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Knowlesi malaria: Human risk factors, clinical spectrum, and pathophysiology

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Abstract

Plasmodium knowlesi is endemic across Southeast Asia, and is the commonest cause of zoonotic malaria. The spectrum of clinical disease from *P. knowlesi* infection ranges from asymptomatic infection, through to severe malaria and death. Over 90% of clinical disease occurs in adults, mostly living in forest edge areas undergoing intensive land use change. With a 24-h asexual life cycle in humans, high parasite counts are possible, but most clinical cases of knowlesi malaria are uncomplicated with low parasitaemia. In co-endemic areas, median parasitaemia in knowlesi malaria is lower than that seen in vivax and falciparum malaria, suggesting a lower fever threshold. Severe malaria occurs in 6–9% of symptomatic adults. Manifestations of severe malaria from *P. knowlesi* are similar to those seen with falciparum malaria, with the notable absence of coma. Age, parasitaemia, cardiovascular comorbidities and delayed diagnosis are risk factors for severe disease and death, which are only seen in adults. Thrombocytopenia is near-universal in adults, likely related to platelet-red cell binding and clearance. Mechanisms underlying the microvascular sludging seen in fatal disease in non-natural primate hosts and the microvascular accumulation of parasites in fatal human disease are not clear. Marked reductions in deformability of both infected and uninfected red blood cells are associated with disease severity in both humans and other non-natural primate hosts, likely contributing to impaired microvascular perfusion and organ dysfunction. Endothelial activation, endothelial dysfunction, glycocalyx degradation and haemolysis are also associated with, and likely contribute to, severe disease and organ dysfunction, particularly acute kidney injury.

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1. Introduction

Plasmodium knowlesi is the commonest cause of zoonotic malaria in humans. A simian malaria parasite, with the natural hosts being the long-tailed macaque and pig-tailed macaque, *Plasmodium knowlesi* was discovered in 1931 by Campbell and Napier in Calcutta, India, in a long-tailed macaque imported from Singapore (Napier and Campbell, 1932). The ability of *P. knowlesi* to infect humans was demonstrated around the same time by Knowles and Das Gutpa, through the inoculation of three volunteer patients (Knowles and Das Gupta, 1932). The first case of naturally acquired human infection with *P. knowlesi* was reported in 1965 in a US national working in Malaysia (Chin et al., 1965), and several years later the experimental mosquito transmission of *P. knowlesi* from man to monkey and man to man was reported (Chin et al., 1968). In 2004, Singh et al. reported a large focus of naturally acquired knowlesi malaria in humans in the eastern Malaysian state of Sarawak (Singh et al., 2004). Human cases of knowlesi malaria have now been reported throughout Southeast Asia, with the greatest number of cases reported from Malaysia, particularly the eastern Malaysian states of Sabah and Sarawak (Cooper et al., 2020; Hussin et al., 2020; Shearer et al., 2016). Knowlesi malaria is unique among the human zoonotic malarias in being able to cause severe and fatal disease (Cox-Singh et al., 2008; Rajahram et al., 2019), with a frequency similar to that occurring following infection with *P. falciparum* (Barber et al., 2013; Grigg et al., 2018a; Rajahram et al., 2016). *Plasmodium knowlesi* is now the commonest cause of death from malaria in Malaysia (Cooper et al., 2020; Rajahram et al., 2019). This chapter reviews the clinical spectrum, human risk factors, and pathophysiology of malaria from *Plasmodium knowlesi*.

2. Transmission in humans

P. knowlesi is predominantly a zoonotic infection, with humans presumed to be incidentally infected when bitten by a mosquito vector which has fed from an infected long-tail or pig-tail macaque reservoir host. While human-mosquito-human transmission has been demonstrated in a laboratory setting (Chin et al., 1968), it has not yet been proven to occur in the natural environment. The predominance of *P. knowlesi* as a zoonosis is further supported by: sequence data of the circumsporozoite protein (*csp*) gene and mtDNA demonstrating shared polymorphisms between human and macaques in Sarawak, and with no haplotypes unique to either species (Lee et al., 2011); mathematical modelling (Imai et al., 2014); and a lack of drug-resistant mutations detected in human isolates (Grigg et al., 2016a). Nonetheless, human cases of *P. knowlesi* have been reported to occur in domestic clusters, with individuals of all ages affected (Barber et al., 2012), and it remains possible that in endemic areas, human-mosquito-human transmission may be occurring to at least some degree. This is reviewed in more detail in the companion chapter by Cuenca et al. (2021).

Human to human transmission of *P. knowlesi* malaria through blood transfusion has also been reported in Thailand and Malaysia (Bird et al., 2016; Traipattanakul et al., 2014). The Thai case was considered probably transfusion-related, and resulted in uncomplicated knowlesi malaria (Traipattanakul et al., 2014). The Malaysian case, confirmed as transfusion-related from a pre-symptomatic donor, occurred in a

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splenectomized thalassaemic patient who developed severe malaria with lactic acidosis and multi-organ failure (Bird et al., 2016).

3. Life cycle in humans

The life cycle of asexual stages of *P. knowlesi* infection in humans is similar to that of *P. falciparum*, with the exception of its 24-h erythrocytic cycle (Coatney et al., 1971). Transmission occurs when a female anopheline mosquito injects saliva containing sporozoites, which travel through the host's blood stream to the liver where they invade hepatocytes, undergo asexual replication and develop into schizonts. Hepatic schizonts rupture and release thousands of daughter merozoites into the blood stream. Merozoites then invade erythrocytes where they develop into young ring parasites and then trophozoites, which undergo further asexual multiplication to form schizonts, containing numerous merozoites. The infected erythrocyte then ruptures, causing fever and an inflammatory response in the host, with released merozoites reinvading uninfected erythrocytes, completing the first erythrocytic replication cycle. The 24-h erythrocytic cycle of *P. knowlesi* is the shortest of any of the *Plasmodium* species causing human malaria (Coatney et al., 1971). Thus, patients with *P. knowlesi* typically experience daily fever spikes. While high parasitaemia may develop rapidly in a minority of patients, most humans have relatively low circulating parasitaemia (Grigg et al., 2018a).

Plasmodium knowlesi does not have the latent hepatic hypnozoite stage seen in *P. vivax, P. ovale* and *P. cynomolgi*, and therefore relapses do not occur (Coatney et al., 1971). Within the erythrocyte some of the merozoites develop into male and female gametocytes. In contrast to their later appearance in *P. falciparum* infection, gametocytes appear early in human infection with *P. knowlesi*. In a recent clinical trial, 85% (82/97) of patients with knowlesi malaria had gametocyte mRNA transcripts detected by PCR on enrolment (Grigg et al., 2016b). Following a blood meal from a macaque host, gametocytes are taken up by a female mosquito and undergo sexual replication within the midgut of the mosquito, to complete the *Plasmodium* life cycle (Coatney et al., 1971). However, while onward mosquito-borne transmission of gametocytes circulating in humans to other humans has been demonstrated experimentally (Chin et al., 1968), the extent to which gametocytes from humans contribute to the transmission of knowlesi malaria in endemic areas is unknown.

4. Human risk factors for symptomatic infection

As *P. knowlesi* is predominantly a zoonotic parasite, infection is generally acquired in forest or forest-fringe areas. Hence, those at risk include farmers, plantation workers, or individuals undertaking other activities in forested areas (Barber et al., 2013; Grigg et al., 2017). Population-level epidemiological risk factors for infection are discussed in detail elsewhere (Cuenca et al., 2021). Symptomatic infection occurs more commonly in males, accounting for 75–85% of cases (Cooper et al., 2020; William et al., 2013, 2014). Clinical disease occurs far more commonly in adults than in children (Cooper et al., 2020; Daneshvar et al., 2009; Grigg et al., 2017, 2018a; William et al., 2013, 2014), with children <15 years accounting for only 9% of all *P. knowlesi* notifications in Sabah, Malaysia, during 2010–14 (Rajahram et al., 2016). More recent Sabah statewide reporting of *P. knowlesi*

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cases (2015–17) showed a continued adult predominance, with children less than 5 years old accounting for only 0.8% (25/3262) of PCR-confirmed infections and children aged 5–13 years accounting for only 5% (170/3262) of PCR-confirmed infections. Nonetheless, with the decline in the human-only *Plasmodium* species, *P. knowlesi* was the most common cause of malaria in children younger than 14 years, accounting for 195 of 253 (77%) cases (Cooper et al., 2020).

Human risk factors for acquiring symptomatic knowlesi malaria have been evaluated in a case-control study conducted in Sabah, Malaysia, involving 229 cases of *P. knowlesi* malaria, 91 cases of other *Plasmodium spp.*, and 953 controls (683 matched to *P. knowlesi* cases, 270 matched to non-*P. knowlesi* cases) (Grigg et al., 2017). Independent risk factors for symptomatic *P. knowlesi* infection included age ≥ 15 years, male gender, plantation work, sleeping outside, travel, being aware of the presence of monkeys in the past 4 weeks, and having open eaves or gaps in walls. Age ≥ 15 years, farming occupation, clearing vegetation, and having long grass around the house increased the risk for *P. knowlesi* infection but not for other *Plasmodium* spp. infection. G6PD deficiency protected against *P. knowlesi*, as did residual insecticide spraying of household walls, and the presence of sparse forest or rice paddies around the house. While adult men working in agricultural areas were at greatest risk of symptomatic knowlesi malaria, peridomestic transmission also occurred (Grigg et al., 2017). Notably, the use of bed nets was not found to be protective, highlighting the differences in control strategies that will likely be required for control of *P. knowlesi* malaria compared to falciparum and vivax malaria.

Travellers from non-endemic countries have acquired *P. knowlesi* infection in Malaysia, Thailand, Indonesia, Myanmar and the Philippines (Barber et al., 2017b; Froeschl et al., 2018; Ozbilgin et al., 2016). The large majority of these cases have been reported in adult males, who have mostly spent time in forested regions.

The extent to which past infection with human-only *Plasmodium* species protects against infection and disease from *P. knowlesi* is not known. Heterologous immunity was first suggested by data from early 20th century malariotherapy studies. Infection was particularly difficult to reproduce in three individuals who had been previously exposed to *P. vivax* (van Rooyen and Pile, 1935). This relative resistance to clinical disease among those previously exposed to malaria was also reported the same year by Nicol (Nicol, 1935), who found that among a total of 76 patients, only 1 of 16 with a previous history of malaria (with *P. vivax* being most commonly used at the time) developed fever following inoculation of *P. knowlesi* compared to 44% of non-immune patients. Heterologous immunity is supported by more recent studies demonstrating inhibition of *P. knowlesi* red cell invasion by *P. vivax* antibodies (Muh et al., 2018, 2020). The possibility that declining cross-protective immunity to *P. vivax* may contribute to rising incidence of knowlesi malaria has important implications for other Southeast Asian countries approaching malaria elimination (Anstey and Grigg, 2019).

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5. Prepatent and incubation periods

Data from experimental human infection and neurosyphilis studies indicate that following intravenous inoculation of *P. knowlesi* the incubation period is usually around 8–12 days, although with a reported range of between 3 and 27 days (Milam and Kusch, 1938; van Rooyen and Pile, 1935). In the first three patients reported by Knowles and Das Gupta, fever and symptoms developed 10, 20 and 23 days after inoculation with parasites appearing in the blood up to 5 days after the first fever was recorded (Knowles and Das Gupta, 1932). The patient with the most severe disease had the shortest incubation time. Later studies confirmed that parasites normally appear in the blood several days after the initial temperature rise. In an early malariotherapy report, Van Rooyen and Pile detailed incubation periods of 3–14 days in 12 neurosyphilis patients inoculated with *P. knowlesi*, with fever recorded for most after 8 days, and patent parasitaemia developing around 10 days (van Rooyen and Pile, 1935). The first experimental transmission of *P. knowlesi* via infected mosquitoes to 7 humans not previously exposed to malaria described a pre-patent period of 9–12 days (Chin et al., 1968).

6. Clinical spectrum in malariotherapy and experimental human infection

In the first human experimental infection by Knowles and Das Gupta, three volunteer patients were infected, the first with “paretic symptoms” of uncertain aetiology, the second with a foot ulcer following a rat bite, and the third with lepromatous leprosy (Knowles and Das Gupta, 1932). Infection was associated with daily fever spikes and two patients experienced mild to moderate disease. However, the patient with the foot ulcer, who received blood passaged through the first patient, became “very seriously ill.” Although reportedly comatose, clinical details were limited, and the patient made a spontaneous recovery (Knowles and Das Gupta, 1932).

Plasmodium knowlesi became widely used as a pyretic agent to treat neurosyphilis, with the first description provided in 1935 (van Rooyen and Pile, 1935). In their report of 12 patients inoculated with *P. knowlesi*, the authors noted the infection to be mild and “eminently suitable for the treatment of elderly debilitated patients.” In the USA, Milam and Kusch noted that the clinical course in European Americans was mostly moderate, with daily fevers lasting about 10 days and with only half experiencing chills. Recovery was generally spontaneous, although 38% later experienced recrudescent infection, half of which were associated with mild clinical illness (Milam and Kusch, 1938). This report also described relative resistance to *P. knowlesi* infection and disease in all six African Americans enrolled (Milam and Kusch, 1938), confirmed by another report the same year of decreased susceptibility to infection and clinical disease in 12 African Americans compared to 30 European Americans (Milam and Coggleshall, 1938). In both these reports several African American patients were described as having subclinical infection, with no parasites seen on blood film and no fever experienced, but with infection produced in monkeys inoculated with their blood. Marked variability in susceptibility to infection and clinical illness was also reported in the UK, with Nicol questioning the utility of *P. knowlesi* for malariotherapy, because even in successfully infected patients, a quarter had low parasitaemias and spontaneously recovered within a week (Nicol, 1935).

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In 1937 Ciuca et al. reported on their use of *P. knowlesi* to treat over 300 patients in Romania (Ciuca et al., 1937a,b). Among these patients, 46% and 80% of immune and non-immune patients respectively developed fever following exposure to *P. knowlesi* (Ciuca et al., 1937a,b). In contrast to Nicol who reported loss of pathogenicity of *P. knowlesi* with repeated passage from person-person (Nicol, 1935), Ciuca et al. later reported increased virulence of infection among patients infected by a single strain of *P. knowlesi* serially passaged 170 times through humans, with high parasite counts of up to 500,000/μL and severe disease necessitating antimalarial treatment (Ciuca et al., 1955). This led to the eventual discontinuation of malariotherapy, which in the meantime had been superseded by penicillin as treatment for neurosyphilis. Increased virulence with successive human transfers was also reported by Milam and Kusch, who noted high parasite counts (>10% infected RBCs) with “disease of serious proportions” in three of 15 (30%) patients infected after up to 22 serial human passages, in contrast to the low parasitaemia (<1%) and “moderate” clinical severity noted in patients treated with low-passage infections (Milam and Kusch, 1938). These authors speculated that the parasite may become better adapted to the human host with successive transfers (Milam and Kusch, 1938).

Chin et al. reported the clinical features of the first experimental sporozoite infections of humans in penitentiary volunteers (Chin et al., 1968). In the two European-American volunteers infected by mosquitoes fed on knowlesi-infected monkeys, the clinical course was reported to be mild, with maximum parasite counts of 1600 and 850/μL, and spontaneous resolution of patent infection after 11–12 days. In contrast, in a second group of volunteers infected with sporozoites from mosquitoes fed on experimentally-infected humans, maximum parasitaemias were higher (3450–6950/μL) and in three of the five infections, including in one African-American, the clinical illness was deemed severe enough to warrant chloroquine rescue therapy (Chin et al., 1968).

7. Clinical spectrum in natural infection

The spectrum of clinical disease from *Plasmodium knowlesi* infection ranges from asymptomatic infection, through to severe malaria and death. All prospective clinical and pathophysiology studies of knowlesi malaria have been performed in Malaysia where the incidence of clinical disease is highest, co-infections are less common than described elsewhere, and where host and parasite factors likely differ from other areas in the region. Accordingly, the clinical spectrum may differ across knowlesi-endemic regions. In studies of symptomatic malaria reported from Malaysia and in reports of returned travellers from all regions, the majority have uncomplicated disease. However, severe and fatal cases can occur, particularly if commencement of antimalarial treatment is delayed (Barber et al., 2021a).

7.1 Asymptomatic infections

Similar to 20th century reports of asymptomatic infection in malariotherapy studies, asymptomatic infection has been reported in natural infection in a number of knowlesi-endemic areas (Jongwutiwes et al., 2011), Vietnam (Marchand et al., 2011), East Malaysia (Fornace et al., 2016; Grignard et al., 2019; Siner et al., 2017), West Malaysia (Noordin et al., 2020), Indonesia (Lubis et al., 2017), Myanmar (Ghinai et al., 2017) and Cambodia

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(Imwong et al., 2019). In the series reported from north of Malaysia, co-infections are common, which may contribute to the high proportion of asymptomatic infections described. With the limited number of community prevalence studies performed and the use of molecular methods with different sensitivities, the true prevalence across knowlesi-endemic regions is not well characterized. In one study of asymptomatic household contacts of symptomatic cases in northeastern Sabah, Malaysia prevalence of asymptomatic infection was 6.9% (Fornace et al., 2016). Interestingly, children aged <15 years and women accounted for a higher proportion of asymptomatic infections compared to symptomatic infections (Fornace et al., 2016). Subsequent larger cross-sectional studies in Sabah found a lower proportion of asymptomatic infections (Fornace et al., 2019; Grignard et al., 2019). Unanswered questions include how long these asymptomatic infections persist, the frequency with which these low-parasitaemia infections spontaneously resolve, their contribution to anaemia in knowlesi-endemic regions and the proportion subsequently progressing to symptomatic disease.

7.2 Symptoms in adults

Common presenting symptoms in knowlesi malaria in the three largest prospective series to date include fever (100%), chills, headache (89–94%), myalgia (47–88%), nausea, vomiting (24–34%), cough (35–56%) and abdominal pain (23–52%) (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a) (Table 1). The median duration of fever is 5 days (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a), comparable with malaria due to falciparum and vivax malaria (Grigg et al., 2018a). The symptoms of knowlesi malaria are non-specific and occur with similar frequency in falciparum and vivax malaria (Grigg et al., 2018a). The non-specific symptoms can also be attributed to other infections in knowlesi-endemic regions such as dengue, leptospirosis, COVID-19, typhoid and typhus. A high index of suspicion of knowlesi malaria is required in endemic areas, as presentations with abdominal pain, diarrhoea or cough can be misdiagnosed as organ-specific infections. Abdominal pain has led to a misdiagnosis of knowlesi malaria as acute gastroenteritis, with consequent delayed commencement of antimalarial treatment, sometimes with fatal outcomes (Cox-Singh et al., 2008; Rajahram et al., 2013).

7.3 Clinical signs in adults

The common signs of knowlesi malaria include fever, tachycardia and tachypnoea (Table 1). In prospective series, splenomegaly and hepatomegaly are present in 6–33% and 24–40% of patients respectively (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a).

Patients can also present with hypotension, jaundice, or respiratory distress (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a), or develop these after commencement of therapy—see “severe knowlesi malaria” below.

In contrast to falciparum malaria (Dondorp et al., 2008; World Health Organization, 2014; Yeo et al., 2007), neurological signs are notably rare in knowlesi malaria. While altered mental state has been rarely reported (Barber et al., 2013), in the three largest prospective series of knowlesi malaria, none of 674 adults had unarousable coma or seizures, including the 76 patients who had severe disease (Barber et al., 2013; Daneshvar et al., 2009; Grigg

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et al., 2018a). In a retrospective case report of a single parasitemic patient presenting with coma (Rajahram et al., 2016), the absence of neuroimaging, lumbar puncture and other diagnostic workup meant alternative diagnoses could not be excluded. Similar diagnostic limitations were present for a knowlesi malaria case presenting with hemiparesis, who had a history of uncontrolled hypertension (Daneshvar et al., 2009).

Retinal haemorrhages were observed on fundal photographs in 3/17 (18%) and 2/12 (17%) of patients hospitalized with uncomplicated and severe knowlesi malaria, respectively. These were associated with thrombocytopenia, but otherwise the retinopathy seen in knowlesi malaria is non-specific and the characteristic retinal whitening seen in severe falciparum malaria has not been reported to date (Govindasamy et al., 2016).

7.4 Laboratory investigations in adults

7.4.1 Haematology—Thrombocytopenia is near-universal in adult knowlesi malaria (Table 2). In the three largest prospective series, thrombocytopenia (platelets $<150 \times 10^3/\mu L$) occurred on presentation in 634/674 (94%) of adults hospitalized with knowlesi malaria (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a) (Table 2), with 29% and 60% of patients at district (Daneshvar et al., 2009) and tertiary referral hospitals (Barber et al., 2013), respectively, having a platelet count of $<50 \times 10/\mu L$. Platelet counts recover quickly, and bleeding is uncommon, occurring in only 5% of those with severe malaria (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a). An absence of thrombocytopenia has been reported in 3 of 7 splenectomized patients (Barber et al., 2013, 2016; Bird et al., 2016; Boo et al., 2016; Cheo et al., 2020).

Anaemia (based on WHO criteria (WHO, 2011)) is found on presentation in 36% of adults with knowlesi malaria (Grigg et al., 2018a), and can develop after treatment. In two randomized controlled trials, 63% (95% CI 55–71%) of artemisinin combination therapy-treated patients with uncomplicated malaria had anaemia at some point throughout 28 days of followup (Grigg et al., 2016b, 2018b). Severe anaemia (<7g/dL) is uncommon in adult knowlesi malaria, reported in only 1.7% and 1.5% overall in district and referral hospital series (Barber et al., 2013; Grigg et al., 2018a), though can occur in up to 28% of those with severe malaria (Grigg et al., 2018a).

The total white cell counts are usually normal although neutrophilia is often seen in severe and fatal disease (Barber et al., 2013, 2017a; Cox-Singh et al., 2011; Willmann et al., 2012) (Table 2). Coagulation studies can also be abnormal. In one series of severe knowlesi malaria, 32% of patients had significant elevations of the prothrombin and partial thromboplastin times, although none were reported to have clinically important bleeding (William et al., 2011).

7.4.2 Parasitaemia—Although *P. knowlesi* can cause high parasitaemia, parasite counts in most cases of knowlesi malaria are low. In the largest series of knowlesi malaria, median parasitaemia in adults presenting to a district hospital with knowlesi malaria (2541 parasites/μL [IQR 478–8585]) was lower than that seen in vivax (3765 [1755–8122]) and falciparum (9924 [2522–22,860]) malaria (Grigg et al., 2018a), suggesting a lower fever threshold in knowlesi malaria. Parasitaemia is strongly correlated with age (Barber et al.,

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2013, 2017a). Parasitaemia is independently associated with severe disease in *P. knowlesi* infections, with median parasitaemia in severe malaria of 42,224 parasites/μL compared with 2044 in non-severe knowlesi malaria (Grigg et al., 2018a). In tertiary hospital studies, the parasitaemias seen severe knowlesi malaria are comparable to those seen in severe falciparum malaria (Barber et al., 2013). Parasitaemia in severe malaria is discussed further below.

7.4.3 Liver function—Mild elevations of liver transaminases are common (Barber et al., 2013; Daneshvar et al., 2009) (Table 2). Hyperbilirubinemia (>42μmol/L) was observed in 3% of district hospital presentations and in 53% of patients with severe knowlesi malaria (Daneshvar et al., 2009; Barber et al., 2013). Hypoalbuminemia occurs more frequently in severe disease compared to uncomplicated disease (Barber et al., 2013) and is universal in fatal cases (Rajahram et al., 2019). Transaminases may also increase after treatment, as has been observed in falciparum and vivax malaria (Odedra et al., 2020; Woodford et al., 2018).

7.4.4 Renal function—Acute kidney injury (AKI) is common in knowlesi malaria. In the largest prospective study to date, AKI of any severity, defined by KDIGO criteria, occurred in 83/437 (19%) adults with knowlesi malaria presenting to a district hospital (Grigg et al., 2018a). In a tertiary referral hospital study, AKI of any severity, by KDIGO criteria (with baseline creatinine estimated by the MDRD equation), was present on admission in 44/154 (29%) non-severe cases, and 40/48 (83%) severe cases (Barber et al., 2018a). A systematic review of fatal cases, showed that 100% of fatal cases had elevated creatinine on presentation (Rajahram et al., 2019). Higher creatinine is positively associated with parasitaemia and neutrophilia (Daneshvar et al., 2018). Hyponatremia is also common in severe malaria, and is universal in fatal cases (Rajahram et al., 2019).

7.5 Severe knowlesi malaria

Severe knowlesi malaria, defined by WHO criteria (WHO, 2014) (Table 3), has been reported in 6–9% of adults presenting to district hospitals (Daneshvar et al., 2009; Grigg et al., 2018a) and in 29% of adults treated at a tertiary referral hospital (Barber et al., 2013). The WHO epidemiological and research definition for severe knowlesi malaria (WHO, 2014) (Table 3) is a modification of that for severe falciparum malaria, with a lower parasite count of 100,000 parasites/μL used as the cut-off to define hyperparasitaemia and a parasitaemia threshold of 20,000/μL in the presence of jaundice (WHO, 2014). In adults, the most common manifestations of severe knowlesi malaria include acute kidney injury, hyperparasitaemia, jaundice, shock, respiratory distress and metabolic acidosis (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a; Singh and Daneshvar, 2013) (Table 3). In contrast to falciparum malaria, coma is not a feature of severe knowlesi malaria, with no case of coma, with exclusion of other causes, yet reported in PCR-confirmed knowlesi malaria. Also in contrast to falciparum malaria, severe disease from *P. knowlesi* has not been reported in children, and no paediatric deaths have been reported (Rajahram et al., 2016, 2019).

Severe acute kidney injury defining severe malaria (creatinine >265/μL) occurs in 24–36% of cases in prospective series of severe knowlesi malaria (Barber et al., 2013; Daneshvar et

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al., 2009; Grigg et al., 2018a) and in up to 94% in retrospective series (William et al., 2011; Willmann et al., 2012). Severe AKI is reported in 70% of fatal cases (Rajahram et al., 2019). Blackwater fever with acute kidney injury has been reported (Barber et al., 2013; William et al., 2011), including in a splenectomized patient with marked intravascular haemolysis (Barber et al., 2016).

Respiratory distress is a common feature in severe malaria, occurring in 7–70% of severe cases (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a; William et al., 2011; Willmann et al., 2012). Acute respiratory distress syndrome (ARDS) has been shown to occur with similar frequency to that seen in severe falciparum malaria (Barber et al., 2013) and is common in fatal cases (Rajahram et al., 2019). Respiratory distress is associated with both parasitaemia (Barber et al., 2013; Daneshvar et al., 2009) and neutrophilia (Barber et al., 2013). Development of respiratory distress has been reported to occur after commencement of antimalarial treatment, with either quinine (Daneshvar et al., 2009; William et al., 2011) or intravenous artesunate (Barber et al., 2013).

Metabolic acidosis, including lactic acidosis, is common in severe malaria, occurring in 11–32% of cases (Barber et al., 2013; Grigg et al., 2018a; William et al., 2011), and 71% of fatal cases (Rajahram et al., 2019). Hematemesis has been reported in patients with severe and fatal disease (Barber et al., 2013; Boo et al., 2016; Cox-Singh et al., 2008). Other severe complications reported in knowlesi malaria include rhabdomyolysis (Takaya et al., 2018) and spontaneous splenic rupture (Chang et al., 2018). As in complicated falciparum malaria (Aung et al., 2018; Nyein et al., 2016), concurrent gram-negative bacteremia can occur in severe knowlesi malaria. Two cases of concurrent *Enterobacter* bacteraemia in severe and fatal disease have been reported (Rajahram et al., 2012; William et al., 2011), and another case of *Neisseria meningitidis* bacteraemia has been reported in uncomplicated knowlesi malaria (Grigg et al., 2018a).

7.6 Clinical and parasitological risk factors for severe disease

Parasitaemia is consistently higher in severe malaria than in uncomplicated knowlesi malaria (Barber et al., 2013, 2017a; Daneshvar et al., 2009; Grigg et al., 2018a; William et al., 2011; Willmann et al., 2012). In a tertiary-referral hospital study, severe disease occurred in 53% of patients with parasite counts of >20,000 and 83% of patients with parasite counts of >100,000/μL (Barber et al., 2013). Parasitaemia is strongly correlated with age (Barber et al., 2013, 2017a). In addition, although older adults have significantly higher parasitaemias and thus are at greater risk of severe disease, age has also been shown to be an independent risk factor for severe disease in a large tertiary hospital series (Barber et al., 2017a). In the largest prospective study of knowlesi malaria, parasitaemia, age, schizont proportion, abdominal pain and dyspnoea were independent predictors of severe disease (Grigg et al., 2018a). In this study a parasitaemia threshold of 15,000/μL had the best combined sensitivity (74%) and specificity (87%) for predicting severe knowlesi malaria, with an area under the curve of 0.80 and a negative predictive value of 98.5%. Thus, while a parasitaemia >100,000/μL has been defined as a WHO research criterion for severe disease in the absence of other severity criteria, in routine clinical practice, intravenous artesunate

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has been recommended for all patients with parasite counts >20,000/μL (WHO, 2014), and more recently >15,000/μL (Anstey et al., 2020; WHO, 2017).

Parasite counts have been found to be higher in farmers and plantation workers compared to other occupations, even when controlling for age and fever duration (Barber et al., 2017a). This may be due to a higher infective inoculum in these occupations. High parasitaemias and severe disease have also been observed in splenectomized patients (Barber et al., 2013, 2016; Bird et al., 2016; Boo et al., 2016; Cheo et al., 2020). HIV infection has been shown to be a risk factor for higher parasitaemia and severe disease in falciparum malaria (Berkley et al., 2009) but it is not known whether HIV infection increases risk of severe disease in *P. knowlesi* infections.

7.7 Deaths from knowlesi malaria

Deaths from knowlesi malaria are well documented (Cox-Singh et al., 2008; William et al., 2011), and *P. knowlesi* is now the most common cause of malaria deaths in Malaysia (WHO, 2020). In an analysis of malaria incidence and fatality data from the Sabah Department of Health from 2010 to 2017, the overall case fatality rate of knowlesi malaria was 2.5/1000 cases. For women, this was 6.0/000 compared to 1.7/1000 for men ($p = 0.01$). Age ≥ 45 years was associated with a fivefold increased risk of death, with a case fatality of 5.8/1000 in this age group, compared to 1.1/1000 for those aged <45 years ($p > 0.001$). On multivariate analysis, age >45 years and female sex were both independent risk factors for fatal disease (Rajahram et al., 2019).

Consistent with these data from Sabah, in a recent systematic review of all reported cases of fatal knowlesi malaria up to August 2018, median age of the 32 cases was 56 years (range 23–84) (Rajahram et al., 2019), and, in contrast to the predominance of males in studies of non-fatal knowlesi malaria (Barber et al., 2013; Grigg et al., 2018a), nearly half of all reported fatal cases were women. There was no difference in parasitaemia or fever duration between males and females, suggesting that the over-representation of females in this series of fatal cases was not due to delay in health-seeking behaviour (Rajahram et al., 2019).

Over a third (34%) of the fatal cases in this series had cardiovascular-metabolic chronic disease, including hypertension, diabetes, morbid obesity, ischemic heart disease and rheumatic heart disease (Rajahram et al., 2019). Abdominal pain was reported in over half of the cases, and elevated serum creatinine, hyponatremia and thrombocytopenia were seen in all. Of severe manifestations, severe acute kidney injury, jaundice and metabolic acidosis were found in >70% of the fatal cases (Rajahram et al., 2019).

Although 30/32 (94%) of the patients in this series met WHO criteria for severe disease at presentation, only 19 (63%) were recognized as having severe malaria by the treating clinician, leading to inappropriate oral therapy and delay in initiation of intravenous artesunate (Rajahram et al., 2019). In addition, species diagnosis on admission microscopy was incorrect in 90% of cases, with 69% diagnosed as *P. malariae*, 14% as *P. falciparum*, and 7% as *P. vivax* (Rajahram et al., 2019). Consistent with earlier reports, the misidentification of *P. knowlesi* by microscopy, particularly as the more benign *P. malariae* (Barber et al., 2013; Lee et al., 2009; Rajahram et al., 2012), likely contributed to inappropriate oral

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therapy and delayed initiation of parenteral therapy (Rajahram et al., 2019). Inadequate access to healthcare in endemic settings was also associated with fatal outcomes, including unavailability of intravenous artesunate despite prompt recognition and diagnosis (Rajahram et al., 2019). In Sabah, Malaysia, a fall in case-fatality rate of PCR-confirmed knowlesi malaria in adults coincided with greater recognition of disease, earlier diagnosis and rollout of artesunate therapy (Rajahram et al., 2016).

7.8 Knowlesi malaria in children

As outlined earlier, knowlesi malaria is predominantly a disease of adults, with prospective clinical series reporting <10% of cases in children (Daneshvar et al., 2009; Grigg et al., 2018a) Clinical presentations in children are usually non-specific, most commonly with a history of fever (Barber et al., 2011). In the first prospective study of clinical presentations in children ($n = 44$), presenting symptoms were similar to those of falciparum and vivax malaria, including fever (100%), rigors (66%), headache (77%), vomiting (32%) and cough (34%) (Grigg et al., 2018a). In this series, abdominal pain was notably more common in knowlesi malaria (43%) than falciparum or vivax malaria. Hepatomegaly (32%) and splenomegaly (21%) occurred with similar frequency as in falciparum and vivax malaria (Grigg et al., 2018a). As in adults, children with knowlesi malaria have lower parasitaemias than malaria from other species. Median parasitaemia in 44 children presenting to a district hospital with knowlesi malaria (1722 parasites/μL [IQR 386-4830]) was lower than that seen in vivax (5967 [1829-13,901]) and falciparum (7392[1462-36,546]) malaria (Grigg et al., 2018a).

In children, thrombocytopenia (platelet count <150,000/μL) occurs frequently (Barber et al., 2011), but is less common in paediatric knowlesi malaria (68%) than in adults (92%) (Grigg et al., 2018a). In Sabah, 84% of children with knowlesi malaria had nonsevere anaemia, with the lowest haemoglobin concentration of 5.1 g/dL occurring in a 4-year old child. In a retrospective series, post-treatment haemoglobin nadir occurred later than that seen in falciparum malaria (Barber et al., 2011). Severe anaemia (haemoglobin of 4.9 g/dL) has been reported in child with microscopy-diagnosed knowlesi malaria, although this was not confirmed with PCR (Barber et al., 2011). While overall clinical illness in the prospective series was milder than in adults, and no cases of severe malaria were reported, children had a 11-fold higher risk of anaemia at presentation and a similar risk of mild-moderate acute kidney injury (by KDIGO criteria) than adults (Grigg et al., 2018a).

It is notable that there have been no cases reported to date of severe PCR-confirmed knowlesi malaria in children (Barber et al., 2011; Grigg et al., 2018a). In contrast to the risk of death in knowlesi malaria in adults, and in contrast to the significant mortality associated with falciparum and vivax malaria in children (Douglas et al., 2014; Marsh et al., 1995), no deaths from knowlesi malaria have been reported among children to date (Rajahram et al., 2016, 2019).

7.9 Knowlesi malaria in pregnancy

In co-endemic areas, the risk of *P. knowlesi* infection in pregnancy appears to be lower than that of *P. falciparum* and *P. vivax* infection (Barber et al., 2015a). This has been

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hypothesized to be due to behavioural changes in pregnancy resulting in less occupational and forest exposure, and differences among species in peridomestic exposure (Barber et al., 2015a). Only seven cases of *P. knowlesi* infection in pregnancy have been reported (Barber et al., 2015a; Rajahram et al., 2019; William et al., 2011). This makes assessment of the risk of adverse maternal and foetal outcomes uncertain. Nevertheless, two of the seven cases presented with severe malaria in the third trimester, with both having acute kidney injury and hypotension, and one with acute respiratory distress syndrome and fatal outcome (Rajahram et al., 2019; William et al., 2011). This suggests that there is an increased risk of severe disease resulting from *P. knowlesi* infection in pregnancy, as also seen in falciparum malaria (WHO, 2014). Adverse foetal outcomes have also been reported, including intrauterine death, preterm delivery and low birth weight (Barber et al., 2015a; Rajahram et al., 2019; William et al., 2011).

7.10 Congenital malaria

Congenital malaria due to *P. falciparum* and *P. vivax* is well-described (Poespoprodjo et al., 2011). The extent to which congenital malaria occurs with *P. knowlesi* is not known. In a baboon model of *P. knowlesi* infection, placental infiltration of parasitized erythrocytes and inflammatory cells was seen but parasitized erythrocytes in cord blood and the foetal-placental region were absent, suggesting that the risk of congenital malaria may be low (Onditi et al., 2015). A possible case of congenital malaria has been reported in Sabah in an anaemic 6 week-old (haemoglobin 7.4g/dL) with no epidemiological risk factors for post-natal infection (Grigg et al., 2018a).

8. Genetic risk factors

8.1 Parasite genetics

P. knowlesi parasites show greater genetic diversity than the two major human malaria species, *P. falciparum* and *P. vivax* (Benavente et al., 2018). Multilocus microsatellite genotyping and whole genome sequencing studies have confirmed the presence of three major subpopulations of *P. knowlesi* in human clinical isolates from Malaysia (Assefa et al., 2015; Divis et al., 2015, 2017). Two highly divergent clusters have been identified in humans from Malaysian Borneo, associated with long-tailed and pig-tailed macaque reservoir hosts respectively, and with significant geographical differentiation occurring between the two subpopulations (Assefa et al., 2015; Divis et al., 2015). A third highly divergent cluster has been identified in clinical isolates from humans and most macaques in Peninsular Malaysia (Divis et al., 2017). Whether these subpopulations are associated with different clinical phenotypes has not yet been reported.

Because disease severity in knowlesi malaria is closely linked with parasitaemia (Barber et al., 2013, 2017a; Grigg et al., 2018a) there has been significant interest in whether parasite genes encoding more efficient erythrocyte invasion are linked to malaria disease severity. Mathematical modelling predicts that parasite adaptation for invasion of older human red cells may be a major factor allowing the high parasitaemias associated with disease severity (Lim et al., 2013). Sequence diversity has been demonstrated in the *P. knowlesi* normocyte binding proteins (*Pknbpxa* and *Pknpbxb*) (Ahmed et al., 2016) that are required for human

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erythrocyte invasion (Moon et al., 2016). Certain alleles of *Pknbpxa* and *Pknbpxb* have been associated with high parasitaemias and other features of disease severity, suggesting a link between invasion gene variants and parasite virulence (Ahmed et al., 2014). Sequence diversity has also been demonstrated in merozoite surface protein-1 (*Pkmsp1*) (Yap et al., 2017), *Pkmsp3* (De Silva et al., 2017) and Duffy binding protein (*PkDBP*) (Putaporntip et al., 2016) and differences in PkDBPαII binding activity with human erythrocytes have been identified (Lim et al., 2017). To date no links with disease severity have been reported for these latter genes.

8.2 Host genetics

Human genetic polymorphisms influencing risk of *P. falciparum* infection and severe disease are well-described (Mackinnon et al., 2005). Much less is known about host genetic factors influencing risk of *P. knowlesi* infection and its disease severity. Glucose-6-phosphate dehydrogenase (G6PD) deficiency linked to mutations in the X chromosome is known to be associated with protection from malaria due to the human-only species *P. falciparum* and *P. vivax* (Leslie et al., 2010; Manjurano et al., 2015). In a large case-control study in Sabah, Malaysia, G6PD deficiency was also found to be associated with protection against acquisition of symptomatic *P. knowlesi* infection in multivariate models, conferring a fivefold lower risk of knowlesi malaria (Grigg et al., 2017).

Southeast Asian ovalocytosis (SAO) is widely distributed in knowlesi-endemic Malaysia and Indonesia (Paquette et al., 2015). *In vitro* studies have shown that human SAO ovalocytes are highly resistant to invasion by *P. knowlesi* merozoites, making it likely that SAO protects against knowlesi malaria (Hadley et al., 1983). This has not yet been shown in case-control studies. It may be anticipated that other genetic erythrocyte abnormalities and haemoglobinopathies prevalent in knowlesi-endemic regions may also confer protection from knowlesi malaria but this remains to be shown.

9. Pathophysiology

The pathophysiology of severe knowlesi malaria in humans is not as well understood as that of severe falciparum malaria. Disease severity in malaria from the human-only species, *P. falciparum* and *P. vivax*, is associated with parasite biomass (Barber et al., 2015b; Dondorp et al., 2005), and this is clearly also the case in knowlesi malaria (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a; William et al., 2011; Willmann et al., 2012). The relative distribution of parasite biomass between the circulating compartment and deep tissue microvasculature in human knowlesi malaria is less well defined. In the following sections, we review pathophysiological mechanisms associated with disease severity in knowlesi malaria.

9.1 Microvascular accumulation of parasites

In rhesus macaques, another non-natural primate host for *P. knowlesi* susceptible to severe disease, infection with *P. knowlesi* is characterized by widespread microvascular accumulation of infected red blood cells, with malarial pigment seen in multiple organs including the small intestine, liver, brain, spleen, heart, lung, kidney, adipose tissue and

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skeletal muscle, and associated with widespread cellular necrosis (Chen et al., 2001; Miller et al., 1971a; Miller and Chien, 1971; Spangler et al., 1978). The occurrence of deep vascular schizogony (the cyclic disappearance of infected red blood cells from the peripheral circulation as the parasite matures), with infected red blood cells accumulating in multiple organs at higher parasitaemia, has also been described in rhesus macaques (Miller et al., 1971a). To-date only a single autopsy in human severe knowlesi malaria has been reported (Cox-Singh et al., 2010). In this report, a lack of peripheral parasitaemia precluded calculation of a sequestration index, but there was widespread marked accumulation of pigmented infected red blood cells in the microvasculature of vital organs including the brain, heart and kidney as well as in the liver sinusoids and the splenic red pulp, without evidence of chronic inflammatory response in the brain or other organs (Cox-Singh et al., 2010). Further investigation with electron microscopy was not able to be conducted.

In macaques, microvascular accumulation of *P. knowlesi*-infected red blood cells appears to occur first in the small intestine (Miller et al., 1971a; Miller and Chien, 1971). Of note, abdominal pain is more common in human knowlesi malaria than malaria from other species, at least in children (Grigg et al., 2018a). Parasitaemia is associated with abdominal pain in adults (Barber et al., 2013; Daneshvar et al., 2009, 2018), and the majority of fatal cases have had abdominal pain, hypothesized to be from microvascular accumulation of parasites in the gut and/or stress ulceration (Rajahram et al., 2019).

The mechanisms underlying the microvascular accumulation of *P. knowlesi*-infected red blood cells are not clear. In *P. falciparum* infection, infected red blood cells express surface knob-like protrusions expressing variant parasite derived proteins of the PfEMP1 *var* family, which cytoadhere to multiple microvascular endothelial cell receptors, including CD36, ICAM-1, and EPCR (Su et al., 1995; Turner et al., 1994), with clear evidence for cytoadherence to activated endothelial cells mediating microvascular sequestration and obstruction (MacPherson et al., 1985; Marchiafava and Bignami, 1894; Taylor et al., 2004; White et al., 2013). In *P. knowlesi*, schizont-infected cell agglutination ligands (SICA) are red blood cell surface proteins encoded by the SICA *var* gene family, and while phylogenetically distinct, share some binding protein motifs with PfEMP1 proteins (Korir and Galinski, 2006), with antigenic variant expression hypothesized to be related to parasite virulence (Barnwell et al., 1983; Howard et al., 1983; Korir and Galinski, 2006). However, evidence for endothelial cytoadherence of *P. knowlesi*-infected red blood cells is limited. Electron microscopy of microvascular *P. knowlesi*-infected red blood cells in severe disease in rhesus macaques does not show the knobs that mediate cytoadherence in *P. falciparum* or *P. coatneyi*, and, while closely associated with the endothelium, *P. knowlesi*-infected red cells “were not held by extensions of the endothelial cells” (Miller et al., 1971a). Lack of surface knobs has been confirmed on atomic force microscopy of circulating *P. knowlesi*-infected red cells from humans with both uncomplicated and severe knowlesi malaria (Barber et al., 2018b). Instead, caveolae pits were observed (Barber et al., 2018b).

In the single human autopsy case, while there was intense accumulation of parasitized red cells within the microvasculature, clear evidence of cytoadherence to endothelial cells was not apparent, with interspersion of infected red blood cells with uninfected red blood cells, and a lack of clear marginalization of parasitized cells (Cox-Singh et al.,

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2010). Furthermore, ICAM-1, which mediates cytoadherence to brain endothelial cells in falciparum malaria (Berendt et al., 1989; Turner et al., 1994), was not detected on brain endothelium (Cox-Singh et al., 2010). A subsequent small study showed that *P. knowlesi*-infected red blood cells could, variably, bind *in vitro* to ICAM-1 and VCAM-1 but not CD36 (Fatih et al., 2012). A paucity of falciparum-like cytoadherence of infected red blood cells in knowlesi malaria is also suggested by the notable lack of coma in reported cases of severe knowlesi malaria to-date (Barber et al., 2013; Cox-Singh et al., 2008; Daneshvar et al., 2009; Grigg et al., 2018a; Rajahram et al., 2012, 2016, 2019; William et al., 2011) and the lack of the specific malarial retinopathy and characteristic retinal whitening seen in severe falciparum malaria (Govindasamy et al., 2016). Thus, important differences likely exist between the pathophysiology of severe knowlesi and severe falciparum malaria, particularly the mechanisms by which infected red blood cells accumulate in the microvasculature.

If not sequestration through falciparum-like endothelial cytoadherence, what other mechanisms might account for the marked accumulation of *P. knowlesi*-infected red cells in the microvasculature? Early 1940s “motion picture” microscopy studies in rhesus macaques demonstrated marked microcirculatory changes in *P. knowlesi*-infected monkeys, with impairment of microvascular flow reported to be a critical factor in fatal outcomes (Knisely and Stratman-Thomas, 1945, 1948). These studies reported coating of parasitized red cells with a “precipitate,” progressing at higher parasitaemias to a further coating of both infected and uninfected red blood cells, binding infected and uninfected red cells together, and changing blood to a “thick, muck-like sludge,” leading to microvascular obstruction and widespread tissue hypoxia (Knisely and Stratman-Thomas, 1945, 1948). Whether such agglutination and sludging contributes to microvascular obstruction and severe disease in human knowlesi malaria is not clear.

Autogglutination and clumping of infected red blood cells is associated with disease severity in falciparum malaria (Pain et al., 2001). Agglutinates of infected red blood cells have been recently observed in blood from a knowlesi malaria patient, in association with platelets (Kho et al., 2018). In falciparum malaria, agglutination of infected red blood cells is mediated by platelet-expressed CD36 (Pain et al., 2001), however, the inability of *P. knowlesi*-infected red blood cells to bind CD36 *in vitro* (Fatih et al., 2012) suggests a distinct mechanism may mediate such agglutination in knowlesi malaria in humans.

Increased viscosity and resistance to flow of red blood cells from *P. knowlesi*- infected macaques has also been demonstrated, suggestive of reduced deformability of RBCs (Miller et al., 1971b). Reduced deformability of red blood cells is a key feature of severe falciparum malaria (Dondorp et al., 1997) (Dondorp et al., 1999) and has recently been reported in human knowlesi malaria (Barber et al., 2018b). In this study, ektacytometry demonstrated overall red blood cell deformability was reduced in Malaysian adults with knowlesi malaria, in proportion to disease severity, and comparable to that seen in severe falciparum malaria (Barber et al., 2018b). Red blood cell deformability was reduced in proportion to parasite biomass and lactate (as a measure of tissue perfusion). Micropipette aspiration confirmed that in humans, *P. knowlesi* infection causes increased stiffness of both infected and uninfected red blood cells, with the latter mostly as a result of echinocytosis (Barber et al., 2018b). Single-event flow cytometry and atomic force microscopy each showed sphericity

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was increased in infected red blood cells *vs* uninfected red blood cells. These findings were in contrast to parallel studies done in the natural host *Macaca fascicularis*, in which the deformability of uninfected red blood cells was not impaired and echinocyte formation was not seen (Barber et al., 2018b). *In vitro* studies using the human red blood cell-adapted *P. knowlesi* A1 strain have shown *P. knowlesi* infection results in a 20% increase in volume of the human red blood cell and an 11% decrease in the surface area to volume ratio, which combine to cause a markedly decreased deformability (Liu et al., 2019). Taken together, findings suggest that in humans, and other non-natural primate hosts of *P. knowlesi*, reduced deformability of both infected and uninfected red blood cells may be a major mechanism contributing to microvascular accumulation of parasites and impaired organ perfusion in severe knowlesi malaria.

In falciparum malaria, additional processes contributing to microvascular sequestration of infected red blood cells and reduced microvascular flow include adherence of infected red blood cells to uninfected red blood cells to form rosettes (David et al., 1988; Kaul et al., 1991; Udomsangpetch et al., 1989), however, rosetting has not been investigated to date in knowlesi malaria.

9.2 Endothelial activation, dysfunction and glycocalyx breakdown

Endothelial activation, impaired nitric oxide (NO) bioavailability and microvascular dysfunction are key features of severe falciparum malaria (Yeo et al., 2007, 2008, 2010, 2013, 2014), with endothelial activation and parasite biomass independent predictors of impaired organ perfusion, severe malaria and malaria mortality (Hanson et al., 2015). Endothelial activation, impaired NO bioavailability and microvascular dysfunction are also associated with severe disease in knowlesi malaria (Barber et al., 2017a, 2018a). In Malaysian adults with knowlesi malaria, parasitaemia and endothelial activation (measured by plasma angiopoietin-2) were independent predictors of severe disease, including acute kidney injury (Barber et al., 2017a). In this study, Barber et al. also found that endothelial activation and microvascular dysfunction were both associated with age, independent of parasitaemia; thus these mechanisms may also contribute to the more severe presentation of knowlesi malaria observed in older adults (Barber et al., 2017a). Because knowlesi malaria affects a wide age range from children through to the elderly, and because the pathogen biomass can be readily quantitated, human knowlesi malaria is an ideal model to evaluate the effects of aging on pathogenic mechanisms in all the human malarias.

The glycocalyx is a gel-like layer lining endothelial cells, essential in maintaining NO-dependent vascular homeostasis (Yeo et al., 2019b). Breakdown of the endothelial glycocalyx is associated with malaria severity, acute kidney injury and mortality in severe falciparum malaria (Yeo et al., 2019a,b). Recent studies show that endothelial glycocalyx degradation is also increased in knowlesi malaria in proportion to disease severity, and associated with impaired NO-dependent microvascular reactivity and acute kidney injury (Barber et al., 2021b). Glycocalyx breakdown is likely an additional mechanism of microvascular dysfunction and acute kidney injury in knowlesi malaria.

Cardiovascular disease and metabolic syndrome are known to be associated with increased endothelial activation, systemic inflammation, albuminuria, glycocalyx degradation and

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microvascular dysfunction (Rabelink and de Zeeuw, 2015). This may contribute to the increased risk of severe and fatal disease from *P. knowlesi* in those with cardiovascular-metabolic disease (Rajahram et al., 2019), as has also been reported in falciparum malaria (Wyss et al., 2017).

9.3 Systemic inflammatory response

As with falciparum malaria, the host inflammatory response has been associated with disease severity in knowlesi malaria, with both inflammatory and anti-inflammatory cytokines increased in severe disease, and with IL-6, IL-10 and IL-ra associated with parasitaemia (Barber et al., 2017a; Cox-Singh et al., 2011). Systemic inflammation (IL-6, and the IL-6/IL-10 ratio) in knowlesi malaria is also associated with age, independent of parasitaemia (Barber et al., 2017a). The causal role of systemic inflammation in pathogenesis is not clear. *Plasmodium knowlesi* infection also impairs protective human immune responses. Recent studies show that *P. knowlesi* infection of adults causes a decline in all subsets of circulating dendritic cells, key activators of the adaptive immune response to infection (Loughland et al., 2021).

9.4 Intravascular haemolysis

In severe falciparum malaria intravascular haemolysis is a key pathogenic mechanism in heme-induced acute kidney injury (Plewes et al., 2017, 2018) and endothelial dysfunction (Yeo et al., 2009). Haemolysis is associated with release of cell-free haemoglobin in proportion to disease severity leading to quenching of NO and endothelial dysfunction (Yeo et al., 2009). The scavenging capacity of haptoglobin and haemopexin are exceeded (Yeo et al., 2009), leading to circulation of free haemoglobin and free heme, which upon filtration through the kidney glomeruli, appear in the urine. Heme is rapidly oxidized from its ferrous (Fe^{2+}) form to ferric (Fe^{3+}) heme. Further oxidation of ferric to ferryl (Fe^{4+}) heme generates lipid radical species, leading to oxidative stress and oxidative injury to glomeruli and renal tubular cells (Reeder and Wilson, 2005). Acute kidney injury in falciparum malaria is associated with both cell-free haemoglobin and measures of lipid peroxidation, supporting the hypothesis that heme-induced lipid peroxidation contributes to acute kidney injury in falciparum malaria (Plewes et al., 2017).

Intravascular haemolysis has been shown to be increased in severe knowlesi malaria, to a greater degree than that seen in falciparum malaria, and is thought to be a key mechanism of acute kidney injury in knowlesi malaria (Barber et al., 2018a). In Malaysian adults with knowlesi malaria, intravascular haemolysis (measured by cell free haemoglobin) is independently associated with clinical measures of disease severity including lactate and acute kidney injury, and with microvascular dysfunction and the endothelial activation markers osteoprotegerin and angiopoietin-2 (Barber et al., 2018a). Given the magnitude of haemolysis in knowlesi malaria, it is likely a significant contributor to impaired tissue perfusion, organ dysfunction and acute kidney injury in severe disease (Barber et al., 2018a).

9.5 Thrombocytopenia

Thrombocytopenia is near-universal in knowlesi malaria (Barber et al., 2013; Daneshvar et al., 2009; Grigg et al., 2018a) but the underlying cause is not certain. The reported absence

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of thrombocytopenia in three of seven splenectomized patients with knowlesi malaria (Barber et al., 2013, 2016; Bird et al., 2016; Boo et al., 2016; Cheo et al., 2020) suggests a role of the spleen in platelet destruction or accumulation. Recent studies in patients with knowlesi malaria have shown that platelets bind to both *P. knowlesi*-infected red blood cells and uninfected red blood cells in the circulation, with platelets killing parasites through a platelet factor 4-mediated mechanism (Kho et al., 2018). Platelet-red blood cell complexes comprised a median of 12.5%, and up to 47.7% of the total circulating platelet pool in knowlesi malaria and are likely to contribute considerably to malaria thrombocytopenia (Kho et al., 2018). Platelets complexed with red blood cells are not recognized in platelet counts generated by automated haematology analyzers and therefore complex formation leads to apparent platelet loss. If there is an accelerated splenic clearance of these complexes this would further add to the contribution of complexes to platelet loss (Kho et al., 2018). It was notable in this study that the proportion of infected red blood cells bound to platelets was significantly higher in *P. knowlesi* infection than infection with *P. falciparum* or *P. vivax*, and that platelet-RBC complexes therefore formed the greatest proportion of the circulating platelet pool in knowlesi malaria (Kho et al., 2018). This is consistent with knowlesi malaria having the highest frequency of thrombocytopenia of all the *Plasmodium* species (Barber et al., 2013; Grigg et al., 2018a).

9.6 Anaemia

Intravascular and extravascular haemolysis of infected red blood cells contributes to anaemia in knowlesi malaria (Barber et al., 2018a). Unlike falciparum and vivax malaria (Jakeman et al., 1999), the relative contribution to anaemia of loss of infected *vs* uninfected red blood cells has not been modelled. Mechanisms underlying the loss of uninfected red blood cells in knowlesi malaria are not clear. Knisely's early "motion picture" microscopy studies in rhesus macaques demonstrated increased phagocytosis by the spleen and liver of "coated" *P. knowlesi*-infected and uninfected red blood cells contributing to anaemia (Knisely and Stratman-Thomas, 1945, 1948). Reduced deformability of red blood cells is associated with severe anaemia in falciparum malaria (Dondorp et al., 1999). The reduced deformability of both infected and uninfected red blood cells seen in both uncomplicated and severe knowlesi malaria (Barber et al., 2018b) likely enhances splenic clearance of both infected and uninfected red blood cells. Reduced red blood cell deformability was associated with haemoglobin nadir, independent of parasitaemia, suggesting a significant contribution to the anaemia of knowlesi malaria (Barber et al., 2018b). In chronic falciparum and vivax malaria, a hidden endosplenic lifecycle of asexual-stage parasites and the splenic accumulation of uninfected red blood cells are both significant contributors to anaemia (Kho et al., 2021a,b). The extent to which this occurs in knowlesi malaria is uncertain, but splenic congestion of both infected and uninfected red blood cells was seen in the single human autopsy case of fatal knowlesi malaria (Cox-Singh et al., 2010). In falciparum and vivax malaria, neutrophil extracellular traps mediate loss of bystander uninfected red blood cells and may be a mechanism contributing to anaemia (Kho et al., 2019). The role of neutrophil extracellular traps in the pathogenesis of anaemia or organ dysfunction in knowlesi malaria is unknown. Barber et al. have shown that concentrations of anti-phosphatidylserine IgM and IgG antibodies are elevated in knowlesi malaria, but unlike *P. falciparum* and *P. vivax*, these are not associated with anaemia (Barber et al., 2019). Loss of red blood cell complement

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regulatory proteins is an age-independent mechanism of loss of uninfected red blood cells in both falciparum and vivax malaria (Oyong et al., 2018), but its contribution to uninfected red blood cell loss and anaemia in knowlesi malaria is not yet known. While platelet-red blood cell complexes contribute significantly to thrombocytopenia in knowlesi malaria, they comprise only a small minority of the total circulating red blood cell pool in knowlesi malaria (up to 1%) and are therefore unlikely to be a significant contributor to anaemia (Kho et al., 2018).

10. Conclusion

Plasmodium knowlesi is the commonest cause of zoonotic malaria, and the only zoonotic malaria to cause severe and fatal disease. The clinical spectrum and risk factors for severe disease and death have been well described in high-incidence settings in Malaysia. While known to be endemic across Southeast Asia, the true burden and spectrum of clinical disease outside Malaysia is not well characterized. As the human-only *Plasmodium* species decline in these regions, the burden of clinical disease from knowlesi malaria will likely increase. Enhanced molecular surveillance of *Plasmodium* species from both symptomatic and asymptomatic infections is required across Southeast Asia. The neonatal and maternal impacts of knowlesi malaria in pregnancy are poorly understood and require further study.

Parasite and host factors associated with the risk of infection, high parasite counts and severe disease need further characterization. While significant advances in our understanding of pathogenesis of human disease have been made over the last decade, further work is needed, particularly in understanding mechanisms of microvascular accumulation of parasitized red blood cells, impaired organ perfusion and organ dysfunction. Such understanding will guide the development of appropriate adjunctive therapies to complement existing strategies to improve clinical outcomes, including rapid and accurate diagnosis and prompt initiation of artemisinin-based antimalarial therapy.

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Table 1

Demographic and clinical features in adults with knowlesi malaria enrolled in prospective studies.

	District hospital studies		**Tertiary referral hospital study**
Patient characteristic	**Grigg et al. (2018a) Sabah, Malaysia *n* =481 (437 adults)**	**Daneshvar et al. (2009) Sarawak, Malaysia *n* =121 (113 adults)**	**Barber et al. (2013) Sabah, Malaysia *n* =130**
Adults (age > 12yrs), (%)	91	93	100
Age, years			
Median (IQR)	35 (25–50)	45 (14.9)[b]	46 (29–58)
Range	13–85	16–79	14–83
Male gender, (%)	79	56	74
Previous malaria (self-reported), (%)	21	26	28
History of chronic disease, (%)	8.0	NR	24
Days of fever, median (IQR)	4 (3–7)	5 (3–7)	5 (3–7)
Symptoms on enrolment, n (%)			
Rigors	82.3	89.7	84
Headache	89.0	94.4	91
Vomiting	24.0	33.6	30
Abdominal pain	23.3	52.3	31
Diarrhoea	8.2	29.0	18
Cough	35.0	56.1	48
Shortness of breath	16.0	NR	17
Myalgia	61.6	87.9	47
Arthralgia	66.1	NR	58
Examination findings on enrolment			
Temperature, °C, median (IQR)	37.4 (37.0–38.1)	37.6 (37–38.5)	37.5 (36.8–38.4)
Fever, temp≥37.5 °C, (%)	49.3	NR	NR
Systolic blood pressure, mmHg, mean (SD)	120 (110–130)[a]	NR	118 (19.7)
Heart rate, beats/min, mean (SD)	88 (77–100)[a]	95 (16)	90 (15.1)
Respiratory rate, breaths/min, mean (SD)	20 (20–24)[a]	26 (22–31)[a]	27 (6.0)

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District hospital studies			Tertiary referral hospital study
Patient characteristic	**Grigg et al. (2018a) Sabah, Malaysia *n* =481 (437 adults)**	**Daneshvar et al. (2009) Sarawak, Malaysia *n* =121 (113 adults)**	**Barber et al. (2013) Sabah, Malaysia *n* =130**
Oxygen saturation, %, median (IQR)	98 (97–99)	NR	97.5 (96–99)
Palpable liver, n (%)	24.0	24.3	40
Palpable spleen, n (%)	5.9	15	33
Rash, n (%)	4.3	NR	NR

[a] Median (IQR)

[b] mean (SD)

NR: not reported

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Table 2

Clinical laboratory features in adults with knowlesi malaria enrolled in prospective studies.

	District hospital studies		Tertiary referral hospital study	
			Barber et al. (2013) Sabah, Malaysia n=130	
Patient characteristic	**Grigg et al. (2018a) Sabah, Malaysia n=481 (437 adults)**	**Daneshvar et al. (2009) Sarawak, Malaysia n=121 (113 adults)**	**Non-severe**	**Severe**
Parasite count, parasites/μL, median (IQR)	2541 (478–8585)	1387 (6–222,570)	4837 (1576–14,641)	80,359 (25874–168,279)
Trophozoite proportion, mean % (SD)	NR	NR	49	42
Schizont proportion, mean % (SD)	2 (5.4)	NR	0 (0–1.25)[a]	0.79 (0–11.1)[a]
Parasite count >20,000/μL, (%)	15	NR	16	79
Haemoglobin, g/dL, mean (SD)	13.2 (12.1–14.3)[a]	13.3 (12–14.3)[a]	12.8 (1.6)[b]	12.1 (1.8)[b]
White blood cell count, ×103/μL, median (IQR)	6.1 (5.1–7.6)	5.6 (4.7–7.0)	6.1 (5.0–7.2)	6.6 (5.6–9.8)
Neutrophil count, ×103/μL, median (IQR)	3.5 (2.6–4.5)	3.7 (1.8)[b]	3.4 (2.5–4.4)	4.7 (3.5–7.6)
Lymphocyte count, ×103/μL, median (IQR)	1.4 (1.0–1.9)	1.5 (1.1–2.0)	1.6 (1.0–2.0)	1.4 (1.0–1.9)
Monocyte count, ×103/μL, median (IQR)	1.0 (0.7–1.4)	NR	0.9 (0.6–1.2)	1.0 (0.7–1.3)
Platelet count, ×103/μL, median (IQR)	70 (50–103)	71 (35)[b]	51 (35–81)	29 (20–49)
Platelet nadir, ×103/μL, median (IQR)	60 (42–83)	NR	42 (25–62)	27 (18–46)
Platelet nadir, median days (IQR)	1 (1–1)	NR	0 (0–1)	0 (0–1)
Thrombocytopenia (platelets <150×103/μL), (%)	92	98	99 (nadir)	100 (nadir)
Creatinine, μmol/L, median (IQR)	88 (75–103)	86 (73–100)	92 (78–110)	141 (101–213)
Sodium, mmol/L, median (IQR)	136 (134–139)	137 (135–140)	134 (3.2)[b]	131 (4.6)[b]
Bilirubin, μmol/L, median (IQR)	17.1 (11.8–24.6)	13 (9–18)	16.6 (12.9–24.8)	42.1 (26–66.8)
Glucose, mmol/L, median (IQR)	6.4 (5.6–7.4)	6.2 (5.3–6.7)	6.7 (5.8–7.8)	7.7 (6.4–9.5)
Albumin, g/dL, median (IQR)	36 (30–40)	36 (33–39)	30.5 (4.7)[b]	26.6 (5.3)[b]
AST, IU/L, median (IQR)	34 (23–47)	NR	39 (26–49)	58 (44–79)
ALT, IU/L, median (IQR)	37 (24–56)	36 (25–54)	35 (21–56)	36 (19–51)
Bicarbonate, mmol/L, median (IQR)	24 (21–27)	NR	24.8 (3.8)[b]	21.6 (4.9)[b]
Acute kidney injury, KDIGO criteria (%)	83 (19)	NR	NR	NR

[a] Median (IQR).

[b] mean (SD).

NR: not reported.

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Table 3

Severe disease[d] in prospective studies of knowlesi malaria in adults.

	Daneshvar et al. (2009) Sarawak, Malaysia (n=10)	Grigg et al. (2018a) Sabah, Malaysia (n=28)	Barber et al. (2013) Sabah, Malaysia Tertiary referral hospital (n=38)
Severe malaria[d] as % of all adult cases	9.3 (3.8–14.8)	6.4 (3.9–8.3)	29.2 (21.4–37)
Age (years) Median (IQR)[c]	63.5	53 (43–64)	55 (47–62)
Range	36–73	13–78	20–74
Male sex (%)	30	68	79
Severe criteria developed after treatment %	20	32	5
WHO severity Criteria[d], (%)			
Hyperparasitaemia (>100,000 parasites/μL)	30	29	47
Respiratory distress	70	7	37
Hypotension	20	18	34
Jaundice	40	29[a]	53[a]
Severe acute kidney injury	30	36	24
Metabolic acidosis	NR	11	11
Severe anaemia	0	29	5
Abnormal bleeding	NR	4	5
Multiple convulsions	NR	0	0
Hypoglycaemia	10	4	0
Coma	0	0	0
Death			
% of severe	20[b]	7.1[c]	0[c]
% of all cases	1.9	0.4	0

[a]With parasite count >20,000 parasites/μL for *P. knowlesi* and/or creatinine >132μmol/L.

[b]Treated with intravenous quinine.

[c]Treated with intravenous artesunate.

[d]Severe malaria defined using WHO research and epidemiological definition of severe malaria (WHO, 2014).