this phenotype and deconvolute the relative contributions of RBC-spleen interactions and
344 genetics [88].

345

346 **Role of the spleen in the transmission of *P. falciparum***

347 Gametocytes, initial sexual forms of *Plasmodium* in RBC, are generated in a small proportion
348 (0.1 to 5%) at each asexual replication stage. They progressively mature from stage I to IV
349 (immature gametocytes) to stage V (mature forms) over 14 days, under the influence of
350 external factors (reviewed in [89]) and the transcription factor PfAP2-G. The deformability of
351 gametocytes is markedly reduced from stages I to IV, then drastically improves [90].
352 Immature stages accumulate in the spleen and in extra vascular spaces of the bone marrow
353 [91,92] and are generally absent from the peripheral circulation, unlike mature stages.
354 Circulation of mature gametocytes is a prerequisite for the parasite to be ingested by
355 *Anopheles* and to maintain the parasite transmission cycle. The deformability of immature
356 gametocytes is regulated in part by the parasite protein STEVOR, kinase A and AMPc [93]. A
357 pharmacological approach aiming at artificially stiffening mature gametocytes has been
358 developed, with the aim to induce their mechanical retention in the spleen, which would
359 subsequently prevent their circulation and make them unavailable to *Anopheles*, hence
360 blocking transmission. This approach has benefited from the technological improvements of

361 microfiltration (Box 1). A large, high-throughput screening campaign to discover stiffening
362 compounds is ongoing [8].

363

364 **Concluding remarks**

365 The spleen has been extensively investigated, in animal models and later in humans, from an
366 immunological perspective. The exploration of how the spleen innately filters normal and
367 altered RBC and eventually eliminates them (predominantly in the red pulp) has received
368 attention from researchers half a century ago. New tools have enabled progress in this field
369 over the last decade. A better understanding of human splenic physiology has shed light on
370 the dual role “Dr. Jekyll and Mr. Hyde-like” of the spleen in human malaria (Table 1, Key
371 Table). The spleen limits the increase in parasite biomass, therefore reducing sequestration
372 and microvascular dysfunction in major target organs like the brain, but concomitantly likely
373 contributes to malarial anemia. Similarly, the spleen-related production of once-infected RBC
374 after treatment with artemisinins reduces RBC loss during therapy but sometimes induces
375 delayed and clinically significant hemolysis. Current and further lines of research (see
376 Outstanding Questions) will investigate the promising field of induction of splenic retention
377 of *Plasmodium* gametocytes, a potentially important contributor to malaria elimination
378 attempts. Not least, new exploratory tools (Box 1) will be of importance to investigate the role
379 of cellular immunity antimalarial defense, a process essentially studied through *in vitro*
380 studies and animal models so far.

381

382 **Acknowledgements**

383 The authors wish to thank Dr Geneviève Milon for her longstanding support and pertinent
384 scientific guidance; Dr Steven Kho & prof. Nicholas Anstey for their comments on this
385 manuscript and fruitful collaboration.

386

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597

598 **Glossary**

599 **Cords:** Circulatory structures specific of the splenic red pulp devoid of endothelial lining. In
600 the cords, circulation of red blood cells is slow, favoring close and prolonged interactions
601 with splenic macrophages which are very abundant in this unique environment. CD34+
602 positive conventional arterioles traverse the red pulp but are not considered a component of
603 the slow and open pathway of the red pulp.

604 **Ektacytometry:** Method to measure RBC deformability. Gradually increasing shear stress is
605 applied to a solution of RBC diluted in a viscous, iso-osmolar medium. Measurement of RBC
606 diffraction pattern enables the calculation of the elongation index, a correlate for RBC
607 deformability.

608 **Erythrophagocytosis:** Phagocytosis of generally altered RBC by effector cells, a process
609 taking place predominantly in the splenic red pulp and operated by red pulp macrophages.

610 **Hyper-reactive malarial splenomegaly (HMS):** Rare form of chronic malaria, occurring in
611 persistently exposed subjects, defined by a gross splenomegaly, elevated total IgM, high titers
612 of anti-*Plasmodium* antibody with a polyclonal immune response pattern, and clinical
613 response to antimalarials.

614 **Inter-endothelial slits (IES):** Narrow spaces (0.2 to 2 microns) between adjacent elongated
615 endothelial cells of sinuses of the splenic red pulp. Venous sinuses do not have a basal

616 membrane but a highly discontinuous helicoid basal fiber. RBC must cross inter-endothelial
617 slits as they navigate from the cords back to the general circulation, which challenges their
618 deformability. Inter-endothelial slits are 2 – 10 times narrower than the smallest capillaries.

619 **Pitting:** Spleen-specific mechanism by which an intraerythrocytic body is extracted from the
620 RBC. The RBC is not lysed by the pitting process and returns to the circulation. Pitting plays
621 a major role in parasite clearance following therapy with artemisinins (but not following
622 treatment with other antimalarial agents).

623 **Post-artesunate delayed hemolysis:** Acute hemolytic anemia, occurring 1 to 2 weeks after
624 treatment with artemisinin derivatives for severe, generally hyper-parasitemic malaria. This
625 adverse event is triggered by the relatively synchronous clearance of pitted RBC.

626 **Rapid diagnostic tests:** Immunochromatographic tests for the diagnosis of *Plasmodium*
627 parasitemia. These tests are performed on capillary blood collected by finger prick and detect
628 the presence of plasmodial antigens, either pan-specific (such as lactico-deshydrogenase), or
629 species-specific (such as the histidine-rich protein-2).

630 **Red pulp:** Part of splenic parenchyma involved in the control of RBC biomechanical and
631 surface properties, erythrophagocytosis and reaction to circulating antigens. It includes cords
632 and venous sinuses.

633 **Venous sinuses:** Circulatory structures collecting blood downstream from the splenic cords
634 and from the closed and fast perifollicular circulation of the spleen. The endothelial lining of
635 venous sinuses is made of parallel, elongated cells lying on discontinuous basal fibers.

636 **White pulp:** Part of splenic parenchyma involved in immune responses, especially against
637 circulating antigens and encapsulated bacteria. It comprises periarteriolar lymphoid sheaths
638 and lymphoid nodules.

639

640 **Box 1. Available tools to assess the human spleen**

641 *Spleen palpation* is the first and the only available assessment in most malaria-endemic
642 settings. Spleen size is best quantified using Hackett's classification. The prevalence of
643 splenomegaly correlates positively with malaria endemicity but lacks specificity.

644 Splenomegaly is frequent in children with malarial anemia in endemic areas, but rare in
645 travelers with acute attacks.

646 *Medical imaging* assesses the size and structure of the spleen, and visualizes splenic
647 abnormalities. Ultrasound coupled with Doppler is the most widely available technique.
648 Splenic contrast-enhanced ultrasonography using microbubbles [94] fine-tunes splenic
649 evaluation and enables the relative quantification of flow in the fast and slow circulations.
650 Scintigraphy using radiolabeled tracers provides a functional assessment: sulphur colloids and
651 sensitized RBC assess macrophage function while heat-stiffened RBC assess the mechanical
652 filtering function [95]. Splenic function can be inferred from the clearance kinetics of labelled
653 RBC, and from the intensity of radioactivity in the splenic area compared to the hepatic or
654 cardiac area.

655 *RBC-related markers of splenic function.* When the filtering and pitting functions are
656 impaired, RBC containing Howell-Jolly bodies (small Giemsa-positive spheres) appear in
657 circulation and can be observed on peripheral blood smears). Pocked RBC contain small
658 intracytoplasmic vesicles, visible using differential interference contrast microscopy. Their
659 proportion in the peripheral blood increases above 2-3% in cases of chronic splenic
660 dysfunction. This accurate marker [96] requires a specific equipment and trained personnel.

661 *Circulating populations of immune cells,* especially IgM memory B cells, have been linked to
662 splenic function. This subpopulation is significantly reduced in splenectomized subjects
663 [97,98].

664 *Ex vivo perfusion of human spleens* retrieved following left pancreateosplenectomy preserves
665 the filtering and phagocytic functions during a few hours [6]. This approach has uncovered
666 the innate, mechanical retention of a proportion of ring-infected RBC [19], a process later
667 confirmed through filtration *in vitro* [7,20] and which may contribute to the intense
668 accumulation of infected RBC in the spleen of chronic carriers.

669 Some *Biomimetic tools* attempt to reproduce splenic function; microspherulites evaluate the
670 ability of RBC to squeeze through narrow slits between metallic beads, and accurately reflects
671 mechanical retention of RBC in the human spleen [7,8,99]. The initial experimental set-up
672 [7] has been adapted to 96 or 384-wells microplates, enabling the parallel analysis of
673 hundreds of samples [8]. Spleen-mimetic microfluidics chips have been recently developed
674 [9,100]. A stage-dependent retention in when chips were infused with infected, labeled RBC.

675

676 **Table 1, Key Table. Dual role of the spleen in human malaria.**

	Acute attacks	In treated patients	Chronic carriage	Transmission
Protective role	Retention of infected RBC	Clearance of infected RBC (pitting)	Limitation of the circulating parasitic load?	Retention of gametocytes
Detrimental role	- Rupture - Contribution to anemia	Post Artesunate Delayed Hemolysis	- Contribution to chronic anemia? - Hyper-Reactive Malarial Splenomegaly - Splenic lymphoma?	Reservoir? (contribution to chronic asymptomatic carriage)

677

678 **Figure 1. Interactions between parasitized and unparasitized RBC, macrophages,**
679 **endothelial cells in the splenic red pulp, across different physiologic and pathologic**
680 **conditions. (A):** schematic view of the splenic microanatomy. PALS: periarteriolar lymphoid
681 sheath. **(B):** Representation of the endothelial lining between splenic cords and venous sinus
682 lumens. Normal RBC must squeeze through inter-endothelial slits (IES) to join venous
683 circulation. Co: cords of Billroth. SL: sinus lumen. **(C):** during acute attacks of malaria,
684 splenomegaly can be observed, and very rarely splenic rupture can occur. RBC infected with
685 mature forms are retained in the spleen and processed by RPM. A proportion of ring-infected
686 RBC crosses IES. Some uninfected RBC become also stiffer and might also be retained. **(D):**
687 in the case of malarial infection in a splenectomized host, parasitemia is higher and mature
688 forms, mostly sequestered in peripheral tissues and sometimes retained in the spleen, appear
689 in the peripheral circulation. **(E):** during chronic parasite carriage, splenomegaly can
690 inconstantly be observed and in rare cases, evolves towards hyper-reactive malarial
691 splenomegaly. Infected RBC might be retained in a similar way as they are in acute attacks, as
692 well as part of uninfected RBC. An intense intrasplenic retention of infected RBC might lead
693 to a fully intrasplenic replication cycle of the parasite. **(F):** during acute malaria attacks
694 treated with artemisinin derivatives, the pitting process occurs in the spleen: dead parasite
695 remnants are expelled from the RBC without lysing it. “Once-infected” RBC harbor parasite
696 proteins (green dotted line) at the inner side of their membrane, allowing them to be identified
697 in the peripheral blood. In some cases, 1 to 2 weeks after acute attacks, synchronous
698 hemolysis of once-infected RBC is responsible for post-artesunate delayed hemolysis
699 (PADH). **(G):** in subjects carrying gametocytes, retention of immature forms (stages I to IV)
700 occurs in the spleen due to their low deformability. After evolution towards mature stages,
701 RBC infected with gametocytes are able to cross IES and therefore circulate in peripheral
702 blood.

