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1

2 Review

# Q4 *Plasmodium malariae*: the persisting mysteries 4 of a persistent parasite

Q6 Q5 Richard Culleton <sup>1,2</sup>, Arnab Pain <sup>3</sup> and Georges Snounou <sup>4,\*</sup>

6 *Plasmodium malariae* is a 'neglected malaria parasite' in as much as the amount  
7 of research conducted on it pales into insignificance when compared to that  
8 pertaining to *Plasmodium falciparum* and *Plasmodium vivax*, its more notorious  
9 and pathogenic cousins. There has, however, been an increase in interest in this  
10 parasite over the past decade. Principally, this is because of the increasing use  
11 of sensitive molecular detection techniques that have revealed a wider than previously  
12 recorded prevalence in some regions (particularly in Africa), and high  
14 numbers of chronic, asymptomatic infections.

## 15 A brief history

Q10 Q9 At the end of 1930 Robert Knowles, Ronald Senior White, and Biraj Mohan Dasgupta published  
17 an extensive monograph [1] in which they reviewed the current knowledge of the global distribu-  
18 tion of the three malaria species that infect humans (*P. falciparum*, *P. vivax*, and *P. malariae*; the  
19 specific status of the recently discovered *Plasmodium ovale* was still in question). Their conclu-  
20 sion: 'Despite the length of this Memoir our final conclusion is that our present-day knowledge  
21 of malaria is remarkably meagre'. Whereas much has been learned since a similar conclusion  
22 could still be justifiably applied to *P. malariae* today. In this review, we present an overview of  
23 the idiosyncrasies of this globally successful species and highlight interesting gaps and possible  
24 misconceptions in our knowledge of its biology and speculate as to their potential significance.

Q11 **Quartan fevers** (see [Glossary](#)), in which febrile episodes recur every fourth day, have been dis-  
26 tinguished from other types of fever since antiquity. Following the acceptance by the Italian  
27 workers of Laveran's discovery in 1880 of the protozoan parasites that cause malaria, Camillo  
28 Golgi was the first in 1885 to associate the parasites' forms in the blood with the evolution of  
29 the **paroxysm**. His detailed microscopical observations led him to differentiate between the par-  
30 asites of the **tertian fever** and the quartan parasites [2], which were then named by Giovanni  
31 Grassi and Raimondo Feletti as *Hæmamoeba vivax* [3] and *Hæmamoeba malariae* [4]  
32 (*Hæmamoeba* = *Plasmodium*).

33 It was noted by early malariologists that quartan fevers were rare and only exceptionally associ-  
34 ated with mortality. The characteristic low-grade parasitaemias (often close to the limit of micro-  
35 scopic detection with peaks rarely exceeding 10 000 parasites/ $\mu$ l) were ascribed to the 72-h  
36 erythrocytic cycle and a low number (from six to 12) of **merozoites** per **schizont**, yet infections  
37 were persistent, confirming the old Roman adage that 'quartana te teneat' (quartan fever is tena-  
38 cious). Indeed, a primary infection often left the patient suffering over many weeks with regular  
39 paroxysms, and recurrent (though shorter) episodes over the ensuing years. In contrast to  
40 *P. vivax* and *P. falciparum*, early attempts at experimental mosquito transmission of *P. malariae*  
41 from one person to another proved elusive [5]. Prevalence was found to be patchy, with specific  
42 localities showing high levels with those close by devoid of any *P. malariae* cases. Such observa-  
43 tions left malariologists perplexed, as pithily stated by Marchoux 'Il existe d'ailleurs au sujet de la

## Highlights

*Plasmodium malariae*, the quartan malaria parasite of man, is a neglected species.

Advances in molecular detection techniques are revealing a much greater prevalence of this species than previously thought.

There remain many mysteries surrounding its biology and epidemiology, including its true prevalence, and the nature of its interactions with coinfecting malaria species.

We review some outstanding mysteries and dogmas concerning the biology and epidemiology of *P. malariae*.

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44 fièvre quarte une sorte de mystère non encore pénétré' [6] (There is a mystery yet to be elucidated  
45 about quartan fever).

## 46 Humans as an experimental model

47 The advent of **malaria therapy** in the early 1920s, whereby a remarkable and unique cure for  
48 **tertiary syphilis** was obtained by multiple malarial paroxysms in patients deliberately infected  
49 with parasites, provided a means to investigate the biology of *P. malariae*, as it did that of the  
50 other species [7,8]. The globally preferred species throughout the malaria therapy era was  
51 **Q12** *P. vivax*, the parasite first used by Wagner-Jauregg to treat **neurosyphilitics**. *P. malariae*, though  
52 tested with success in 1924, was **used** mainly to obtain infection in patients who were or became  
53 immune to *P. vivax*, and in the **USA** to infect patients of African descent who were found to be  
54 broadly insusceptible to infection by *P. vivax*. Nonetheless, the observations on experimental  
55 *P. malariae* spanned more than 40 years (1924–1968); of the 67 biologically informative articles  
56 we identified 11 from England, 22 from Romania, and 22 from the **USA**. Mosquito transmissions  
57 having finally been achieved in 1933, these records include cases of induced infections by **sporo-**  
58 **zoites** as well as by infected blood inoculation. Successful adaptation of *P. malariae* to New World  
59 monkeys (*Aotus* and *Saimiri*) in the late 1960s provided further scope for observations. Two other  
60 quartan species, *Plasmodium brasilianum* of South American primates and *Plasmodium rodhaini*  
61 of African Apes, which are both considered to be synonymous with *P. malariae*, provided additional  
62 **Q13** scope for experimentation (**Box 1**). Altogether, these data have provided the basis on which the  
63 clinical and epidemiological reports on *P. malariae* are currently perceived.

64 Thus, *P. malariae* is presented as a parasite of humans, with an overall low – though unevenly  
65 variable – prevalence [9–13], that is potentially zoonotic in Africa as *P. rodhaini* and in

### b0.2 **Box 1. The *P. malariae* parasites of nonhuman primates**

b1.3 The genomes of *P. malariae* parasites collected from humans harbour much lower levels of pairwise nucleotide diversity than  
b1.4 those identified in the so-called malariae-like parasite collected from chimpanzees in Gabon ( $3.2 \times 10^{-4}$  vs.  $6.5 \times 10^{-3}$ ) [17], a  
b1.5 situation akin to that noted for *P. falciparum* and *P. reichenowi* [90]. This Gabonese parasite may correspond to *P. rodhaini*, a  
b1.6 species found in gorillas and chimpanzees that is indistinguishable morphologically from *P. malariae* [91].

b1.7 Further analysis of *P. malariae* type sequences from both captive and wild African apes revealed the presence of three  
b1.8 clades: (i) *P. malariae* of humans (sometimes also found infecting captive, but not wild apes), (ii) a *P. malariae*-like parasite  
b1.9 of wild apes (the Gabonese parasite from above), closely related to but distinct and separate from the human infecting line-  
b1.10 age, and (iii) a distinct and distantly related species infecting chimpanzees and gorillas in west and central Africa, with the  
b1.11 proposed name *Plasmodium celatum* [92].

b1.12 The above analyses also revealed that the genetic diversity of the *P. malariae* clade infecting humans globally is significantly  
b1.13 lower than that of the clade infecting wild chimpanzees and apes in Africa. This suggests that *P. malariae* became a par-  
b1.14 asite of humans as the result of a host-switch from African nonhuman Hominidae.

b1.15 Investigations of *P. brasilianum*, another species restricted to New World monkeys that is morphologically identical to  
b1.16 *P. malariae*, found that their genomic sequences were almost identical [19]. This supports the view that *P. brasilianum* rep-  
b1.17 resents the outcome of a host-switch of *P. malariae* from humans to a lower primate. Detailed analysis of a larger collection  
b1.18 of samples should help to establish whether *P. malariae* and the two malaria-like species above are part of a single species  
b1.19 or are distinct *Plasmodium* species. This is of some interest because humans are susceptible to infection by all three par-  
b1.20 asite types, as are the African apes and the New World monkeys to *P. malariae* [93,94]. Further targeted genomic analy-  
b1.21 ses, for example, comparisons of the repertoire of proteins implicated in red blood cell invasion might illuminate the origin  
b1.22 and the dynamics of the zoonotic infections [95].

b1.23 The availability of complete reference genomes has made it possible, for the first time, to determine a true phylogeny for the  
b1.24 human-infecting malaria species [17]. A maximum-likelihood tree based on 1000 evolutionary conserved single-copy pro-  
b1.25 teins from each of the twelve *Plasmodium* genomes provided distinct evidence that *P. malariae* forms a unique clade sep-  
b1.26 arate from those of the other primate- and rodent-infective species. Although this phyletic position contrasts with that  
b1.27 derived from previous phylogenomic datasets [16,96,97], it concords with one based on apicoplast sequences [98].

## Glossary

**Gametocyte:** the sexual stage of malaria parasites.

**Hypnozoite:** latent liver stage of malaria parasites.

**Malaria therapy:** the treatment of neurosyphilis, a syphilis complication affecting the central nervous system, and other neurological disorders by induction of fever through controlled inoculation of malaria parasites and their subsequent clearance by drug treatment.

**Merozoite:** red blood cell-invasive form of the malaria parasite.

**Normocyte:** mature red blood cell.

**Oocyst:** form of the malaria parasite resulting from the encystation of an ookinete on the midgut epithelium of the mosquito. A replicating stage that results in the production of numerous sporozoites.

**Paroxysm:** a sudden and acute attack of a disease. Malaria paroxysms are typically associated with fever onset.

**Pyrogenic threshold:** the density of parasites in the blood sufficient to cause fever onset.

**Quartan fever:** a fever that recurs every third day. Associated with malaria parasites, such as *Plasmodium malariae*, that replicate in the blood every 72 h.

**Reticulocyte:** immature red blood cell, characterised by the presence of a reticular network of ribosomal RNA.

**Schizont:** multinucleate, replicating stage of the malaria parasite, producing numerous merozoites.

**Sporogonic cycle:** the part of the malaria parasite's developmental cycle that occurs in mosquitoes, initiated by the fusion of male and female gametes and ultimately resulting in the production of sporozoites.

**Sporozoite:** the form of the malaria parasite inoculated into vertebrates via the bite of infected mosquitoes.

**Syphilis:** a sexually transmitted disease caused by the spirochete bacterium *Treponema pallidum*.

**Tertian fever:** a fever that recurs every second day. Associated with malaria parasites such as *Plasmodium falciparum*, *P. vivax*, and *P. ovale* that replicate in the blood every 48 h.

**Tertiary syphilis:** the third phase of infection with *Treponema pallidum* in which severe damage to the central nervous system (neurosyphilis) and internal organs (cardiovascular syphilis) occurs.

86 South America as *P. brasilianum*. The hepatic phase is lengthy by comparison with the other  
87 species, with a minimum of 15 days required for maturation. *P. malariae* is considered not to  
88 form **hypnozoites**, though this has been recently questioned [14,15]. Following a variable,  
89 often lengthy, prepatent period, clinical symptoms appear when the parasitaemia is very low  
90 (<500 parasites/ $\mu$ l) and can persist for months in primary infections of naïve subjects. The **py-**  
91 **rogenic threshold** is the lowest among the species that infect humans, with the first rise in  
92 fever or clinical signs often declaring when the parasites are barely detectable (500 parasites/ $\mu$ l  
93 or less). Parasite levels rarely peak above 10 000 parasites/ $\mu$ l, and when untreated the parasite  
94 can persist for months at low and often submicroscopically detectable levels. Infections lasting  
95 asymptotically for several decades have been recorded in persons who had migrated from an  
96 endemic area to a malaria-free country; however, the proportion of those where the infection  
97 was extinguished without radical cure is unknown. The characteristically low parasite burdens  
98 are ascribed to a 72-h erythrocytic cycle, the low number of merozoites per schizont (from six to  
99 12), and a strict preference for mature erythrocytes. *P. malariae* is reported as non-sequestering  
100 since both young and mature forms can be seen in peripheral blood samples. The infection is  
101 considered benign in that directly attributed mortality or clinical severity has been rarely reported,  
102 but it has been associated with quartan nephropathy, whose aetiology remains to be elucidated.  
103 Mosquito transmission is hindered by low **gametocyte** numbers, few **oocysts**, and a rather  
104 lengthy extrinsic period of development. A critical survey of the available observations suggests  
105 to us that the strength of some of these notions on *P. malariae* might deserve reappraisal. Recently,  
106 large-scale and whole-genome sequences of the *malariae* group of parasites have become  
107 available [16–19], but the functional significance of tantalising differences within and between  
108 other *Plasmodium* species remains speculative. Knowledge of the genetic diversity of this  
109 species still lags far behind that of *P. falciparum* and *P. vivax* (Box 2), but it is expected to  
110 expand in future.

111 Rich as they might be, the observations from induced infections in humans (mainly in  
112 malariotherapy but also a few in volunteers) should be interpreted with some caveats at hand.  
113 In order to deal with the repeated malarial paroxysms, the patients selected for infection had to  
114 be in good health, and were monitored daily and protected from further infectious bites. The par-  
115 asite strains used were selected to be of moderate pathogenicity and susceptible to the available  
116 antimalarials; moreover, they were often maintained by repeated human-to-human blood pas-  
117 sages. The majority of data reported derives from six *P. malariae* strains acquired locally (one in  
118 Germany and The Netherlands, two in the USA, and three in England and Romania). Thus, the  
119 data are biased towards *P. malariae* parasites from temperate endemic regions. The only recent  
120 reanalyses of malariotherapy data on *P. malariae* were based solely on infections by a single strain  
121 [20–23]. Finally, the follow-up periods were rarely of sufficient length and frequency to address the  
122 longevity of the infection. Thus, the published observations on *P. malariae* infections that could  
123 illuminate the natural history of the parasite are rather disparate, partial, and difficult to interpret.  
124 Whereas the available observations provide sufficient support for the current views on  
125 *P. malariae*, they are equally consistent with, but not sufficient to dismiss, alternative views. We  
126 feel that a paucity of data should promote rather than hinder speculation. In this context, some  
127 of the *P. malariae* canons merit further appraisal.

## 128 Of paradigms and dogmas

### 129 Do reticulocytes matter?

130 The notion that the malarial merozoite might favour younger rather than older red blood cells was  
131 first made for *P. vivax* in 1934 [24]. The first investigation on red blood cell selectivity [25] actually  
132 led to the conclusion that, given an equal availability of either cell type, the penetration of  
133 a **reticulocyte** by *P. malariae* would have occurred 9.5 times as frequently as for a mature

### Q3 Box 2. The dawn of *P. malariae* genomic diversity

b2.3 Investigations of the diversity, evolutionary history, and population structure of *P. malariae* in field-collected samples have  
 b2.4 been, until recently, restricted to a few genes [99–107]. These studies indicated a degree of genetic diversity and variation  
 b2.5 akin to those observed for the other malaria parasite species of humans, including a high level of the multiplicity of infection  
 b2.6 in areas of high endemicity.

b2.7 In 2016–2017, two independent studies made the genome of this neglected malaria parasite available [16,17]. Ansari and  
 b2.8 colleagues offered the first insight into the genome of *P. malariae* with a particular focus on the architecture of the ex-  
 b2.9 panded gene families across seven complete human-infecting *Plasmodium* genomes that also included the zoonotic ma-  
 b2.10 laria parasites *P. knowlesi* and *P. cynomolgi*. In the following year, a more complete reference-quality genome of  
 b2.11 *P. malariae* was published [17]. The *P. malariae* genome has a larger nuclear genome compared to other *Plasmodium* spe-  
 b2.12 cies (by about 30%; ~31.9–33.6 Mb with > ~6500 protein-coding genes). The core *P. malariae* genome is by and large  
 b2.13 shared with that of other species, but there are unique, and in some cases, massive expansions of some gene families –  
 b2.14 for example, of the *pir*, *phist*, *surfin*, and *fam-a* (later denoted as *fam-l* and *fam-m*) gene families, which suggests an in-  
 b2.15 creased diversity of proteins predicted to be exported to the surface of infected red blood cells (RBCs) [16]. The large pro-  
 b2.16 portion of ‘pseudogenized’ members of the *pir* family is a distinct characteristic, as is the presence of gene doublets from  
 b2.17 the *fam-i* and *fam-m* at the subtelomeric boundaries of the chromosomes, with some evidence that these two families  
 b2.18 coevolved [16], and might be related to a major malaria red cell invasion ligand, RH5, in *P. falciparum* [108]. The potential  
 b2.19 to uncover the functional significance of these genes is restricted by the inability to cultivate *P. malariae* *in vitro*.

b2.20 Over the past 5 years, genome-enrichment techniques, such as selective whole-genome amplification (sWGA), have been  
 b2.21 developed and optimised for the *Plasmodium* parasites of humans [109–112]. This makes it possible to extend analyses to  
 b2.22 field samples hitherto excluded by low parasitaemias or the presence of mixed-species infections. A proof-of-concept  
 b2.23 study that applied an sWGA protocol to 18 *P. malariae* clinical samples [112] yielded a total of 28 899 core-genome SNPs  
 b2.24 that were subsequently used to establish a robust phylogeny. With careful handling of the starting material during DNA  
 b2.25 extraction, high-molecular-weight DNA preparations are now amenable to sWGA-based amplification protocols for  
 b2.26 long-read sequencing using the PacBio or the ONT platforms. Thus, much-larger-scale population-genetics-driven  
 b2.27 studies on *P. malariae* are now possible. When coupled with recent state-of-the-art ultrasensitive nucleic acid detection  
 b2.28 technologies, such as the clustered regular interspaced short palindromic repeats (CRISPR)-based SHERLOCK (specific  
 b2.29 high-sensitivity enzymatic reporter unlocking) platform [113], a quantum leap in our knowledge of *P. malariae* biology and  
 b2.30 epidemiology has become possible.

134 erythrocyte. This was later contradicted by two investigations [26,27] that sealed the notion that  
 135 *P. malariae* is restricted to mature red blood cells, because in the samples collected, parasites  
 136 were nearly all observed in normocytes. One possible explanation for these discrepant observa-  
 137 tions might lie in the time at which the blood samples are collected. Robert William Hegner based  
 138 his conclusion of a reticulocyte preference on a single slide obtained soon after the chill, that is,  
 139 soon after invasion. Given their lengthy cycle, the *P. malariae* ring stage extends for a long period,  
 140 during which the reticulocyte would have had the time to mature [11]. An accelerated transition  
 141 from reticulocyte to normocyte has been demonstrated for *P. vivax* [28], and it is likely that  
 142 such a phenomenon would occur for all malaria parasites that invade reticulocytes. We propose  
 143 that *P. malariae* might actually have a predilection for reticulocytes. This would account for the low  
 144 parasite burdens and could explain the failure to achieve *in vitro* cultivation [29–31] where eryth-  
 145 rocytic maturation occurred but with little re-invasion of the plentiful mature red blood cells.

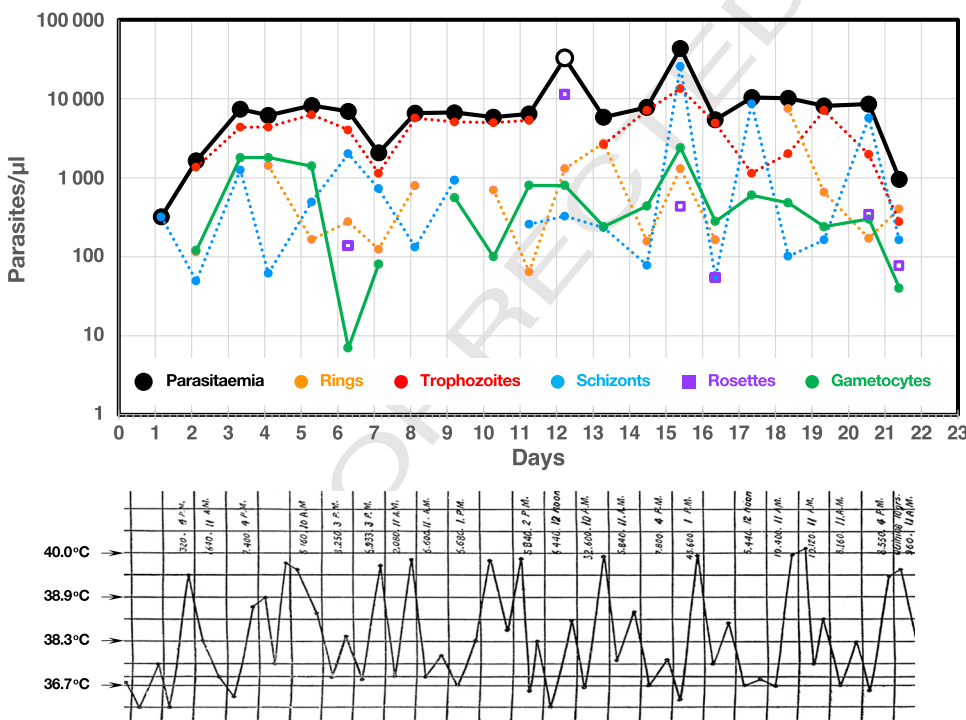
### 146 Does sequestration play a role?

147 Sequestration, defined here as a withdrawal from the peripheral circulation for a period during the  
 148 erythrocytic cycle, is stated not to occur for *P. malariae* parasites. This conclusion is based on ob-  
 149 servations of parasites at all developmental stages in blood smears. The question arises as to  
 150 whether this represents a valid demonstration that sequestration does not occur. The matter pri-  
 151 marily rests on what observers define as a schizont as well as on the actual duration and extent of  
 152 sequestration, should it occur. The term ‘schizont’ has often been used loosely, and rarely de-  
 153 fined, to encompass the whole or only the ultimate period following the initiation of nuclear divi-  
 154 sion. In *P. falciparum*, nearly all the parasites sequester from 18–36 h post-invasion, with  
 155 multinucleate schizonts usually absent from the peripheral circulation. However, should



Q14 sequestration occur for only the last few hours of the cycle, concern only a proportion of the parasites, or a combination of both, then all parasite stages would be observed at any one time in the peripheral blood. Evidence for sequestration would require careful analysis of the parasite stages at close intervals throughout successive erythrocytic cycles in a synchronous infection.

From the data on the course of infection in naturally and experimentally inoculated persons (mostly for the initial episode) we could only identify 19 patients where parasitaemia was presented over time before treatment (Figure 1, and see Figures S1–S3 in the supplemental information online): in a single volunteer inoculated with sporozoites [32], in three naturally infected persons during the chronic phase [33,34], and in 15 patients or volunteers inoculated with infected blood [10,12,22,32,34]. These data are clearly insufficient to derive an estimate of multiplication rates [35]. To our knowledge, the only record of daily differential counts of parasite stages [33] was obtained from a neurosyphilitic patient inoculated with infected blood and followed up daily for 3 weeks (Figure 1). Two aspects are noteworthy. First, whereas the parasitaemia remained at relatively constant levels (5000–10 000 parasites/ $\mu$ l), there were two curious sharp peaks when parasite numbers increased five- to sixfold in 24 h and reverted to the original levels the next day. On neither occasion was the fever particularly high. Second, fully mature schizonts, Q15 known as rosettes, were observed on only 6 of the 21 days, mostly as a very minor fraction of the



Trends in Parasitology

Q2 Figure 1. Daily course of parasitaemia in a case of blood-induced *Plasmodium malariae* infection. The course of infection with daily differential counts of parasite forms over a period of 21 days, derived from a neurosyphilitic patient of Q1 35 years of age, who was inoculated intramuscularly with 5 ml of *P. malariae*-infected blood '...showed a fair number of rings and growing trophozoites and scanty schizonts and gametocytes' on 17 February. Scanty parasites were observed on 4 March, the first temperature rise was recorded on 5 March (D0 in the graph) and full patency was observed from 6 March onwards. The patient was administered an antimalarial only after 29 March. The authors define the parasite stages as follows: 'rings, still preserving the ring outline, and with the vacuole present; growing trophozoites, with chromatin undivided, schizonts, showing the division of the chromatic, mature rosettes in which the merozoites have developed fully, and are distinguishable as different individuals, gametocytes'. The graph is derived from the data presented in [33].

Q16 total parasitaemia. Interestingly, free merozoites were seen only during the first peak when they accounted for ca. 60% of the total number of parasites. To our knowledge, only one other author distinguished fully mature schizonts (the characteristic daisy-head rosette) from maturing schizonts in his observations. In an article summarizing 4 years of observations on *P. malariae* in Panama [36], William McCully James specifically notes that he has found 'the perfect segmenting form to be uncommon in the peripheral blood'. He further remarks that, in well-marked paroxysms, 'the youngest form of the parasite is first observed about two hours after the temperature begins to rise' with their number increasing over the next hours. These observations suggest that the mature or nearly mature *P. malariae* parasites sequester for a few hours, with reinvasion taking place away from the peripheral circulation. This would also be consistent with the paucity of observations of *P. malariae* rings in reticulocytes.

The first of these two observations is consistent with a cryptic cycle in which a significant proportion of the *P. malariae* biomass multiplies in the vasculature of internal organs away from the peripheral circulation. A single observation from a post-mortem examination of a chronic *P. malariae* case (who died accidentally) seems to support this possibility [37]. The concept of such a cryptic cycle has been recently made evident for *P. vivax* and *P. falciparum* [38,39] and it would seem logical to assume that the same phenomenon could occur for *P. malariae*.

#### 212 The vexed question of dormancy

213 The longevity of *Plasmodium* infections is not novel per se, as the parasites have often been found to persist throughout the life of their animal natural hosts. The longevity of *P. vivax* infections is ascribed to the activation of persisting dormant liver stages, hypnozoites, that also occur in a few other species (*P. ovale*, and *Plasmodium cynomolgi*, *Plasmodium fieldi*, and *Plasmodium simiovale* in macaques). The persistence of *P. malariae* over many years, and that of *P. falciparum* [40], is ascribed to continued erythrocytic parasite multiplication since neither species is considered to produce hypnozoites, based on the fact that relapses are not observed in sporozoite-induced *P. falciparum* or *P. malariae* sporozoite infections following chemotherapeutic clearance of the primary episode. However, a recent report of a traveller treated successfully for *P. falciparum* in Germany, who then experienced a *P. malariae* episode 4 months later, led the authors to propose the existence of *P. malariae* hypnozoites [14]. Similar cases of such late recurrences of *P. malariae* a few months after return to Germany despite adequate prophylaxis had been reported [41–43], prompting the authors to re-evaluate the question of dormant stages in *P. malariae*.

226 It should be noted that *in vivo* investigations of the liver stages of *P. malariae* are very limited indeed. The first set of observations was made when sporozoites obtained from naturally infected humans in Liberia were inoculated into four recipient chimpanzees [44], and the pre-erythrocytic stages were sought in liver biopsies taken over the next 8–12.5 days, when about 1500 nuclei were noted in the oldest schizonts. The second study was made using the sporozoites from a Romanian strain (VS) that were inoculated into a splenectomised chimpanzee [45]. The resulting liver forms were examined from days 12 to 14 by which time an estimated 15 000 merozoites in mature schizonts were observed, and their rupture on day 15. A liver biopsy, made on day 87, proved negative for parasites. Finally, liver biopsies from a person who died accidentally 3 months after inoculation with the same *P. malariae* VS strain also failed to reveal any parasite forms [37]. These meagre observations are clearly insufficient to exclude the possibility of a *P. malariae* hypnozoite, not least as the number of sporozoites inoculated in the human subject was probably too low to allow meaningful interpretation (the bite of 50 mosquitoes that were not uniformly infected).

239 Two other possibilities exist. First, the notion of a secondary exoerythrocytic cycle. The hypothesis that some hepatic merozoites invade a fresh hepatocyte to maintain a liver cycle was

241 proposed by Henry Edward Shortt and Percy Cyril Claude Garnham in 1948 [46] and was only  
242 abandoned with the discovery of the hypnozoite in 1980 [47]. This hypothesis remains unex-  
243 plored. A single observation of growing *Plasmodium chabaudi* and *Plasmodium yoelii* hepatic  
244 schizonts in the livers of naturally infected *Thamnomys rutilus* [48,49] supports a secondary exo-  
245 erythrocytic cycle, though it is insufficient as proof. Second, the existence of quiescent erythro-  
246 cytic forms, which was invoked in the 1890s by Italian malariologists to explain latent malaria.  
247 Investigations of rodent malaria parasites suggested the existence of latent merozoites [50]. Initial  
248 observations of free parasites in the lymphatic circulation of infected mice [51] and intact merozo-  
249 ites within lymphocytes [52] led to the discovery of merophores, a term coined to denote groups  
250 of intact merozoites, often enclosed in a sack or within a lymphocyte, which were found mainly  
251 in the spleen and the lymphatic circulation of infected mice [53]. The occurrence of parasites  
252 in a state of arrested development was suggested by observations on *in vitro*-cultured  
253 *P. falciparum* subjected for a few days to fully inhibitory doses of pyrimethamine but that then  
254 reappeared when the drug was removed [54]. A similar phenomenon was discovered for the  
255 ring stages of *P. falciparum* in artesunate-exposed cultures [55], and it was later confirmed that  
256 such parasites could remain 'dormant' for up to 25 days [56]. There is no biological impediment  
257 to discount the possibility that such quiescent, possibly metabolically minimally active, *P. malariae*  
258 parasites could remain viable for substantially lengthier periods.

### 259 An unexplored epidemiology

#### 260 Transmission with discretion

261 The initial difficulties in obtaining mosquito transmission [5] led the early malariologists to propose  
262 that *P. malariae* could thrive only in some special anopheline species. However, further attempts  
263 showed this to be incorrect but also revealed that the mean number of oocysts that can be ob-  
264 tained through experimental feeding rarely exceeds ten and the sporozoite output is conse-  
265 quently low (e.g., [57–62]). The sporogonic cycle at 25°C is about 5 days longer than that of  
266 *P. falciparum* and *P. vivax* and about 3 days longer than that of *P. ovale*. These features associ-  
267 ated with a relative paucity of gametocytes have contributed to considering that *P. malariae* trans-  
268 mission is somewhat inefficient, which could incidentally also account for the patchy distribution.  
269 However, it is not clear that the paucity of gametocytes should be taken as an indication of poor  
270 transmissibility. Indeed, a common feature of those very few reports of experimental transmission  
271 is the lack of correlation between the number of gametocytes in the blood and the success of  
272 mosquito infection. Moreover, infectivity to mosquitoes could be obtained during the lengthy  
273 chronic infection [44,63]. One could legitimately suggest that *P. malariae* transmission to mosqui-  
274 toes occurs continuously, albeit at low levels, which would ensure a constant pool of infective  
275 mosquitoes throughout the transmission season. Ultimately, there are woefully few detailed ob-  
276 servations to confirm or invalidate this view.

#### 277 The unavoidable underestimation of prevalence

278 The conundrum of the distinctly mosaic nature of *P. malariae* prevalence that challenged early  
279 malariologists [1,6] remains unsolved, and it is still not clear today whether *P. malariae* is in fact  
280 a rare or a common parasite [13,64]. Sole reliance on microscopic detection is not adequate to  
281 capture those harbouring parasites that hover close to the limit of detection over time. When  
282 combined with variations due to differing levels of acquired immunity, and to changing transmis-  
283 sion intensity, it is not surprising that wide variations in prevalence are observed in cross-sectional  
284 surveys. Moreover, *P. malariae* is most likely to be overlooked, even by an experienced micros-  
285 copist, in areas where infections with other parasite species that lead to higher burdens are pres-  
286 ent. A potential resolution of this question has become theoretically feasible with the development  
287 of molecular amplification techniques that provide a high level of specificity and detection sensi-  
288 tivity [65]. These brought to the fore the pervasiveness of asymptomatic infections. However,



289 the amplification techniques that are routinely used are not sufficient to provide an accurate pic-  
290 ture of *P. malariae* epidemiology. Whereas most amplification protocols can detect a single par-  
291 asite, the true limit of detection is determined by the volume of blood from which the aliquot of  
292 DNA is added to the assay. A parasitaemia below 1000 parasite/ml is likely to be missed in an  
293 assay of 1  $\mu$ l of blood. Higher limits of detection can be achieved [66], but larger blood volumes  
294 requiring special processing would be required. Ultimately, a mere routine cross-sectional survey  
295 will, by definition, lead to an underestimate of the true prevalence, and in the case of *P. malariae*  
296 substantially so. Simply put, an infection in a person who is sampled monthly but whose parasites  
Q18 are detectable for only 10 days over 3 months, is more likely to be missed than if sampling  
Q19 were performed weekly. To quote Leonard Bruce-Chwatt 'The usual single, cross-section  
299 malarimetric survey gives about as much information of the natural history of malaria in an  
300 endemic area as a few 'frames' taken out at random from a reel of film would of its plot'. Thus,  
301 the actual prevalence of *P. malariae* is likely to remain elusive. Given that nearly all cross-  
Q20 sectional molecular surveys brought to light higher numbers of *P. malariae* than were detected  
303 by microscopy, we suggest that, in all regions of moderate to high endemicity, *P. malariae* should  
304 be considered to have a significant prevalence unless proven otherwise.

#### 305 Immunity and premunition

306 Little is known concerning the acquisition of immunity to *P. malariae*. The current consensus seems  
307 to be that it is established faster, is more effective, and lasts longer than that of the other species.  
308 Nearly all the relevant observations were conducted in Romania [67–69] where the infections were  
309 induced by inoculation of erythrocytic parasites into neurosyphilitic patients from endemic areas.  
310 The Romanian workers, nonetheless, noted that sterilising immunity required more frequent repeat  
311 infections than for *P. falciparum* or *P. vivax*. To what extent the acquisition of immunity differs from  
312 that in the other species remains to be determined, as is the molecular basis of antigenic variation.

313 Edmond Sergent, Louis Parrot, and André Louis Donatien in 1924 proposed the term premuni-  
314 tion to describe a state of relative refractoriness to disease and high parasitaemias conferred  
315 by the continued presence of a subclinical/subpatent infection when subjected to new infection  
316 [70,71]. Given the particularly lengthy chronic nature of *P. malariae* infections, one might specu-  
317 late whether premunition plays a significant, if not predominant, role in the control of this parasite  
318 in its human host, and the rapid and long-lasting acquisition of immunity against it.

#### 319 A predominance of mixed infections... with consequences

320 Given their global distribution, *P. malariae* parasites invariably circulate in endemic areas where  
321 other species predominate. Thus, it is highly likely that *P. malariae* occurs frequently in mixed-species infec-  
322 tions. This is equally probable even for areas of low or seasonal endemicity because of the persistence  
323 of the asymptomatic malariae infection. It is recognised that microscopic diagnosis is particularly ill-  
324 adapted in determining the prevalence of mixed infections with *P. malariae*. The limitation imposed  
325 by low parasite numbers is confounded by the morphological similarities with other species,  
326 *P. falciparum* rings in particular and *P. knowlesi*, and by the 'flexible stopping rule' in which examina-  
327 tion of slides ceases following the discovery of a single parasite or following the enumeration of a set  
328 number of fields or cells in a blood smear [1,72]. The extent to which this prevalence can be  
329 underestimated was shown in one of the first epidemiological surveys where diagnosis by amplifica-  
330 tion revealed *P. malariae* in 44/107 positive samples of which 43 were a mixed infection [73]. Since  
331 then, the increasing number of molecular surveys in diverse endemic settings are confirming that  
332 the significant prevalence of *P. malariae* is predominantly represented by mixed infections.

333 Although vertebrate hosts, including humans, are naturally subjected to infections by multiple  
334 species, simultaneously or successively, the biological or pathological consequences remain

335 speculative, with only a few experimental investigations addressing this aspect [74]. Acquired im-  
336 munity is highly species-specific, and thus, unlikely to contribute significantly to interactions be-  
337 tween concurrent *Plasmodium* species. Seasonal alternation of species prevalence and some  
338 infections due to an unsuspected cryptic species suggested that some form of 'mutual exclusion'  
339 might occur when two species are present in the same host. Support for this was provided by the  
340 first (and possibly only) detailed description of the course of parasitaemia in two patients moni-  
341 tored over 140 days following simultaneous inoculation of *P. falciparum* and *P. vivax* by mosquito  
342 bite [75]. In both cases, *P. vivax* appeared only after the resolution of the primary peak(s) that were  
343 fully dominated by *P. falciparum*. It is interesting to note that both species coexisted during the  
344 chronic phase in one of the patients but not the other. However, although observations in England  
345 confirmed this early dominance, they also indicated that the suppression of *P. vivax* does not  
346 occur if this species is introduced a few days before *P. falciparum* [76]. The unique observation  
347 of a concurrent *P. vivax* and *P. malariae* experimental inoculation, using infected blood, showed  
348 a dominance of *P. vivax* in nine of 12 cases where both parasites appeared in the blood, but of  
349 *P. malariae* in three others, while in the four remaining patients, three showed only *P. vivax* and  
350 one only *P. malariae* [77]. Controlled studies involving the infection of nonhuman primates [78]  
351 and rodents [79,80] demonstrate that malaria parasites interact in mixed-species, as well as  
352 mixed-strain, infections and that this significantly affects the course of parasitaemia, disease se-  
353 verity, and parasite transmission. However, at present, it is not possible to predict how and to  
354 what extent this will occur, and the underlying mechanisms remain speculative.

355 In nature, *P. malariae* infections occur under highly variable environmental, epidemiological, and  
356 host conditions. A truthful insight into the interplay between coinfecting species will require  
357 large-scale longitudinal studies with standardised protocols for sample collection and highly sen-  
358 sitive parasite-detection methods. Available surveys have been predominantly cross-sectional  
359 and have employed variable sampling and detection strategies, making it difficult to derive a co-  
360 herent picture. Nonetheless, these have provided important indications worthy of further investi-  
361 gation. A preliminary study on paediatric malaria in an Ivory Coast village revealed an inverse  
362 relationship between mixed *P. falciparum*-*P. malariae* infection and fever in infected children  
363 [81]. Similar and additional interesting observations were recently made in two cross-sectional  
364 surveys of school children using the same PCR diagnostic approach in the Democratic Republic  
365 of Congo [82] and Nigeria [83]. *P. malariae* was less likely to be observed in children with symp-  
366 tomatic malaria than in those who were asymptomatic (ca. three times less in the Democratic Re-  
367 public of Congo and ten times less in Nigeria). In both studies, *P. malariae* was almost exclusively  
368 found in coinfections with *P. falciparum*, and it is assumed that symptoms were caused by the  
369 *P. falciparum* component of the mixed infection. Furthermore, both studies revealed that parasite  
370 carriage, including that of *P. malariae*, was higher in rural as compared to urban residents. This  
371 illustrates the heterogeneous nature of parasite prevalence through space, the basis of which  
372 in environmental, vector, or socioeconomic factors remains to be explored. It will also be useful  
373 to establish whether such patterns also occur in areas of low endemicity.

374 The differential distribution of *P. malariae* between asymptomatic and symptomatic infections  
375 where *P. falciparum* dominates might suggest a role of the former species in moderating clinical  
376 symptoms, which are likely to be due to the higher *P. falciparum* parasitaemias. However, al-  
377 though mathematical modelling predicts that an ongoing *P. malariae* infection could reduce the  
378 parasitaemia of a subsequent *P. falciparum* infection by up to 50% [84], other field observations  
379 on the pathological consequence of *P. falciparum* + *P. malariae* mixed infections suggest an in-  
380 crease in anaemia [85]. Alternatively, it is possible that the faster-growing *P. falciparum* sup-  
381 presses *P. malariae* either through *P. falciparum*-induced nonspecific immune responses that  
382 would reduce the already low *P. malariae* burdens or through competition for host cells (which

would be consistent with the preference for reticulocytes proposed above). If this were the case, then *P. malariae* prevalence will likely increase in areas where that of *P. falciparum* becomes reduced. This was observed in Tanzania in the early 1960s [86], and more recently in Uganda [87]. A further potential consequence of this mixed-species infection is a modulation of gametogenesis, which would alter the transmission dynamics of the coinfecting species. Indeed, analysis of malariotherapy patients infected by a *P. malariae* strain (USPHS) and subsequently by a *P. falciparum* strain (McLendon), indicated a significant increase in *P. falciparum* gametocyte production [23]. This observation was mirrored in studies in Kenya and Tanzania [88], and Burkina Faso [89] where *P. falciparum* gametocyte carriage was higher in persons with concurrent *P. malariae* parasites.

### Concluding remarks

Observations of *Plasmodium* in both vertebrate and anopheline hosts, and of the clinical course and outcome of infections, guides the nature and focus of subsequent laboratory investigations and underlies our perception of a particular species. *Plasmodium malariae* by virtue of its discrete and long-lived parasitaemias remained under the radar over the decades. This was further exacerbated by a global distribution that overlaps that of other malaria species, making it even less noticeable among the higher parasite loads of *P. vivax* and *P. falciparum*. The application of sensitive and reliable molecular diagnostic techniques is now helping to bring this species to the attention of the research community. Furthermore, the realisation that it is far more prevalent than previously thought has also attracted the notice of the malaria-control community. In the absence of a routine *in vitro* cultivation method, or a practical experimental laboratory model, all investigations, including those employing state-of-the-art technology, remain reliant on field and clinical sampling.

The comparative paucity of observations on *P. malariae* parasites has by equal measure provided the scope for assertions that evolved into dogmas, and speculation as to alternative interpretations, such as some of those presented here. It is hoped that this review will stimulate future investigations (see Outstanding questions) that will not only confirm or invalidate some of these speculations but bring forth new insights into this overlooked parasite species.

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### Declaration of interests

The authors declare no competing interests.

### Supplemental information

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### References

- Knowles, R. et al. (1930) Studies in the parasitology of malaria. *Indian Med. Res. Mem.* No 18, 1–436
- Golgi, C. (1889) Sul ciclo evolutivo dei parassiti malarici nella febbre terzana. Diagnosi differenziale tra i parassiti endoglobulari malarici della terzana e quelli della quartana. *Arch. Sci. Med. (Torino)* 13, 173–196
- Grassi, G.B. and Feletti, R. (1890) Ancora sui parassiti malarici negli uccelli. Seconda Nota preliminare. *Bull. Mens. Accad. Gioenia Sc. Nat. Catania* 14, 2–7
- Feletti, R. and Grassi, G.B. (1890) Sur les parasites de la malaria. 1ère et 2ème notes préliminaires. *Arch. Ital. Biol.* 13, 287–296
- Gordon, R.M. and Davey, T.H. (1932) *P. malariae* in Freetown, Sierra Leone. *Ann. Trop. Med. Parasitol.* 26, 65–84
- Marchoux, E. (1926) *Paludisme (Nouveau Traité de Médecine et de Thérapeutique)*, Vol. 5. Baillière et Fils
- Wagner-Jauregg, J. (1918) Über die Einwirkung der Malaria auf die progressive Paralyse. *Psych.-neur. Wochenschr.* 20, 132–134

### Outstanding questions

What is the true prevalence of *P. malariae* globally?

Does *P. malariae* preferentially invade reticulocytes?

Do certain forms of *P. malariae* sequester?

Are there dormant tissue stages of *P. malariae*?

Does *P. malariae* modulate the severity or transmissibility of *P. falciparum* pathology?

- 441 8. Snounou, G. and Pérignon, J.-L. (2013) Malariotherapy – insanity  
442 at the service of malariology. *Adv. Parasitol.* 81, 223–255
- 443 9. Coatney, G.R. *et al.* (1971) *Plasmodium malariae*. In *The Primate*  
444 *Malarias*, pp. 209–229, U.S. Government Printing Office
- 445 10. Collins, W.E. and Jeffery, G.M. (2007) *Plasmodium malariae*:  
446 parasite and disease. *Clin. Microbiol. Rev.* 20, 579–592
- 447 11. Garnham, P.C.C. (1966) Human quartan malaria parasite.  
448 In *Malaria Parasites and Other Haemosporidia*, pp. 257–284,  
449 Blackwell Scientific Publications
- 450 12. Kitchen, S.F. (1949) Quartan malaria. In *Malariology. A Comprehen-*  
451 *sive Survey of All Aspects of This Group of Diseases from a Global*  
452 *Standpoint* (Boyd, M.F., ed.), pp. 1017–1026, W.B. Saunders
- 453 13. Mueller, I. *et al.* (2007) *Plasmodium malariae* and *Plasmodium*  
454 *ovale* – the ‘bashful’ malaria parasites. *Trends Parasitol.* 23,  
455 278–283
- 456 14. Franken, G. *et al.* (2012) Why do *Plasmodium malariae* infec-
- 457 tions sometimes occur in spite of previous antimalarial medica-
- 458 tion? *Parasitol. Res.* 111, 943–946
- 459 15. Sutherland, C.J. (2016) Persistent parasitism: the adaptive  
460 biology of malariae and ovale malaria. *Trends Parasitol.* 32,  
461 808–819
- 462 16. Ansari, H.R. *et al.* (2016) Genome-scale comparison of ex-
- 463 panded gene families in *Plasmodium ovale wallikeri* and  
464 *Plasmodium ovale curtisi* with *Plasmodium malariae* and with  
465 other *Plasmodium* species. *Int. J. Parasitol.* 46, 685–696
- 466 17. Rutledge, G.G. *et al.* (2017) *Plasmodium malariae* and *P. ovale*  
467 genomes provide insights into malaria parasite evolution. *Nature*  
468 542, 101–104
- 469 18. Rutledge, G.G. *et al.* (2017) Genomic characterization of recr-
- 470 descent *Plasmodium malariae* after treatment with artemether/  
471 lumefantrine. *Emerg. Infect. Dis.* 23, 1300–1307
- 472 19. Talundzic, E. *et al.* (2017) First full draft genome sequence of  
473 *Plasmodium brasilianum*. *Genome Announc.* 5, e01566–01516
- 474 20. Collins, W.E. and Jeffery, G.M. (1999) A retrospective examination  
475 of sporozoite- and trophozoite-induced infections with *Plasmodium*  
476 *falciparum* in patients previously infected with heterologous species  
477 of *Plasmodium*: effect on development of parasitologic and clinical  
478 immunity. *Am. J. Trop. Med. Hyg.* 61, 36–43
- 479 21. Collins, W.E. and Jeffery, G.M. (1999) A retrospective examination  
480 of secondary sporozoite- and trophozoite-induced infections with  
481 *Plasmodium falciparum*: development of parasitologic and clinical  
482 immunity following secondary infection. *Am. J. Trop. Med. Hyg.*  
483 61, 20–35
- 484 22. McKenzie, F.E. *et al.* (2001) *Plasmodium malariae* blood-stage  
485 dynamics. *J. Parasitol.* 87, 626–637
- 486 23. McKenzie, F.E. *et al.* (2002) *Plasmodium malariae* infection  
487 boosts *Plasmodium falciparum* gametocyte production. *Am.*  
488 *J. Trop. Med. Hyg.* 67, 411–414
- 489 24. Eaton, P. (1934) Susceptibility of red cells to malaria. A prelimi-  
490 nary note. *Am. J. Trop. Med.* 14, 431–437
- 491 25. Hegner, R.W. (1938) Relative frequency of ring-stage  
492 *Plasmodia* in reticulocytes and mature erythrocytes in man  
493 and monkey. *Am. J. Hyg.* 27, 690–718
- 494 26. Kitchen, S.F. (1939) The infection of mature and immature  
495 erythrocytes by *Plasmodium falciparum* and *Plasmodium*  
496 *malariae*. *Am. J. Trop. Med.* 19, 47–62
- 497 27. Chwatt, L.J. (1948) Infection of reticulocytes by *Plasmodium*  
498 *falciparum* and *Plasmodium malariae* in hyperendemic indige-  
499 nous malaria. *Ann. Trop. Med. Parasitol.* 42, 101–112
- 500 28. Malleret, B. *et al.* (2015) *Plasmodium vivax*: restricted tropism  
501 and rapid remodeling of CD71-positive reticulocytes. *Blood*  
502 125, 1314–1324
- 503 29. Chowdhuri, A.N.R. *et al.* (1979) Simultaneous propagation of  
504 *P. malariae* and *P. falciparum* in a continuous culture. *Indian*  
505 *J. Med. Res.* 70, 72–78
- 506 30. Lingnau, A. *et al.* (1994) Evidence for 6-day cultivation of human  
507 *Plasmodium malariae*. *Parasitol. Res.* 80, 265–266
- 508 31. Siddiqui, W.A. *et al.* (1972) *In vitro* cultivation of *Plasmodium*  
509 *malariae*. *J. Parasitol.* 58, 804
- 510 32. Mackerras, M.J. and Ercole, Q.N. (1948) Observations on the  
511 life-cycle of *Plasmodium malariae*. *Aust. J. Exp. Biol. Med.*  
512 *Sci.* 26, 515–519
- 513 33. Knowles, R. and Das Gupta, B.M. (1930) Studies in untreated  
514 malaria. I. A case of experimentally induced quartan malaria.  
515 *Ind. Med. Gaz.* 65, 301–310
34. Knowles, R. and Das Gupta, B.M. (1932) Clinical studies in malaria  
516 by cultural and enumerative methods. Second series. *Ind. Med.*  
517 *Gaz.* 67, 432–446
- 518 35. Nganngon, B. *et al.* (2021) Deconstructing the parasite multipli-  
519 cation rate of *Plasmodium falciparum*. *Trends Parasitol.* 37,  
520 922–932
- 521 36. James, W.M. (1910) Quartan malaria and its parasite. With  
522 especial reference to the occurrence of this form of malaria in  
523 the Canal Zone. *Proc. Canal Z. Med Assoc.* 3, 29–58
- 524 37. Lupascu, G. *et al.* (1968) Parasitological and clinical investiga-  
525 tions on infections with the VS Romanian strain of *Plasmodium*  
526 *malariae* transmitted by *Anopheles labranchiae atroparvus*. *Bull.*  
527 *World Health Organ.* 38, 61–67
- 528 38. Kho, S. *et al.* (2021) Evaluation of splenic accumulation and  
529 colocalization of immature reticulocytes and *Plasmodium vivax*  
530 in asymptomatic malaria: a prospective human splenectomy  
531 study. *PLoS Med.* 18, e1003632
- 532 39. Kho, S. *et al.* (2021) Hidden biomass of intact malaria parasites  
533 in the human spleen. *N. Engl. J. Med.* 384, 2067–2069
- 534 40. Ashley, E.A. and White, N.J. (2014) The duration of *Plasmodium*  
535 *falciparum* infections. *Malar. J.* 13, 500
- 536 41. Hess, F.I. *et al.* (1993) Problems in the therapy of mixed malarial  
537 infections: a case of infection with *Plasmodium falciparum* and  
538 *P. malariae* treated with mefloquine and halofantrine. *Trans. R.*  
539 *Soc. Trop. Med. Hyg.* 87, 688
- 540 42. Müller-Stöver, I. *et al.* (2008) *Plasmodium malariae* infection in  
541 spite of previous anti-malarial medication. *Parasitol. Res.* 102,  
542 547–550
- 543 43. Teo, B.H.-Y. *et al.* (2015) Delayed onset of symptoms and  
544 atovaquone-proguanil chemoprophylaxis breakthrough by  
545 *Plasmodium malariae* in the absence of mutation at codon  
546 268 of *pmc1b*. *PLoS Negl. Trop. Dis.* 9, e0004068
- 547 44. Bray, R.S. (1960) Studies of malaria in chimpanzees. VIII. The  
548 experimental transmission and pre-erythrocytic phase of *Plas-*  
549 *modium malariae*, with a note on the host-range of the parasite.  
550 *Am. J. Trop. Med. Hyg.* 9, 455–465
- 551 45. Lupascu, G. *et al.* (1967) The late primary exo-erythrocytic  
552 stages of *Plasmodium malariae*. *Trans. R. Soc. Trop. Med.*  
553 *Hyg.* 61, 482–489
- 554 46. Shortt, H.E. and Garnham, P.C.C. (1948) The pre-erythrocytic  
555 development of *Plasmodium cynomolgi* and *Plasmodium*  
556 *vivax*. *Trans. R. Soc. Trop. Med. Hyg.* 41, 785–795
- 557 47. Krotoski, W.A. *et al.* (1980) Relapses in primate malaria: discov-  
558 ery of two populations of exoerythrocytic stages. Preliminary  
559 note. *Br. Med. J.* 280, 153–154
- 560 48. Landau, I. and Chabaud, A.G. (1968) Schizogonie hépatique  
561 secondaire dans le paludisme spontané des rongeurs. *C. R.*  
562 *Acad. Sci. Hebd. Seances Acad. Sci. D* 266, 1730–1733
- 563 49. Landau, I. *et al.* (1968) Morphologie et évolution des schizontes  
564 hépatiques secondaires dans le paludisme spontané des rongeurs  
565 de La Maboké. *Cahiers de La Maboké* 6, 63–72 (in French)
- 566 50. Landau, I. and Chabaud, A.G. (1994) Latency of *Plasmodium*  
567 merozoites and drug-resistance. A review. *Parasite* 1, 105–114
- 568 51. Landau, I. *et al.* (1995) Circulation in the lymphatic system and  
569 latency of *Plasmodium* merozoites. Preliminary note. *Parasite*  
570 2, 185–186
- 571 52. Boulard, Y. *et al.* (1996) Dendritic leucocytes as possible carriers  
572 of murine *Plasmodium* merozoites. Preliminary note. *Parasite* 3,  
573 383–386
- 574 53. Landau, I. *et al.* (1999) Survival of rodent malaria merozoites in  
575 the lymphatic network: potential role in chronicity of the infec-  
576 tion. *Parasite* 6, 311–322
- 577 54. Nakazawa, S. *et al.* (1995) *Plasmodium falciparum*: recrudesc-  
578 ence of parasites in culture. *Exp. Parasitol.* 81, 556–563
- 579 55. Kyle, D.E. and Webster, H.K. (1996) Postantibiotic effect of  
580 quinine and dihydroartemisinin on *Plasmodium falciparum*  
581 *in vitro*: implications for a mechanism of recrudescence. In  
582 *XIVth International Congress for Tropical Medicine and*  
583 *Malaria*
- 584 56. Teuscher, F. *et al.* (2010) Artemisinin-induced dormancy in  
585 *Plasmodium falciparum*: duration, recovery rates, and implica-  
586 tions in treatment failure. *J. Infect. Dis.* 202, 1362–1368
- 587 57. Boyd, M.F. and Stratman-Thomas, W.K. (1933) A note on the  
588 transmission of quartan malaria by *Anopheles quadrimaculatus*.  
589 *Am. J. Trop. Med.* 13, 265–271
- 590



591 58. Collins, W.E. *et al.* (1975) Studies on human malaria in *Aotus*  
592 monkeys. VI. Infectivity of *Plasmodium malariae* to different  
593 Anophelines. *J. Parasitol.* 61, 941–943

594 59. Collins, W.E. *et al.* (1993) Experimental infection of *Anopheles*  
595 *gambiae* s.s., *Anopheles freeborni* and *Anopheles stephensi*  
596 with *Plasmodium malariae* and *Plasmodium brasilianum*.  
597 *J. Am. Mosq. Control Assoc.* 9, 68–71

598 60. Shute, P.G. and Maryon, M.E. (1951) Studies in the transmission  
599 of *Plasmodium malariae* by *Anopheles* mosquitoes. *Parasitology*  
600 41, 292–300

601 61. Siddons, L.B. (1944) The experimental transmission of quartan  
602 malaria by *Anopheles culicifacies* Giles. *J. Mal. Inst. India* 5,  
603 361–373

604 62. Young, M.D. and Burgess, R.W. (1947) The transmission of  
605 *Plasmodium malariae* by *Anopheles maculipennis freeborni*.  
606 *Am. J. Trop. Med.* 27, 39–40

607 63. Constantinescu, P. and Negulici, E. (1967) The experimental  
608 transmission of *Plasmodium malariae* to *Anopheles labranchiae*  
609 *atroparvus*. *Trans. R. Soc. Trop. Med. Hyg.* 61, 182–188

610 64. Oriero, E.C. *et al.* (2021) *Plasmodium malariae*, current knowl-  
611 edge and future research opportunities on a neglected malaria  
612 parasite species. *Crit. Rev. Microbiol.* 47, 44–56

613 65. Snounou, G. *et al.* (1993) High sensitivity of detection of human  
614 malaria parasites by the use of nested polymerase chain reac-  
615 tion. *Mol. Biochem. Parasitol.* 61, 315–320

616 66. Imwong, M. *et al.* (2014) High-throughput ultrasensitive molec-  
617 ular techniques for quantifying low-density malaria parasitemias.  
618 *J. Clin. Microbiol.* 52, 3303–3309

619 67. Ciuca, M. (1938) Contribution à l'étude de la fièvre quarte  
620 d'inoculation étude comparée de deux souches de *Pl. malariae*.  
621 *Bull. Sec. Scient. Acad. Roum.* 21, 1–11 (in French)

622 68. Ciuca, M. *et al.* (1943) Contribution à l'étude de l'immunité dans  
623 l'infection paludéenne expérimentale. Observations recueillies  
624 sur 41 sujets immunisés. *Arch. Roum. Pathol. Exp. Microbiol.*  
625 13, 45–122 (in French)

626 69. Ciuca, M. *et al.* (1934) Immunity in malaria. *Trans. R. Soc. Trop.*  
627 *Med. Hyg.* 27, 619–622

628 70. Sergeant, E. *et al.* (1924) Une question de terminologie: immuniser  
629 et prémunir. *Bull. Soc. Pathol. Exot.* 17, 37–38 (in French)

630 71. Sergeant, E. *et al.* (1926) Prémunition et protozooses sanguines.  
631 *Bull. Soc. Pathol. Exot.* 19, 532–540 (in French)

632 72. Knowles, R. and White, R.S. (1930) Studies in the parasitology  
633 of malaria. *Indian Medical Research. Memoirs* 18

634 73. Snounou, G. *et al.* (1993) The importance of sensitive detection  
635 of malaria parasites in the human and insect hosts in epidemiol-  
636 ogy studies, as shown by the analysis of field samples from  
637 Guinea Bissau. *Trans. R. Soc. Trop. Med. Hyg.* 87, 649–653

638 74. Richie, T.L. (1988) Interactions between malaria parasites in-  
639 fecting the same vertebrate host. *Parasitology* 96, 607–639

640 75. Boyd, M.F. and Kitchen, S.F. (1937) Simultaneous inoculation  
641 with *Plasmodium vivax* and *Plasmodium falciparum*. *Am.*  
642 *J. Trop. Med.* 17, 855–861

643 76. Shute, P.G. (1946) Latency and long-term relapses in benign  
644 tertian malaria. *Trans. R. Soc. Trop. Med. Hyg.* 40, 189–200

645 77. Mayne, B. and Young, M.D. (1938) Antagonism between spe-  
646 cies of malaria parasites in induced mixed infections (Preliminary  
647 note). *Public Health Rep.* 53, 1289–1291

648 78. Voller, A. and Rossan, R.N. (1969) Immunological studies on  
649 simian malaria parasites. IV. Heterologous superinfection of  
650 monkeys with chronic *Plasmodium knowlesi* infections. *Trans.*  
651 *R. Soc. Trop. Med. Hyg.* 63, 837–845

652 79. Snounou, G. *et al.* (1992) Assessment of parasite population  
653 dynamics in mixed infections of rodent plasmodia. *Parasitology*  
654 105, 363–374

655 80. Tang, J. *et al.* (2019) The consequences of mixed-species ma-  
656 laria parasite co-infections in mice and mosquitoes for disease  
657 severity, parasite fitness, and transmission success. *Front.*  
658 *Immunol.* 10, 3072

659 81. Black, J. *et al.* (1994) Mixed infections with *Plasmodium*  
660 *falciparum* and *P. malariae* and fever in malaria. *Lancet* 343, 1095

661 82. Nundu, S.S. *et al.* (2021) Malaria parasite species composition  
662 of *Plasmodium* infections among asymptomatic and symptom-  
663 atic school-age children in rural and urban areas of Kinshasa,  
664 Democratic Republic of Congo. *Malar. J.* 20, 389

83. Abdurraheem, M.A. *et al.* (2022) High prevalence of  
665 *Plasmodium malariae* and *Plasmodium ovale* in co-  
666 infections with *Plasmodium falciparum* in asymptomatic  
667 malaria parasite carriers in southwestern Nigeria. *Int.*  
668 *J. Parasitol.* 52, 23–33

669 84. Mason, D.P. *et al.* (1999) The blood-stage dynamics of mixed  
670 *Plasmodium malariae-Plasmodium falciparum* infections.  
671 *J. Theor. Biol.* 198, 549–566

672 85. May, J. *et al.* (2000) Impact of subpatent multi-species and  
673 multi-clonal plasmodial infections on anaemia in children from  
674 Nigeria. *Trans. R. Soc. Trop. Med. Hyg.* 94, 399–403

675 86. Bruce-Chwatt, L.J. (1963) A longitudinal survey of natural ma-  
676 laria infection in a group of West African adults. Part I. *West*  
677 *African Med. J.* 12, 141–173

678 87. Betson, M. *et al.* (2018) Emergence of nonfalciparum  
679 *Plasmodium* infection despite regular artemisinin combina-  
680 tion therapy in an 18-month longitudinal study of Ugandan  
681 children and their mothers. *J. Infect. Dis.* 217, 1099–1109

682 88. Bousema, J.T. *et al.* (2008) Increased *Plasmodium falciparum*  
683 gametocyte production in mixed infections with *P. malariae*.  
684 *Am. J. Trop. Med. Hyg.* 78, 442–448

685 89. Gnémé, A. *et al.* (2013) *Plasmodium* species occurrence, tem-  
686 poral distribution and interaction in a child-aged population in  
687 rural Burkina Faso. *Malar. J.* 12, 67

688 90. Otto, T.D. *et al.* (2014) Genome sequencing of chimpanzee ma-  
689 laria parasites reveals possible pathways of adaptation to  
690 human hosts. *Nat. Commun.* 5, 4754

691 91. Reichenow, E. (1921) Ueber das Vorkommen der Malaria-  
692 parasiten des Menschen bei den afrikanischen Menschenaffen. *Centralbl.*  
693 *Bakt. Parasitenk. Infektionsk. 1. Abt. Orig.* 85, 207–216  
694 (in German)

695 92. Plenderleith, L.J. *et al.* (2022) Zoonotic origin of the human ma-  
696 laria parasite *Plasmodium malariae* from African apes. *Nat.*  
697 *Commun.* 13, 1868

698 93. Garnham, P.C.C. (1966) *Malaria Parasites and Other Haemo-*  
699 *sporidia*, Blackwell Scientific Publications

700 94. Coatney, G.R. *et al.* (1971) *The Primate Malariae*, U.S. Government  
701 Printing Office

702 95. Carlton, J.M.-R. and Sullivan, S.A. (2017) A feast of malaria pa-  
703 rasite genomes. *Cell Host Microbe* 21, 310–312

704 96. Sutherland, C.J. *et al.* (2010) Two nonrecombining sympatric  
705 forms of the human malaria parasite *Plasmodium ovale* occur  
706 globally. *J. Infect. Dis.* 201, 1544–1550

707 97. Schaefer, J. *et al.* (2013) High diversity of West African bat malaria  
708 parasites and a tight link with rodent *Plasmodium* taxa. *Proc.*  
709 *Natl. Acad. Sci. U. S. A.* 110, 17415–17419

710 98. Arisue, N. *et al.* (2012) The *Plasmodium* apicoplast genome:  
711 conserved structure and close relationship of *P. ovale* to rodent  
712 malaria parasites. *Mol. Biol. Evol.* 29, 2095–2099

713 99. Tahar, R. *et al.* (1998) Heterogeneity in the circumsporozoite  
714 protein gene of *Plasmodium malariae* isolates from sub-  
715 saharan Africa. *Mol. Biochem. Parasitol.* 92, 71–78

716 100. Bruce, M.C. *et al.* (2000) Cross-species interactions between  
717 malaria parasites in humans. *Science* 287, 845–848

718 101. Bruce, M.C. *et al.* (2007) Characterization and application of  
719 multiple genetic markers for *Plasmodium malariae*. *Parasitology*  
720 134, 637–650

721 102. Guimarães, L.O. *et al.* (2012) The genetic diversity of *Plasmodium*  
722 *malariae* and *Plasmodium brasilianum* from human, simian and  
723 mosquito hosts in Brazil. *Acta Trop.* 124, 27–32

724 103. Fuehrer, H.-P. *et al.* (2014) High prevalence and genetic  
725 diversity of *Plasmodium malariae* and no evidence of  
726 *Plasmodium knowlesi* in Bangladesh. *Parasitol. Res.* 113,  
727 1537–1543

728 104. Lalremruata, A. *et al.* (2015) Natural infection of *Plasmodium*  
729 *brasilianum* in humans: man and monkey share quartan ma-  
730 laria parasites in the Venezuelan Amazon. *EBioMedicine* 2,  
731 1186–1192

732 105. Lalremruata, A. *et al.* (2017) Species and genotype diversity of  
733 *Plasmodium* in malaria patients from Gabon analysed by next  
734 generation sequencing. *Malar. J.* 16, 398

735 106. Saralamba, N. *et al.* (2018) Genetic polymorphisms in the  
736 circumsporozoite protein of *Plasmodium malariae* show a geo-  
737 graphical bias. *Malar. J.* 17, 269

738



- 739 107. Srisutham, S. *et al.* (2018) Genetic diversity of three surface pro-  
740 tein genes in *Plasmodium malariae* from three Asian countries.  
741 *Malar. J.* 17, 24
- 742 108. Crosnier, C. *et al.* (2011) Basigin is a receptor essential for eryth-  
743 rocyte invasion by *Plasmodium falciparum*. *Nature* 480, 534–537
- 744 109. Oyola, S.O. *et al.* (2016) Whole genome sequencing of *Plasmodium*  
745 *falciparum* from dried blood spots using selective whole genome  
746 amplification. *Malar. J.* 15, 597
- 747 110. Cowell, A.N. *et al.* (2017) Selective whole-genome amplification  
748 is a robust method that enables scalable whole-genome se-  
749 quencing of *Plasmodium vivax* from unprocessed clinical  
750 samples. *mBio* 8, e02257–02216
111. Benavente, E.D. *et al.* (2019) Whole genome sequencing  
751 of amplified *Plasmodium knowlesi* DNA from unpro-  
752 cessed blood reveals genetic exchange events between  
753 Malaysian Peninsular and Borneo subpopulations. *Sci.*  
754 *Rep.* 9, 9873
112. Ibrahim, A. *et al.* (2020) Selective whole genome amplification of  
755 *Plasmodium malariae* DNA from clinical samples reveals in-  
756 sights into population structure. *Sci. Rep.* 10, 10832
113. Lee, R.A. *et al.* (2020) Ultrasensitive CRISPR-based diagnostic for  
757 field-applicable detection of *Plasmodium* species in symptomatic  
758 and asymptomatic malaria. *Proc. Natl. Acad. Sci. U. S. A.* 117,  
759 25722–25731
- 760  
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Supplementary file

***Plasmodium malariae*: the persisting mysteries of a persistent parasite**

Richard Culleton<sup>1,2</sup>, Arnab Pain<sup>3</sup>, Georges Snounou<sup>4\*</sup>

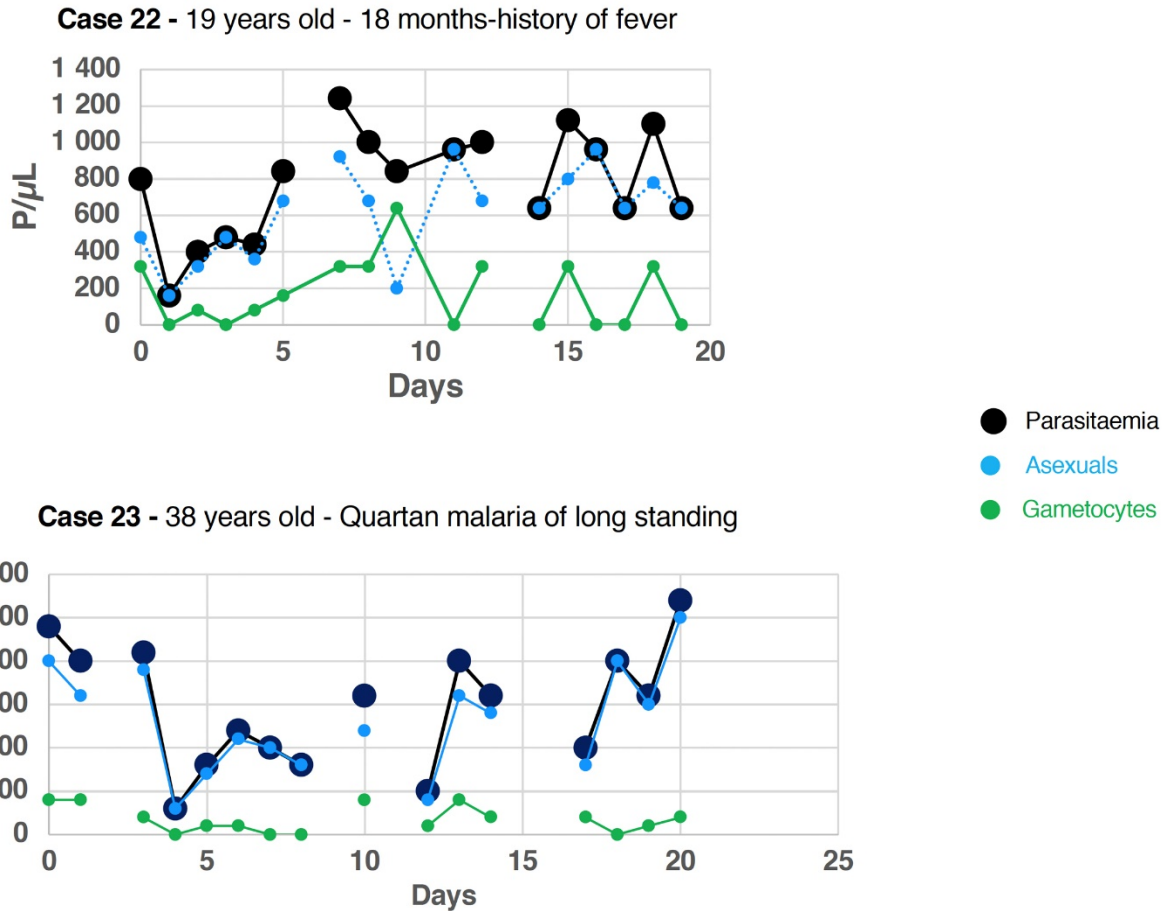
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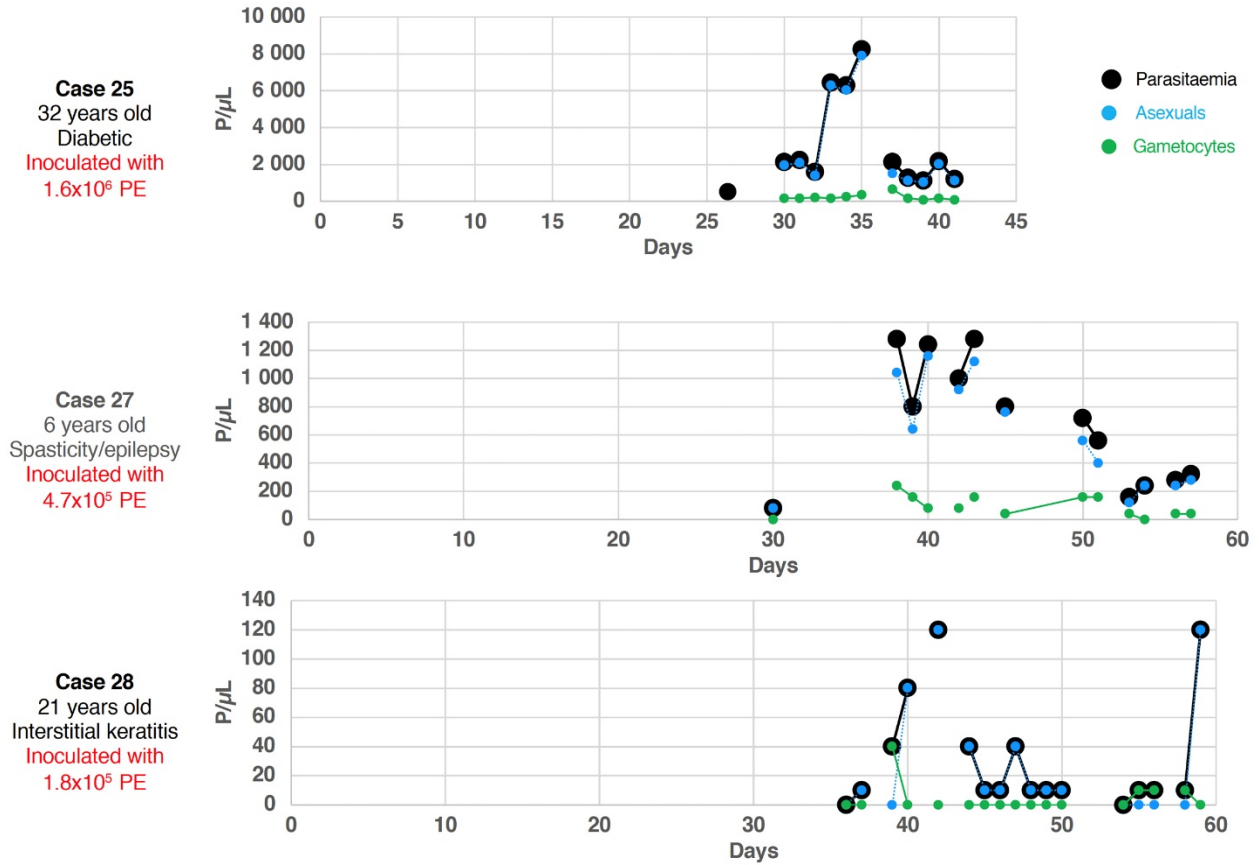
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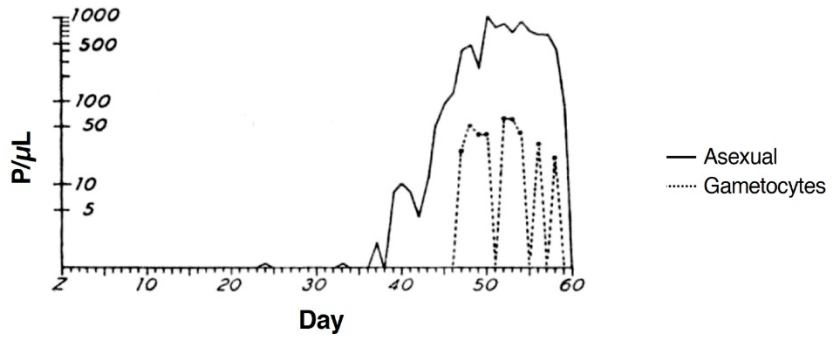


**Figure S1. Parasitological course of chronic naturally acquired *P. malariae* infections.** Two naturally infected patients with chronic *P. malariae* followed up after admission (Day 0) for 20 days prior to initiation of anti-malarial treatment. P/μL = parasites/μL. P/μL = Parasites/μL. The data are derived from [S1].

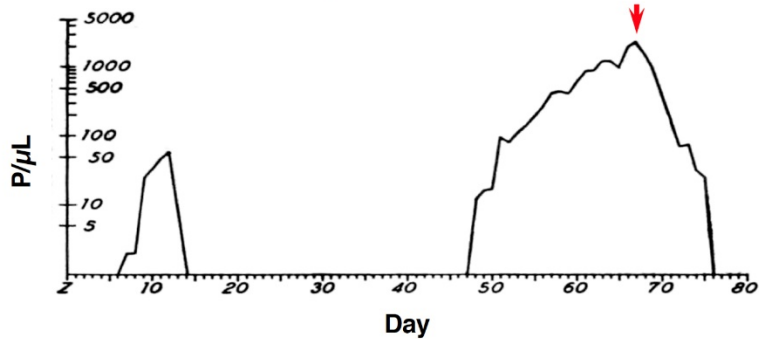


**Figure S2. Course of infection following *P. malariae*-infected blood inoculation.** Three patients were subjected to malariotherapy through inoculation of *P. malariae*-infected parasitised erythrocytes (PE) on Day 0 and then allowed to experience several paroxysms. The parasitaemias presented are that of the follow-up before the initiation of anti-malarial treatment. P/μL = Parasites/μL. The data are derived from [S1].

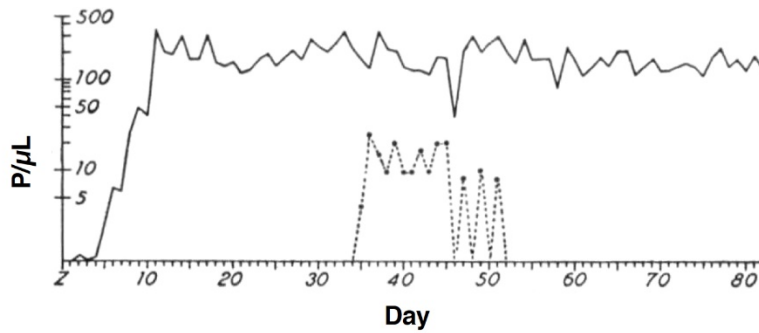
**Volunteer 1**  
Bitten by 3 infected  
mosquitoes over a period  
of 10 days



**Volunteer 2**  
Sub-inoculated with  $1.8 \times 10^6$   
PE derived from Volunteer 1



**Volunteer 3**  
Sub-inoculated with  $6.4 \times 10^6$   
PE derived from Volunteer 1



**Figure S3. Variation in the course of infections induced by a Papua New Guinea *P.***


***malariae* strain.** Three volunteers were experimentally infected with *P. malariae*. Volunteer 1 was infected through the bites of mosquitoes that had fed on a *P. malariae* carrier from Papua New Guinea. The two other volunteers sub-inoculated from blood subsequently collected from Volunteer 1. Only Volunteer 2 required anti-malarial treatment (red arrow) before the termination of the follow-up. P/μL = Parasites/μL. Adapted from [S2].



## References

- S1. Knowles, R. and Das Gupta, B.M. (1932) Clinical studies in malaria by cultural and enumerative methods. Second series. *Ind. Med. Gaz.* 67, 432-446.
- S2. Mackerras, M.J. and Ercole, Q.N. (1948) Observations on the life-cycle of *Plasmodium malariae*. *Aust. J. Exp. Biol. Med. Sci* 26, 515-519.

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