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Biological concepts in recurrent *Plasmodium vivax* malaria

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Abstract

A curious aspect of the evolution of the hypnozoite theory of malarial relapse is its transmogrification from theory into 'fact', this being of historical, linguistic, scientific and sociological interest. As far as it goes, the hypnozoite explanation for relapse is almost certainly correct. I contend, however, that many of the genotypically homologous, non-reinfection, relapse-like *Plasmodium vivax* recurrences that researchers ascribe to hypnozoite activation are probably hypnozoite-independent. Indeed, some malariologists are starting to recognize that homologous *P. vivax* recurrences have most likely been overattributed to activation of hypnozoites. Hitherto identified, non-hypnozoite, possible plasmodial sources of recurrence that must be considered, besides circulating erythrocytic stages, include parasites in splenic dendritic cells, other cells in the spleen (in addition to infected erythrocytes there), bone marrow (importantly) and the skin. I argue that we need to take into account the possibility of a dual or multiple extra-vascular origin of *P. vivax* non-reinfection recurrences, not arbitrarily discount it. The existence of a *P. vivax* reservoir(s) is a topical subject and one of practical importance for malaria eradication. Pertinent drug-associated matters are also discussed, as is the dormancy-related significance of clues provided by blood-stage-induced malarial infection.

Introduction

The malaria parasite *Plasmodium vivax*, like *P. malariae*, *P. ovale curtisi* and *P. o. wallikeri*, exhibits the phenomenon of 'persistent parasitism' (Sutherland, 2016), which is increasingly being encountered during the course of epidemiological investigations (Betson *et al.* 2018). One of the forms in which *P. vivax* persists is the 'hypnozoite' (Markus, 1978, 2011*a*). This hepatic stage is conventionally thought to be the source of relapse in *P. vivax* malaria (Markus, 2011*b*, 2016*a*), and the indirect evidence for the validity of the hypothesis is compelling, despite the theory remaining parasitologically unproven in humans. As regards hypnozoite-independent recurrences, it was concluded approximately 7 years ago (Table 1) that there might be more to *P. vivax* recurrences biologically than is readily apparent (Markus, 2011*c*, 2012*a*,*b*). Additional suggestions made since then are covered in this paper. Concepts involved are summarized below and attention is called to contrasting styles of discourse.

'Recurrence' means that the cause of renewed clinical illness or peripheral blood parasitaemia is a recrudescence, a relapse, or a reinfection. In this article, the last-mentioned cause is excluded by the description 'non-reinfection recurrence'. A 'recrudescence' has a merozoite source, whereas the origin of a 'relapse' is hypnozoite activation. For further details regarding these malaria-related definitions, see Box 1 in Markus (2015) and Box 1 in Markus (2017). The combined information in the two cited publications is a comprehensive terminological explanation that includes judgemental comments.

Evolution of the hypnozoite theory of relapse

The character of discourse on the hypnozoite hypothesis of relapse and latency in malaria has changed over time. The factual way in which parasitologists talk today about what hypnozoites allegedly do was also my attitude up until a few years ago (Markus, 2011*b*). By contrast, malariologists in the 1980s did not always rationalize so rigidly, particularly those who carried out hypnozoite-associated studies. Knowing more about malarial recurrence than their colleagues and being cognizant of past mistakes (Shortt and Garnham, 1948; Pays, 2012), they sometimes seemed reluctant to state firmly that relapses are hypnozoite-mediated. One is hard-pressed to find this earlier type of qualified statement in today's publications. The scientific wait-and-see attitude has all but disappeared, the greater caution evident in some of the old literature having been discarded. Current, almost exclusively factual statements about hitherto unproven aspects of malarial relapse are, however, not necessarily wrong. On the contrary, the dogma that hypnozoites are the source of relapse in *P. vivax* and *P. ovale sensu lato* malaria is almost certainly correct, at least for *P. vivax* (*P. ovale* falls outside the scope of this article). Nevertheless, Richter *et al.* (2016), when writing open-mindedly about their idea of graded dormancy,

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Table 1. Relapse in malaria: main related events and hypotheses

Year	Details	Reference(s)
Pre-1948	The most prevalent theory before 1948 was that the origin of relapses was parasites in the reticulo-endothelial system	Corradetti (1982)
1948	Discovery of hepatic schizogony in the life cycle of *Plasmodium*. This led to malarial relapse being explained as the consequence of continuous cycles of schizogony taking place in the liver (assumed to be the source of parasites for renewed erythrocytic schizogony)	Shortt and Garnham (1948)
1976	Discovery of the apicomplexan hypnozoite (non-malarial) by ultrastructural recognition of its sporozoite-like nature	Mehlhorn and Markus (1976)
1976	Occurrence of hypnozoites in the life cycle of *Plasmodium* predicted on the basis of non-plasmodial research results (by extrapolation)	Markus (1976)
1978	Coining of the term 'hypnozoite' and its adoption for *Plasmodium* (in advance of and in anticipation of the future discovery of malarial hypnozoites)	Markus (1978, 2011*a*)
1980	Discovery of the malarial hypnozoite, resulting in the hypnozoite hypothesis of relapse and latency in malaria becoming established	Krotoski *et al.* (1980)
2011	First proposal that there might be one or more hypnozoite-independent, non-bloodstream sources of homologous (specifically) *Plasmodium vivax* parasites in recurrences. Such recurrences would be recrudescences, not relapses. The suggestion is that *P. vivax* recurrences are being overattributed to hypnozoite activation	Markus (2011*c*, 2012*a*, *b*)

expressed surprise at '… how rapidly the scientific world switched to the hypnozoite concept'. Schmidt (1986) was similarly puzzled by the '… promptness with which the hypnozoite concept was accepted …'. The current dogma is reminiscent of an earlier (but probably erroneous) conviction that prevailed between 1948 and 1980 about the origin of relapse (Table 1). That concept was usually worded as a fact. Textbooks and journal articles would emphatically state that sequential cycles of schizogony take place in the liver during *P. vivax* malaria. In regard to malarial relapse, we find a clash between what has been accepted historically and what has seemingly been correct. This prompted Corradetti (1982) to remark that he considered it '… safer to doubt any theory until it has been clearly demonstrated'. He was referring primarily, but not solely, to pre-1948 relapse dogma (Table 1), which also appeared 'factually' in textbooks and elsewhere. Of relevance to the elucidation of the biology of *Plasmodium* in general is the observation by Pays (2012) that '… deference towards scientific orthodoxy and a degree of reluctance to question and to criticise can delay advances in knowledge'.

As mentioned above, relapse in malaria was between 1948 and 1980 (Table 1) explained in terms of repeated cycles of hepatic schizogony (Shortt and Garnham, 1948). In writing about the concept, Desowitz (2000) noted that the arguments '… are now fading with the years, as the graduate students and associates … (of Shortt and Garnham) also now fade into history'. This is true for the post-1980 hypnozoite concept of relapse (Table 1) as well, which in the view of most malariologists has replaced the previous theory. Note from the illustrative early (mainly 1980s) quotations below how the hypnozoite hypothesis became transmogrified into a 'fact', this being virtually the only way the concept is referred to by authors today. As I have already indicated, tentative or qualified phrasing on this topic is almost completely absent from present-day researchers' publications.

The earlier discourse style enabled hypnozoite researchers to avoid 'putting their heads on any blocks', so to speak. Bray and Garnham (1982) used the words 'believe' and 'belief' when talking about the hypnozoite concept of relapse. Furthermore, they cautiously wrote that sporozoites of particular (so-called 'non-relapsing') primate plasmodial species '… are *thought* not to differentiate into hypnozoites' (italics added). Bray (1984) began a statement tentatively: 'If one accepts the hypnozoite theory of relapse and long-term delayed primaries …'. Garnham (1988) also hedged his discourse: '… the hypnozoite and its probable course in the life cycle …'; and '… on the assumption that the presence of hypnozoites is responsible for latency'. In the following year, Krotoski (1989) cautioned: 'It must be emphasized … that additional work … is necessary in order to establish the (hypnozoite) concept fully …'. Soon after the discovery by Mehlhorn and Markus (1976) of the apicomplexan (but non-malarial) hypnozoite (Table 1), Krotoski *et al.* (1980) reported the serendipitous recognition in hepatic tissue of malarial hypnozoites (Table 1). This stage was first seen on 13 June 1979 by Dr David Martin, who was working in Krotoski's laboratory. Writing a decade later, Mons and Sinden (1990) offered a scientifically flawless opinion similar to that of Krotoski (1989), quoted above. They said that the hypnozoite is widely accepted as the source of true relapses but warned that '… more research is needed to prove beyond doubt that this parasite is not a re-invaded merozoite, but a dormant, sporozoite-derived parasite'. No relevant life cycle work took place during the next couple of decades. Investigations of the recommended type (Krotoski, 1989; Mons and Sinden, 1990) have been carried out only very recently, and provide strong support for the validity of the hypnozoite theory of malarial relapse (Mikolajczak *et al.* 2015; Soulard *et al.* 2015; Cubi *et al.* 2017; Voorberg-van der Wel *et al.* 2017). Publications that contain the results of important biological and related research since 2013 on *P. cynomolgi* are listed by Voorberg-van der Wel *et al.* (2017).

Hypnozoite-independent speculations

To explore the presumed hypnozoite basis of relapse, Imwong *et al.* (2012) undertook a study that had the primary objective of distinguishing between single and separate parasite inoculations. They found that the first recurrences in very young children are frequently homologous (i.e. genetically closely related). Epidemiologically, it is indeed considered that a predominance of homologous recurrences early in life suggests bites by only one or a few infective mosquitoes (Koepfli and Mueller, 2017). By contrast, more heterogeneity in this respect is thought to reflect greater hypnozoite diversity, resulting from multiple infective mosquito bites. To add to this likely scenario, we might ask whether the ubiquity of genotypic homology in recurrent *P. vivax* malaria in individuals of various ages indicates that there can be, apart from a presumed hypnozoite origin, another non-circulating source or sources of homologous, relapse-like *P. vivax* recurrences (Table 1). If so, these would be recrudescences, not relapses.

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Research has validated the question by revealing that our understanding of the survival of plasmodial sporozoites and merozoites in the mammalian skin and lymphatic system is incomplete (Landau *et al.* 1999; Gueirard *et al.* 2010; Wykes *et al.* 2011; Voza *et al.* 2012; Wykes and Horne-Debets, 2012; Ménard *et al.* 2013). Since 2010, there has accordingly been some speculation as to whether quiescent *P. vivax* sporozoites or extra-erythrocytic merozoites might occur in dermal or lymphatic sites (Fig. 1) and be associated with recurrences (Gueirard *et al.* 2010; Markus, 2011*c*, 2012*a*,*b*, 2016*b*; Ménard *et al.* 2013). Furthermore, it should be noted that erythrocytes are not the only cells in peripheral blood from humans and non-human primates in which forms of *Plasmodium* (that are not necessarily reproductively active) have been seen (Landau *et al.* 1999; Wykes and Horne-Debets, 2012; Markus, 2017). There is also the question I raised (Markus, 2015) of whether drug-induced, temporary blood-stage-parasite dormancy can sometimes be correlated with *P. vivax* recrudescences – an aspect of the recurrence puzzle that is covered in the penultimate section of this paper. However, more obvious possible erythrocytic-stage sources that might be associated with homologous instances of recurrent *P. vivax* malaria are bone marrow and the spleen, a subject dealt with in one of the sections below.

Significance of blood-stage-induced malarial infections

Simian *P. cynomolgi* malaria is regarded as a disease model for human *P. vivax* malaria (Waters *et al.* 1993; Fonseca *et al.* 2017; Joyner *et al.* 2017), and recrudescences are often a feature of untreated, blood-stage-initiated *P. cynomolgi* malaria (Corradetti, 1966). It follows that recurrent *P. cynomolgi* parasitaemia in sporozoite-infected monkeys might occasionally, or perhaps even frequently, have the same non-hypnozoite origin or origins (whatever it or they may be) as recrudescences in monkeys infected by inoculation of erythrocytic parasites (Markus, 2017). Prevailing hypnozoite-related dogma obfuscates this probability. Thus, recurrences in sporozoite-transmitted *P. cynomolgi* malaria might regularly have a hypnozoite-independent origin, like that of recrudescences of erythrocytic-stage-induced *P. malariae* infections in humans, which frequently take place in the long term (Corradetti, 1966). Such *P. malariae* recurrences cannot be relapses (even if hypnozoites do occur in the life cycle of *P. malariae*) because the evidence is that hypnozoites are derived from sporozoites, which are not present in post-passage, *P. malariae* blood-stage inocula. When extrapolated to *P. cynomolgi*'s sister species *P. vivax*, these comparative arguments support my contention that some homologous *P. vivax* recurrences in naturally acquired human infections are erroneously being ascribed to hypnozoite activation (Table 1).

As for hypnozoite formation, it must be emphasized that, as stated above, it is thought to be only sporozoite-induced (not blood-stage-associated) infections that result herein; malarial hypnozoites apparently being directly sporozoite-derived (Markus, 2012*b*). This was the original extrapolative interpretation from the results of my non-malarial, laboratory-based apicomplexan biological investigations (Table 1; Markus, 1975, 1976), as acknowledged by Garnham (1985), and which strongly influenced the thinking of malarial hypnozoite researchers around 1980 (R. Killick-Kendrick, personal communication). By this time, I

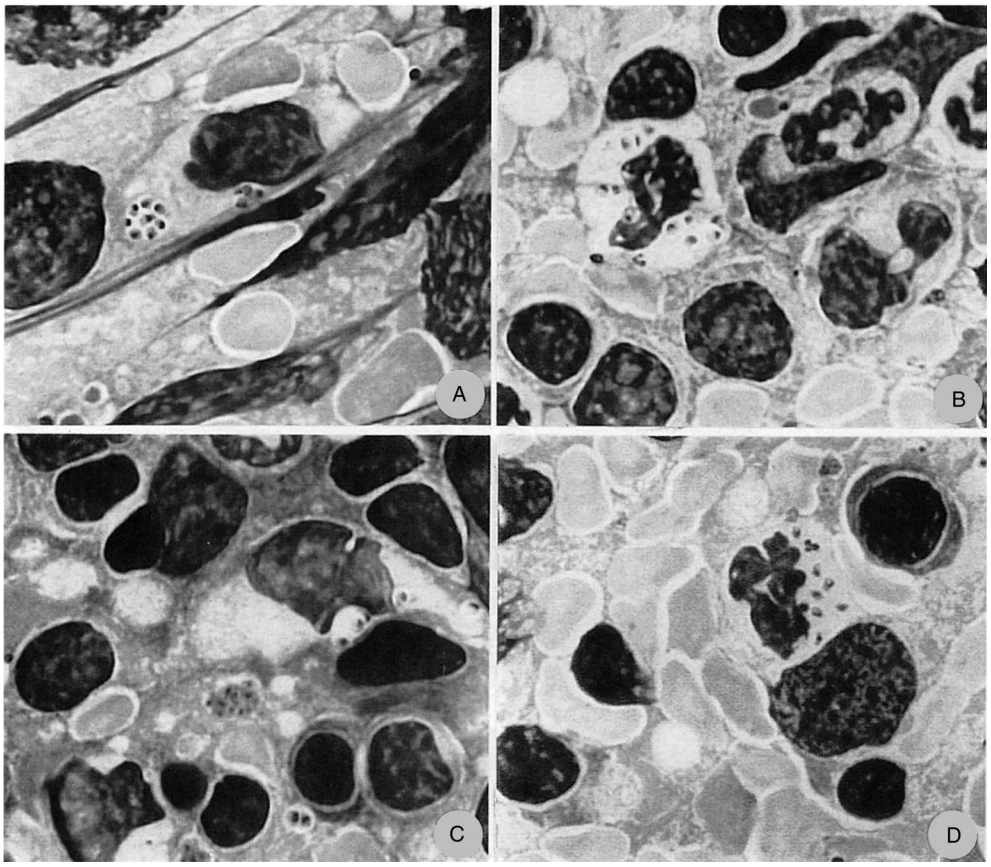

Fig. 1. 'Merophores' of *Plasmodium vinckei petteri* in murine splenic impression smears (from Landau *et al.* 1999). Stain: Giemsa-colophonium. Merophores can possibly be associated with rodent malarial recrudescences. We need to determine whether such parasites that are not destroyed intracellularly occur in the life cycles of any primate plasmodial species. (A) Merophore sack inside a macrophage, day 7 post-inoculation (p.i.). At this time during the course of a murine malarial infection, the average size of macrophages in the spleen is approximately 23 μm. (B) Merophore leucocyte (polymorphonuclear cell), 8 days p.i. (C) Merophore leucocyte (macrophage), 8 days p.i. (D) Merophore leucocyte (polymorphonuclear cell), 8 days p.i. This historical plate was originally published at a magnification of ×1750 (no scale bar provided). Reproduced with the permission of the Société Française de Parasitologie, obtained *via* Jean-Lou Justine, current Editor-in-Chief of the journal *Parasite*.

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was no longer at Imperial College London, where much of the malarial and other research took place.

In summary, non-reinfection, recurrent *P. vivax* malaria has a bimodal origin, the parasite source being either merozoites (even if only in the bloodstream) or, presumably, hypnozoites. Homologous (as opposed to heterologous) recurrences are highly suggestive of a merozoite origin, although not exclusively so. The point I am making here is that many authors are probably over-attributing homologous *P. vivax* malarial recurrences to the activation of hypnozoites. Close genetic relatedness of the parasites in a *P. vivax* recurrence to those that were present in peripheral blood during the initial bout of parasitaemia could be an indication that the recurrence is a recrudescence (merozoite origin) and not, in fact, necessarily a relapse (hypnozoite origin). One newly recognized, possible parasite source that might initiate or contribute to homologous, hypnozoite-independent *P. vivax* recurrences is bone marrow (Malleret *et al.* 2015; Fonseca *et al.* 2017; Markus, 2017), as discussed below.

Erythrocytic parasites in bone marrow and the spleen

The idea that human bone marrow may harbour plasmodial parasites goes back more than 125 years (Franken *et al.* 2017). This aside, the recorded occurrence of *P. vivax* in bone marrow was discussed by Baird (2013) and Barber *et al.* (2015) in regard to malarial severity. Furthermore, bone marrow has been mentioned in the context of recrudescence of *P. falciparum* malaria (Shanks, 2015), a subject related to the bone marrow-associated concept summarized here for *P. vivax*. Erythrocytic forms of *P. vivax* are not seen exclusively in cells *in vivo* that are obviously reticulocytes. They can also be present in what seem morphologically to be normocytes, but which could be reticulocytes with parasite-induced loss of their reticular material (Lim *et al.* 2016; Malleret *et al.* 2017). Reports of the occurrence of *P. vivax* in red blood cells of whatever ages in bone marrow (which is reticulocyte-rich) and the spleen have appeared from time to time in the literature. For example, see Siqueira *et al.* (2012), Baird (2013), Barber *et al.* (2015), Malleret *et al.* (2015) and Baro *et al.* (2017). Asexual multiplication of *P. vivax* must surely take place in the bone marrow, especially considering the low peripheral parasitaemias that are characteristic of *P. vivax* malaria.

It is generally assumed that *P. vivax* malarial recrudescences are the result of asexual reproduction of circulating stages. But is the bone marrow a source of parasites in recrudescent *P. vivax* malaria (Malleret *et al.* 2015)? The question as to whether *P. vivax* parasites in bone marrow are ever the origin of or contribute to homologous (specifically) recurrent peripheral parasitaemia was raised for the first time recently (Markus, 2017). Life cycle research on *Plasmodium cynomolgi* needs to be done in relation to bone marrow, as commented by Fonseca *et al.* (2017) and Markus (2017). It will, in addition, be important to ascertain whether *P. vivax*'s erythrocytic forms can be present in the bone marrow of appropriately humanized mice. *Plasmodium vivax* has been seen in human bone marrow when it has not been detected microscopically in the bloodstream (Ru *et al.* 2009; Malleret *et al.* 2015). We need to know more about this, using molecular diagnosis (Gruenberg *et al.* 2018), because the occurrence of *P. vivax* in bone marrow probably has implications for the eradication of malaria (Malleret *et al.* 2015; Markus, 2017).

When examining primate or murine organs/tissues such as bone marrow or the spleen for stages of *Plasmodium*, Giemsa-stained impression smears should be used (Markus, 1975; Mehlhorn and Markus, 1976; Landau *et al.* 1999), not only haematoxylin and eosin-stained sections. When apicomplexan parasites are found in the former, interpretation of their nature is easier, as compared with the use of the latter. Giemsa is helpful for staining *Plasmodium* and related coccidian parasites in tissue sections as well (Markus *et al.* 1974).

Lastly, whether erythrocytic parasites in the spleen can multiply or are always destroyed is at present unknown for both *P. vivax* trophozoites and splenic *P. falciparum* ring stages (Safeukui *et al.* 2008; Fig. 2 in Markus, 2016*b*). Is the spleen a source of recrudescent malarial parasites?

Drug-associated considerations in recurrence

It is well known that both blood schizontocides and the supposedly radically curative drug primaquine are not always effective for *P. vivax* malaria (e.g. Campo *et al.* 2015; Carmona-Fonseca, 2015; Zuluaga-Idarraga *et al.* 2015; Commons *et al.* 2017). Recurrences can therefore be a consequence of failure to clear all parasites. Drug resistance might be the problem in many instances. However, in the absence of systems for *P. vivax* that enable reproducible testing of the effects of compounds (van Schalkwyk *et al.* 2017), little is actually known about *P. vivax* drug resistance (Chaorattanakawee *et al.* 2017). More pharmacogenetic studies would probably facilitate the evaluation of anti-*P. vivax* drug efficacy (Milner *et al.* 2016; Silvino *et al.* 2016; Baird *et al.* 2018; Pewkliang *et al.* 2018).

Such considerations aside, primaquine is known to be merozoitocidal and gametocytocidal, in addition to being, apparently, hypnozoitocidal. There was early direct microscopic *in vivo* parasitological evidence for the last-mentioned effect (Garnham, 1988), based on some findings for *P. cynomolgi* in rhesus monkeys *Macaca mulatta*. To complicate interpretational matters further in respect of treated individual *P. vivax*-infected humans (as opposed to presumed reduction by primaquine of the collective hypnozoite load in a whole group of patients), it has yet to be determined whether primaquine will kill parasites with equal efficacy in all parts of the body where they might occur (Markus, 2015). The need for such information is indicated by the fact that whereas primaquine eliminates rodent plasmodial stages in the liver, it is either less effective or ineffective against the same murine parasites when they are present dermally (Gueirard *et al.* 2010; Voza *et al.* 2012). There is yet another drug factor that we should take into account in regard to recurrences. I have recently debated whether relapse-like clinical and parasitaemic recrudescences can sometimes follow temporary, drug-induced, *P. vivax* erythrocytic-stage quiescence (Markus, 2015), something that might occur *in vitro* (Thomson-Luque *et al.* 2017). The phenomenon has been clearly shown *ex vivo* for *P. falciparum* in experiments that are ongoing (Gray *et al.* 2016; Dembélé *et al.* 2017; Zhang *et al.* 2017).

Moreover, the efficacy of drugs for clearing *P. vivax* from bone marrow, haemopoietic or otherwise, might prove to be important if bone marrow is a significant reservoir site for the parasites. It is worth noting that Joyner *et al.* (2017) recorded the presence of haemozoin in bone marrow of a rhesus monkey with *P. cynomolgi* malaria, but they made no mention of the presence of parasites there (or elsewhere). However, the animal had received subcurative antimalarial treatment 48 h earlier to reduce parasitaemia. If *P. cynomolgi* is normally readily detectable in the bone marrow of infected, untreated rhesus monkeys, the absence of *P. cynomolgi* parasites in this instance could indicate that rapid clearance of stages from bone marrow by means of drugs is possible. Future research will undoubtedly shed light on the matter.

An outstanding question is whether or not recrudescences of *P. vivax* malaria can result from interplay between immunity and any drugs, such as the range of compounds for which Pukrittayakamee *et al.* (2000) reported therapeutic responses. In relation to the current malaria eradication research agenda

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(Rabinovich *et al.* 2017), it is a subject that should receive attention. This type of interaction has been found to affect total clearance or otherwise of *P. yoelii* parasites by artesunate (Claser *et al.* 2017), so follow-up is needed. Interpretation for *P. vivax* malaria might not always be straightforward. Boyd and Kitchen (1944) found that *P. vivax* recurrences took place in treated artificial infections that had been sporozoite-induced, but usually not when the initial infection was allowed to run its course and become self-terminating. Intuitively, this situation is perhaps not what one would have expected.

Concluding remarks

The uncertainties to which I have drawn attention make the confident categorization of individual homologous *P. vivax* recurrences as hypnozoite-associated relapses difficult to impossible, irrespective of whether they are short-term or long-term recurrences. A long-term homologous *P. vivax* recurrence in a naturally infected person is not necessarily a relapse, even if the patient has been treated with both primaquine and a blood schizontocide such as chloroquine (considering the frequency of drug failure). Pending greater understanding of recurrent *P. vivax* malaria, an out-of-the-box (in terms of hypnozoite-related dogma) range of non-hypnozoite explanations for homologous, relapse-like *P. vivax* recurrences should be considered – along the lines of, for example, the analytical approach adopted for a patient with recurrent *P. malariae* infection (Rutledge *et al.* 2017). It is considered that selective whole-genome amplification might be helpful for future characterization of *P. vivax* recurrences (Cowell *et al.* 2017), determination of the origin of which is particularly problematic in endemic areas (Popovici *et al.* 2018). Research topics to address some of the issues surrounding recurrent *P. vivax* malaria have been suggested (Markus, 2012*b*, 2015, 2016*b*, 2017).

Finally, elucidation of the mechanisms of activation (hypnozoites) or reactivation (merozoites) of hidden *P. vivax* parasites could be important in the practical context of attempted malaria eradication. In respect of the induction of hypnozoite latency, one possibility is that phosphorylation of the α subunit of eukaryotic translation initiation factor 2 is involved (Holmes *et al.* 2017). If reactivated quiescent merozoites do facilitate transmission of *P. vivax* malaria, then they are collectively (like hypnozoites, probably) an obstacle to eradication of the disease. Whatever the parasite source in a *P. vivax* recurrence, renewed peripheral parasitaemia will normally be accompanied by the formation of gametocytes which, if ingested by mosquitoes, will frequently lead to ongoing transmission of malaria.

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