

Reduced red blood cell deformability in *Plasmodium knowlesi* malaria

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Key Points

- RBC-D is reduced in humans with *knowlesi* malaria in proportion to disease severity.
- In humans, but not the macaque hosts, deformability of uRBCs is reduced and is related to the presence of echinocytes.

The simian parasite *Plasmodium knowlesi* can cause severe and fatal human malaria. However, little is known about the pathogenesis of this disease. In falciparum malaria, reduced red blood cell deformability (RBC-D) contributes to microvascular obstruction and impaired organ perfusion. In *P knowlesi* infection, impaired microcirculatory flow has been observed in *Macaca mulatta* (rhesus macaques), unnatural hosts who develop severe and fatal disease. However, RBC-D has not been measured in human infection or in the natural host *M fascicularis* (long-tailed macaques). Using ektacytometry, we measured RBC-D in adults with severe and non-severe *knowlesi* and falciparum malaria and in healthy controls. In addition, we used micropipette aspiration to determine the relative stiffness of infected RBCs (iRBCs) and uninfected RBCs (uRBCs) in *P knowlesi*-infected humans and *M fascicularis*. Ektacytometry demonstrated that RBC-D overall was reduced in human *knowlesi* malaria in proportion to disease severity, and in severe *knowlesi* malaria, it was comparable to that of severe falciparum malaria. RBC-D correlated inversely with parasitemia and lactate in *knowlesi* malaria and HRP2 in falciparum malaria, and it correlated with hemoglobin nadir in *knowlesi* malaria. Micropipette aspiration confirmed that in humans, *P knowlesi* infection increased stiffness of both iRBCs and uRBCs, with the latter mostly the result of echinocytosis. In contrast, in the natural host *M fascicularis*, echinocyte formation was not observed, and the RBC-D of uRBCs was unaffected. In unnatural primate hosts of *P knowlesi*, including humans, reduced deformability of iRBCs and uRBCs may represent a key pathogenic mechanism leading to microvascular accumulation, impaired organ perfusion, and anemia.

Introduction

The simian parasite *Plasmodium knowlesi* occurs throughout Southeast Asia and is the most common cause of malaria in humans in Malaysia^{1,2} and in regions of western Indonesia.^{3,4} *P knowlesi* can cause severe and fatal disease, with the risk of severe disease in adults at least as high as that of *P falciparum*.⁵ However, few studies have examined the pathogenic mechanisms of human disease.⁶

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In falciparum malaria, severe disease is characterized by cytoadherence of infected red blood cells (iRBCs) to activated endothelium, leading to microvascular sequestration and impaired organ perfusion.⁷ Decreased deformability of both iRBCs and uninfected RBCs (uRBCs) is an additional key contributor to microvascular obstruction.^{8,9} In severe knowlesi malaria, a single autopsy report revealed widespread microvascular accumulation of iRBCs.¹⁰ However, endothelial cytoadherence was not clearly evident, and intercellular adhesion molecule-1, which mediates cytoadherence to brain endothelial cells in falciparum malaria,^{11,12} was not detected. A paucity of *P falciparum*-like endothelial cytoadherence and sequestration is also suggested by the notable lack of coma attributable to *P knowlesi* to date and lack of specific malarial retinopathy.¹³ Thus, mechanisms by which *P knowlesi* accumulates in the microvasculature likely differ from those for *P falciparum*.

Early studies in rhesus macaques (*Macaca mulatta*), an unnatural host for *P knowlesi*, demonstrated marked circulatory changes in infected monkeys, with impairment of microvascular flow reported to be a critical factor in fatal outcomes.¹⁴ Increased viscosity and resistance to flow of RBCs from *P knowlesi*-infected rhesus macaques has also been demonstrated, suggesting reduced RBC deformability (RBC-D).¹⁵ We hypothesized that in human knowlesi malaria, reduced deformability of RBCs may contribute to microvascular obstruction and organ dysfunction.

We used a laser-assisted optical rotational cell analyzer (LORCA) to measure the RBC-D in fresh whole blood from patients with severe and non-severe knowlesi malaria and compared the results with those of patients with falciparum malaria and healthy controls. Micropipette aspiration was used to assess the relative contributions of iRBCs and uRBCs to the overall RBC-D as measured by LORCA. We also assessed the effect of *P knowlesi* on the RBC-D of *M fascicularis*, the natural host and primary reservoir of *P knowlesi*.

Patients and methods

Study participants

Patients included in the LORCA study were enrolled as part of a prospective observational study of all malaria patients admitted to Queen Elizabeth Hospital, an adult tertiary referral hospital in Sabah, Malaysia.⁵ Patients were included if they had *P knowlesi* or *P falciparum* mono-infection confirmed by polymerase chain reaction (PCR) and were ≥ 12 years old, within 18 hours of starting treatment for malaria, not pregnant, and with no major comorbidities. Patients were included from November 2010 to May 2011 and from September 2011 to April 2012, coinciding with the local availability of LORCA. Clinical details of a subset of these patients have been previously reported.^{5,6} Severe malaria was defined according to modified World Health Organization criteria for severe falciparum malaria, as previously described.¹⁶ Healthy controls were visitors with no history of fever in the past 48 hours and with blood film negative for malaria parasites who were accompanying malaria patients.

Standardized history and physical examinations were documented. Hematology, biochemistry, acid-base parameters, lactate (by bedside i-STAT blood analysis), and HRP2 (in falciparum patients) were obtained on enrollment. Parasitemia was determined by microscopy, and parasite species were confirmed by PCR.^{17,18} Because thalassemia can affect RBC-D,¹⁹ hemoglobin electrophoresis was performed on blood samples collected on enrollment. Fresh whole blood collected in a tube with heparin

anticoagulant underwent LORCA assessment of RBC-D (see below) and was cryopreserved in Glycerolyte 57 (Baxter) before being stored in liquid nitrogen for later assessment of RBC rigidity by micropipette aspiration (see below).

Patients were treated according to hospital guidelines, as previously described.⁵ Written informed consent was provided by study participants or their relatives. Approvals were obtained from the ethics committees of the Malaysian Ministry of Health and Menzies School of Health Research.

Measurement of RBC-D

LORCA (RR Mechatronics, Zwaag, The Netherlands) was used to measure RBC-D in freshly collected whole blood on enrollment and on day 3 in a subset of patients with knowlesi malaria. With this method, whole blood is added to a highly viscous medium (5% polyvinylpyrrolidone in phosphate-buffered saline [PBS]), and the RBC suspension is sheared between 2 concentric rotating cylinders at a constant temperature of 37°C.²⁰ The increasing rotation of the outer cylinder leads to a shear stress that causes the RBCs to elongate and align themselves in the fluid layer. A laser beam is directed through this fluid layer and forms a diffraction pattern on the screen behind it. This diffraction pattern undergoes computer analysis to produce an elongation index (EI); a lower EI indicates reduced RBC-D. RBC-D was assessed at shear stresses of 1.7 Pa and 30 Pa. Shear stresses of 1.7 Pa are encountered in the capillaries.²¹ Shear stresses of 30 Pa provide information on cell geometry, in particular ratios of surface area to volume²² and approximate values encountered by RBCs passing through intercellular gaps in splenic sinusoids.²³

Collection of blood from *M fascicularis*

To compare the effect of *P knowlesi* infection on RBC-D in the human host with that in its natural simian reservoir, we measured RBC stiffness in blood collected from *P knowlesi*-infected *M fascicularis* monkeys. All procedures were approved by the Institutional Animal Care and Use Committee, University of Malaya. *M fascicularis* monkeys were bred and grown in animal facilities in a malaria-free environment in Vietnam (Nafovanny) and were 2 years old when they became part of our experiment. Approximately 4×10^6 thawed *P knowlesi* UM01 strain parasites²⁴ suspended in PBS were inoculated intravenously into each macaque. At ~ 8 days after inoculation, the parasitemia reached $\sim 3\%$ with predominantly late trophozoite stages. A total of 2 mL of whole blood was drawn from the infected macaques, and parasites were allowed to mature ex vivo to obtain the stage required for micropipette aspiration. Isolates were cryopreserved using the same method (Glycerolyte 57 [Baxter]) as that used for the human *P knowlesi* isolates.

Micropipette aspiration, imaging flow cytometer, and atomic force microscopy

Because RBC-D derived from LORCA provides an overall RBC population measurement, we used micropipette aspiration to determine the relative contributions of iRBCs and uRBCs to the overall RBC-D. Cryopreserved iRBCs and uRBCs from whole human and *M fascicularis* blood were assessed by using a modified previously published methodology²⁵ (supplemental Methods).

Table 1. Epidemiologic and clinical features and RBC-D of malaria patients and controls enrolled at Queen Elizabeth Hospital

	Controls (n = 15)			Plasmodium knowlesi			Plasmodium falciparum		
	Non-severe (n = 63)	Severe (n = 19)	P (severe vs non-severe)	Non-severe (n = 82)	Severe (n = 8)	P (severe vs non-severe)			
Age, y									
Median (IQR)	36 (22-45)	50 (38-61)	.014	28 (20-39)	22 (17-34)	.391			
Range	16-71	20-69		13-62	13-50				
Male sex, n (%)	47 (75)	17 (89)	.325	64 (78)	5 (63)	.321			
Parasite count, parasites per μ L	2544 (930-14,056)	141 689 (39 564-292 007)	<.0001	9210 (2154-32 267)	25 468 (4307-103 852)	.234			
Parasite stage, %									
Rings	39 (4-93)	83 (38-98)		100 (100-100)	100 (100-100)				
Trophozoites	56 (4-92)	3 (0-59)		0 (0-0)	0 (0-0)				
Schizonts	0 (0-4)	1 (1-17)		0 (0-0)	0 (0-0)				
Hemoglobin, g/dL	14.4 (13.3-15.4)*	12.9 (12.2-13.7)	.930	13.4 (11.3-14.4)	13.1 (9.1-14.1)	.399			
MCV	86.3 (79.5-89.1)*	81.9 (75.1-85.1)	.419	80.4 (75.6-85.7)	78.8 (71.6-84.1)	.523			
Nadir hemoglobin, g/dL	11.6 (10.7-12.8)	10.4 (9.1-11.4)	.0009	11.8 (10.5-12.8)	10.1 (8.9-11.6)	.059			
Platelets $\times 10^9/L$	56 (40-83)	23 (15-33)	<.0001	74 (42-134)	29 (23-56)	.007			
Creatinine, μ mol/L	88 (75-110)	129 (113-215)	<.0001	87 (72-105)	113 (76-246)	.158			
Lactate, μ mol/L	1.17 (0.84-1.39)	1.67 (1.15-2.30)	.001	1.18 (0.92-1.55)	1.53 (1.2-2.04)	.061			
Bilirubin, μ mol/L	16 (12-24)	49 (26-95)	<.0001	18 (12-30)	54 (24-75)	.009			
RBC-D									
SS 1.7 Pat	0.203 (0.178-0.222)	0.192 (0.168-0.223)	.175	0.180 (0.163-0.197) †	0.195 (0.177-0.214)	.171			
SS 30 Pa§	0.583 (0.576-0.590)	0.551 (0.477-0.569)	.010	0.520 (0.475-0.558)¶	0.510 (0.496-0.539)#	.949			
Time from start of antimalarials, h	4.8 (0-10.1)	11.0 (4.3-13.6)	.033	8.0 (0-13.0)	2.1 (0-7.4)	.152			

Data are median (interquartile range [IQR]) unless otherwise indicated.

MCV, mean corpuscular volume; SS, shear stress.

*Data were missing for 3 controls.

†For difference among controls, non-severe malaria, and severe malaria, using Kruskal-Wallis test $P = .227$ for *P. knowlesi* and $P = .033$ for *P. falciparum*.

$P = .018$ vs controls.

§For difference among controls, non-severe malaria, and severe malaria, using Kruskal-Wallis test, $P = .0001$ for *P. knowlesi* and $P = .0002$ for *P. falciparum*.

|| $P = .0001$ vs controls.

¶ $P < .0001$ vs controls.

$P = .005$ vs controls.

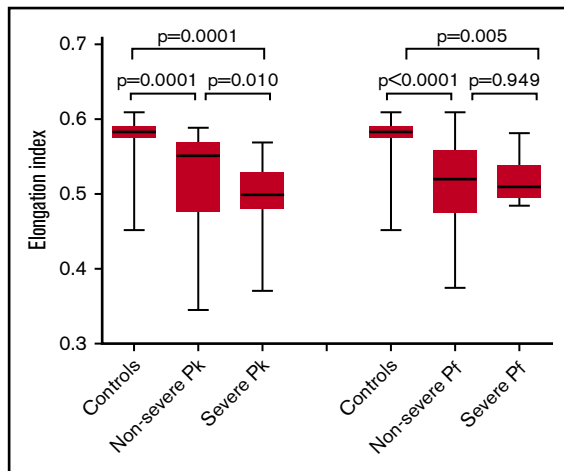


Figure 1. RBC-D in patients with knowlesi and falciparum malaria. (A) Deformability profile of whole peripheral RBCs from patients with knowlesi malaria (*Plasmodium knowlesi* [Pk]; 19 severe, 63 non-severe), falciparum malaria (*Plasmodium falciparum* [Pf]; 8 severe, 82 non-severe), and healthy controls (n = 15) as measured by LORCA at a shear stress of 30 Pa. An increase in EI reflects an increase in overall RBC-D. Error bars indicate range, boxes indicate interquartile range, and horizontal black bars indicate median values.

Geometry is a major component of RBC-D. Thus, an imaging flow cytometer (Amnis) was used to assess sphericity of cryopreserved iRBCs and uRBCs from human and *M. fascicularis* blood, as previously described.²⁶ Results are reported as an aspect ratio, which reflects the ratio of the diameter of a cell and hence sphericity. Greater sphericity (and hence greater RBC stiffness) is indicated by values trending to 1. Sphericity of iRBCs from monkeys and humans infected with the UM01 strain of *P. knowlesi* was also assessed by atomic force microscopy (supplemental Methods).

Measurement of echinocytes

Because the micropipette aspiration demonstrated that the increased stiffness of iRBCs and uRBCs in humans with knowlesi malaria seemed to relate to echinocytosis, additional experiments were performed to confirm and quantify echinocytosis in fresh whole blood from patients with knowlesi malaria. For these experiments, an additional 42 patients with PCR-confirmed knowlesi malaria and 25 healthy controls were enrolled at district hospitals in Sabah from November 2015 to September 2016 (enrollment criteria as above). Venous blood was collected in EDTA, and 50 μ L was transferred to a 2-mL Eppendorf tube. Fifty μ L of PBS and 2.5 μ L of Giemsa stain were added, and the mixture was inverted 5 times. After 15 minutes, 7.5 μ L was placed onto a glass slide with a cover slip, and the slide was immediately examined and photographed by a research microscopist using $\times 100$ magnification with an oil immersion lens. For each participant, echinocytes and normocytes were counted in a minimum of 10 photographed fields. The photographs from 5 participants (50 photographs) were cross-checked by one of the study authors (B.R.) who was blinded to the participant's malaria status. There was good concordance (agreement, 84% [κ statistic, 0.73; 95% confidence interval, 0.58-0.89]) when the number of echinocytes was expressed in multiples of 10 and with a maximum of 100.

Statistical analysis

Stata v10.1 was used for analysis. For continuous variables, the Kruskal-Wallis test was used to compare differences across

3 groups (controls, non-severe malaria, severe malaria), and the Mann-Whitney *U* test was used for 2-group comparisons. Categorical variables were compared using the χ^2 test. Associations between RBC-D and other variables were examined using Spearman's correlation coefficient. Confounding variables were adjusted for using partial correlation or multiple linear regression, with variables log-transformed when necessary. Paired longitudinal measurements of RBC-D were compared by using the Wilcoxon signed-rank test.

For reporting the membrane shear modulus (MSM), the average (geometric mean) MSM was first calculated for each monkey or human participant. An overall average of these values was then calculated for each parasite stage within each species. Mixed-effects linear regression with log-transformed MSM and adjusting for human or monkey participants was used to assess the association between MSM and parasite stage, to compare MSM between human echinocytes and normocytes at each parasite stage, and to compare MSM between healthy participants and uninfected echinocytes and normocytes. Mixed effects linear regression adjusting for monkey and human participants was also used to compare aspect ratio (measured by imaging flow cytometer) between species.

Results

Demographic and clinical characteristics of patients enrolled at Queen Elizabeth Hospital

A total of 82 patients with knowlesi malaria (63 non-severe, 19 severe), 90 patients with falciparum malaria (82 non-severe, 8 severe), and 15 healthy controls were enrolled. Baseline clinical and laboratory details are listed in Table 1. Among the 19 patients with severe knowlesi malaria, the most common severity criteria included hyperparasitemia (n = 12 [63%]), jaundice (n = 10 [53%]), respiratory distress (n = 8 [42%]), hypotension (n = 4 [21%]), and acute kidney injury (n = 4 [21%]). In falciparum malaria, severity criteria included hypotension (n = 4 [50%]), jaundice (n = 4 [50%]), respiratory distress (n = 3 [38%]), and metabolic acidosis (n = 3 [38%]). No patient from either species had coma, and no deaths occurred.

The mean corpuscular volume of RBCs did not differ significantly between patients with severe or non-severe knowlesi malaria or between patients with severe or non-severe falciparum malaria. Thirty patients (37%) with knowlesi malaria and 44 (49%) with falciparum malaria had microcytosis (mean corpuscular volume, <80 fl). Of these, results of hemoglobin electrophoresis were available in 23 patients (77%) with knowlesi malaria and 40 patients (91%) with falciparum malaria, with β thalassemia carrier state detected in 4 (17%) of 23 and 6 (15%) of 40. Three patients were heterozygous for hemoglobin E (HbE) (1 with knowlesi and 2 with falciparum malaria), and 3 had results suggestive of α thalassemia trait (2 with knowlesi and 1 with falciparum malaria).

RBC-D

***P. knowlesi* malaria.** At a shear stress of 30 Pa, median RBC-D was reduced in patients with severe (EI, 0.499; IQR, 0.481-0.529) and non-severe (EI, 0.551; IQR, 0.477-0.569) knowlesi malaria compared with controls (EI, 0.583; IQR, 0.576-0.590; *P* = .0001 for both comparisons) and was reduced in severe compared with

Table 2. MSM of RBCs in monkeys and humans with knowlesi malaria and in healthy monkey and human controls

	Average MSM of RBCs (pN/μm)				P (normocyte vs echinocyte)
	Healthy monkey controls (n = 3)	Monkeys with <i>P knowlesi</i> (n = 3)	Healthy human controls (n = 5)	Humans with <i>P knowlesi</i> (n = 4)	
uRBCs	4.2 4.0, 4.3, 4.5 (N = 8, 8, 7)	5.0 4.9, 5.1, - (N = 10, 10, 0)	3.2 1.2, 2.6, 4.2, 4.9, 5.4 (N = 4, 8, 10, 8, 10)	5.1 7.0, 5.1, 3.6, 5.2 (N = 4, 6, 5, 5)	14.3 136, 15.0, -, - (N = 5, 10, 0, 0)
Rings	8.7 7.7, 9.9, - (N = 9, 7, 0)	8.9 10.1, 5.8, 12.1, - (N = 7, 1, 5, 0)	8.9 10.1, 5.8, 12.1, - (N = 7, 1, 5, 0)	8.9 10.1, 5.8, 12.1, - (N = 7, 1, 5, 0)	5.6 5.6, 6.5, 4.8, 2.6* (N = 5, 9, 5, 2)
Trophozoites	13.8 22.6, 12.6, 9.2 (N = 4, 6, 6)	13.8 22.6, 12.6, 9.2 (N = 4, 6, 6)	18.2 12.3, 16.9, 29.1, - (N = 4, 7, 1, 0)	18.2 12.3, 16.9, 29.1, - (N = 4, 7, 1, 0)	7.3 7.0, 5.7, 9.8, - (N = 6, 1, 4, 0)
Schizonts	16.9 21.9, 20.7, 10.7 (N = 3, 6, 6)	16.9 21.9, 20.7, 10.7 (N = 3, 6, 6)	28.4 34.1, 22.9, 29.3, - (N = 4, 5, 9, 0)	28.4 34.1, 22.9, 29.3, - (N = 4, 5, 9, 0)	43.6 47.5, 43.0, 40.6, - (N = 8, 8, 20, 0)

The average of averages is presented in bold. Average for each participant is calculated first and is presented in the second row of each cell. Monkeys and humans with malaria are listed in the order given in the supplemental Figure (ie, subject 1, subject 2, subject 3). HHCs and monkey controls are ordered from lowest to highest. N is the number of RBCs examined for each participant, in respective order. P values are calculated by using mixed effects regression and adjusting for monkey and human participants and with MSM log-transformed. Using this analysis, MSM of uninfected echinocytes from humans with knowlesi malaria was 4.2-fold higher than MSM of RBCs from HHCs ($P < .0001$). MSM of uninfected normocytes from humans with knowlesi malaria was 1.5-fold higher than the MSM of RBCs from HHCs ($P = .14$). There was no significant difference in the MSM of RBCs from uninfected monkeys and the MSM of uRBCs from infected monkeys ($P = .22$).

*Not included in the average of averages presented in bold or in the regression analyses because of the absence of data for other parasite stages.

non-severe knowlesi malaria ($P = .010$) (Table 1; Figure 1). The difference in RBC-D between patients with severe and non-severe knowlesi malaria remained significant after exclusion of patients with microcytosis ($P = .019$), as did the difference in RBC-D between controls and patients with non-severe knowlesi malaria ($P = .014$) and between controls and patients with severe knowlesi malaria ($P = .006$).

In knowlesi malaria, RBC-D at 30 Pa was inversely correlated with parasite count ($r = -0.37$; $P < .001$), percentage of schizonts ($r = -0.26$; $P = .022$), and lactate ($r = -0.26$; $P = .021$) and was positively correlated with platelet count ($r = 0.24$; $P = .027$) and hemoglobin nadir ($r = 0.30$; $P = .006$). The association with hemoglobin nadir remained significant after controlling for parasitemia ($r = 0.22$; $P = .049$).

At a lower shear stress of 1.7 Pa, the difference in median RBC-D between patients with severe knowlesi malaria, non-severe knowlesi malaria, and controls was not statistically significant (Table 1). However, RBC-D at 1.7 Pa was inversely correlated with parasite count ($r = -0.37$; $P \leq .001$) and lactate ($r = -0.27$; $P = .019$) and positively correlated with hemoglobin nadir ($r = 0.31$; $P = .004$). After controlling for parasitemia, the correlation coefficients for lactate and hemoglobin nadir were $r = -0.22$ ($P = .058$) and $r = 0.23$ ($P = .035$), respectively.

In patients with severe and non-severe knowlesi malaria who had RBC-D reassessed on day 3, there was no significant improvement in RBC-D at 30 Pa from baseline EI of 0.515 (IQR, 0.454-0.565) to day 3 EI of 0.539 (IQR, 0.502-0.564) in non-severe knowlesi malaria ($n = 29$; $P = .247$) and from baseline EI of 0.498 (IQR, 0.485-0.519) to day 3 EI of 0.508 (IQR, 0.493-0.536) in severe knowlesi malaria ($n = 11$; $P = .241$).

All but one patient with severe knowlesi malaria was enrolled after antimalarial treatment was started. In patients with non-severe knowlesi malaria, there was a trend toward increased RBC-D in patients enrolled after, compared with before, beginning antimalarial treatment (supplemental Table).

P falciparum malaria. At a shear stress of 30 Pa, median RBC-D was lower in severe (EI, 0.510; IQR, 0.496-0.539) and non-severe (EI, 0.520; IQR, 0.475-0.558) falciparum malaria compared with controls (EI, 0.583; IQR, 0.576-0.590; $P = .005$ and $P < .0001$, respectively; Table 1; Figure 1). However, the difference between patients with severe and non-severe falciparum malaria was not significant. At a lower shear stress of 1.7 Pa, median RBC-D was also lower among patients with severe (EI, 0.195; IQR, 0.177-0.214) and non-severe (EI, 0.180; IQR, 0.163-0.197) falciparum malaria compared with controls (EI, 0.203; IQR, 0.178-0.222), although only the difference between patients with non-severe falciparum malaria and controls was significant ($P = .018$).

In patients with falciparum malaria, there was no association between RBC-D and parasite count; however, RBC-D was inversely correlated with HRP2 at 30 Pa ($r = -0.24$; $P = .020$). At 1.7 Pa, but not at 30 Pa, RBC-D was associated with hemoglobin nadir ($r = 0.32$; $P = .002$). In falciparum malaria, there was no significant association between RBC-D and lactate.

RBC-D was lower in non-severe falciparum malaria compared with non-severe knowlesi malaria at 1.7 Pa ($P = .032$). In severe malaria, the impairment in RBC-D was comparable between the two species (Figure 1).

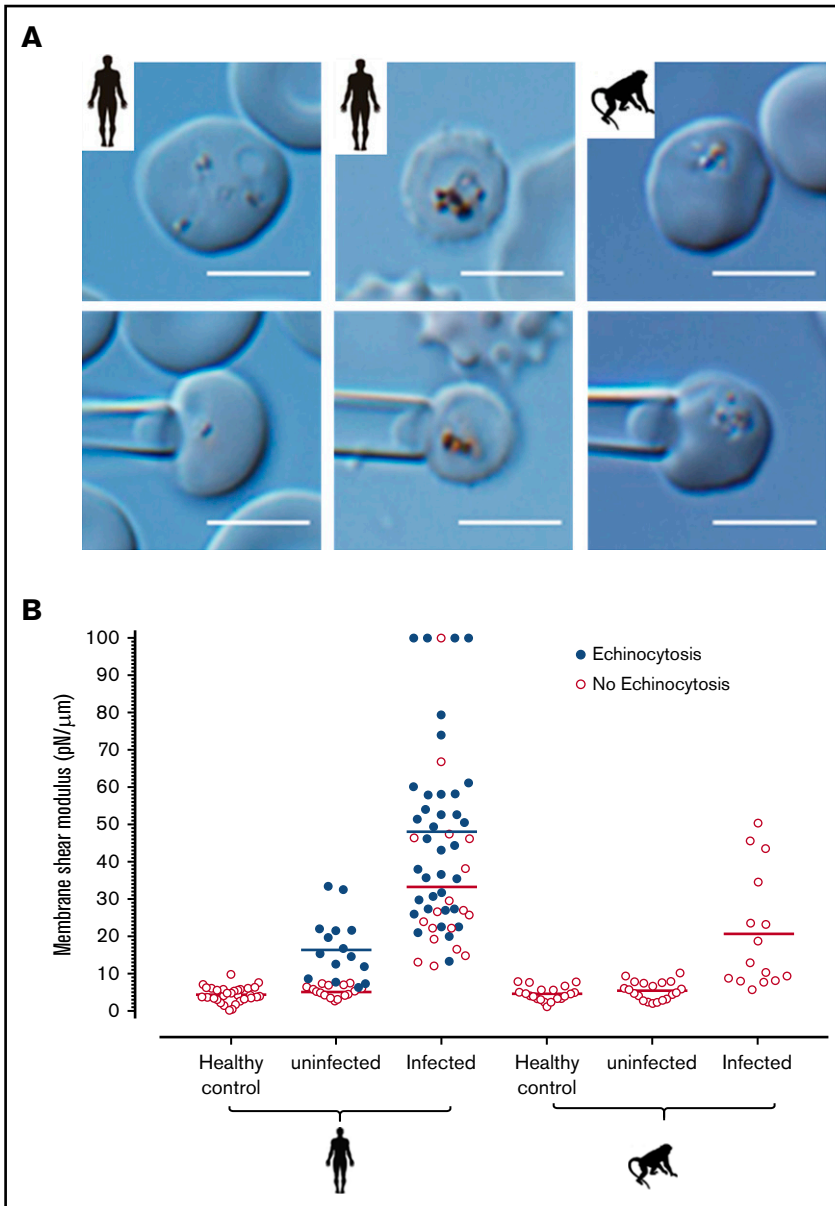


Figure 2. Comparison of the deformability of *P knowlesi* iRBCs from humans and *M fascicularis*. (A) Human iRBCs (first two columns) and *M fascicularis* iRBCs (last column) before (upper row) and after (lower row) micropipette aspiration. (B) Deformability, as measured by micropipette aspiration, of iRBCs and uRBCs from *P knowlesi*-infected humans and *M fascicularis* monkeys compared with healthy human and monkey controls. An increase in MSM reflects a decrease in deformability. Scale bar = 5 μm .

Micropipette aspiration, imaging flow cytometer, and atomic force microscopy

Micropipette aspiration was performed on RBCs from 3 monkeys infected with *P knowlesi*, 3 uninfected monkeys, 5 healthy human controls (HHCs), and 4 patients with knowlesi malaria (parasitemias <4% iRBCs). The total number of RBCs examined for each monkey or human participant at each parasite stage is listed in Table 2.

In monkeys, there was no significant difference in the resistance to membrane extension (as measured by MSM) of the RBCs of uninfected monkeys (average MSM, 4.2 pN/ μm) compared with the uRBCs of infected monkeys (average MSM, 5.0 pN/ μm ; $P = .22$). However, in infected monkeys, MSM was higher in ring iRBCs compared with uRBCs (average MSM, 8.7 vs 5.0 pN/ μm ; $P = .003$), and it increased with each subsequent parasite stage ($P < .0001$; Table 2; supplemental Figure).

In humans with knowlesi malaria, MSM was increased in both iRBCs and uRBCs compared with HHCs (Table 2; Figure 2). However, the increase in MSM related in part to the presence of echinocytes (Figures 2 and 3). In the case of uRBCs, normocytes from knowlesi malaria patients demonstrated only a minimal increase in resistance compared with HHC RBCs (average MSM, 5.1 vs 3.2 pN/ μm ; $P = .14$). However, when echinocytes were examined, the median MSM of uRBCs was greater than fourfold that of the RBCs from healthy controls (average MSM, 14.3 vs 3.2 pN/ μm ; $P < .0001$). We also observed that MSM increased with parasite maturation, but again this was influenced by the presence of echinocytes. In normocytes, there was a linear increase in MSM with increasing parasite stage ($P < .0001$) (supplemental Figure). However, when only echinocytes were considered, the MSM of ring- and trophozoite-infected echinocytes was lower than that of uninfected echinocytes from malaria patients (5.6 and 7.3 vs 14.3 pN/ μm ,

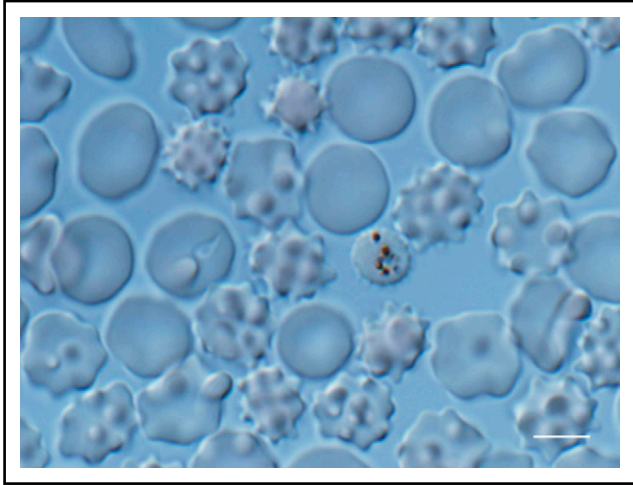


Figure 3. Representative photograph of RBCs from a patient with severe knowlesi malaria. The unstained sample was observed on a heated stage (~37°C) in isotonic saline. Although most of the RBCs observed are noninfected, 1 infected RBC containing a mature parasite with hemazoin can be seen. Many of the RBCs are echinocytes. Scale bar = 5 μ m.

respectively; $P \leq .001$ for both comparisons), with the MSM then increasing in the schizont stage. Thus, although echinocytes were more rigid than normocytes in the uninfected and schizont stage, the reverse was true in the ring and trophozoite stages.

Data from the imaging flow cytometer (Amnis) on 3 monkey and 3 human blood samples infected with *P knowlesi* showed that in both primate species, sphericity was increased in iRBCs compared with uRBCs ($P < .0001$ for both comparisons; Figure 4A). Significantly increased sphericity was also observed in human iRBCs compared with monkey iRBCs ($P < .0001$), although there was no difference in sphericity between human uRBCs and monkey uRBCs (Figure 4A). The sphericity of the mature *P knowlesi* iRBCs in humans was also evident on atomic force microscopy images (Figure 4C).

Echinocytosis

A notable finding from the micropipette aspiration experiments was the marked increase in echinocytes in thawed cryopreserved blood from humans with knowlesi malaria (Figure 3) compared with HHCs in whom echinocytes were rarely observed (<1%). To confirm that echinocytosis was not an artifact arising from the cryopreservation-thawing process, wet preparations of fresh whole blood from an additional 31 patients with knowlesi malaria and 22 controls were analyzed after excluding samples in which time from blood collection to the end of blood examination exceeded 90 minutes. Malaria patients had a median parasite count of 2211 (IQR, 552-7648) parasites per μ L. The median proportion of echinocytes in patients with knowlesi malaria was 96% (IQR, 15%-99%) compared with 1% (IQR, 0%-31%) for controls ($P = .0002$). Echinocytes made up >10% of RBCs in 81% of malaria patients (25 of 31) compared with 36% of controls (8 of 22) ($P = .001$). Although there was a greater delay from blood collection to examining the wet preparation in malaria patients compared with controls (73 minutes [IQR, 66-81 minutes] vs 63 minutes [IQR, 59-69 minutes]; $P = .0005$), there was no correlation between time

to examination and the proportion of echinocytes observed. The median proportion of echinocytes also remained higher in malaria patients compared with controls ($P = .0002$) after excluding samples in which time from blood collection to the end of blood examination exceeded 75 minutes. Notably, studies in thawed and fresh blood isolated from *M fascicularis* infected with *P knowlesi* did not show evidence of echinocyte formation.

Discussion

The RBC diameter exceeds the capillary diameter by several micrometers. Therefore, the ability of RBCs to deform as they pass through capillaries is a critical factor in maintaining normal microvascular flow. In this study, we demonstrated that in human adults with knowlesi malaria, deformability of RBCs is reduced in proportion to disease severity. In addition, we demonstrated that in human knowlesi malaria, the reduction in deformability occurs in both iRBCs and uRBCs, with the latter related primarily to the presence of echinocytes. Finally, we showed that in *M fascicularis* (the natural host of *P knowlesi*), echinocytosis does not occur, and the deformability of uRBCs is unaffected. These results suggest that the formation of echinocytes and the consequent reduction in deformability of uRBCs may be a key feature contributing to the pathogenesis of human knowlesi malaria. Our finding of reduced RBC-D in knowlesi malaria is consistent with previous studies involving adults and children with falciparum malaria, in whom RBC-D is also reduced in severe disease and is independently associated with severe anemia, hyperlactatemia, and mortality.^{9,27-29} In this study, no deaths occurred; however, in patients with knowlesi malaria, impairment of RBC-D was associated with increased lactate and with nadir hemoglobin, suggesting that as with falciparum malaria, reduced RBC-D may contribute to anemia and impaired organ perfusion.

Our findings of reduced RBC-D in patients with knowlesi malaria are consistent with early studies in non-natural primates (*M mulatta* [rhesus macaques]). In one such study, Miller et al¹⁵ measured viscosity and filterability of RBC suspensions in *P knowlesi*-infected rhesus macaques, in which infection results in severe disease and death. Viscosity and resistance to flow of infected blood was increased, suggesting reduced RBC deformability. Resistance to flow increased with increasing parasitemia and with parasite maturation. Furthermore, RBCs infected with *P knowlesi* schizonts were excluded from rouleaux, further suggesting rigidity of these cells.¹⁵ In other early simian studies, Knisely et al^{14,30} observed that iRBCs and uRBCs were bound together by an adhesive precipitate that changed blood to thick muck-like sludge that resisted flow through the microvasculature. This sludge resulted in impaction of small vessels, which seemed to underlie coma and death in rhesus macaques.³⁰ Although coma attributable to knowlesi infection has not been definitively reported in the 21st century literature of human knowlesi malaria, accumulation of iRBCs within cerebral vessels was noted in the autopsy report of a single human infected with *P knowlesi*,¹⁰ and it is possible that reduced RBC-D and consequent impaired microvascular flow contributes to organ dysfunction in human knowlesi malaria. In falciparum malaria, additional processes that contribute to reduced microvascular flow include adherence of iRBCs to activated endothelium,^{11,31} uRBCs that form rosettes,³²⁻³⁴ and other iRBCs in platelet-mediated clumps.³⁵ These processes

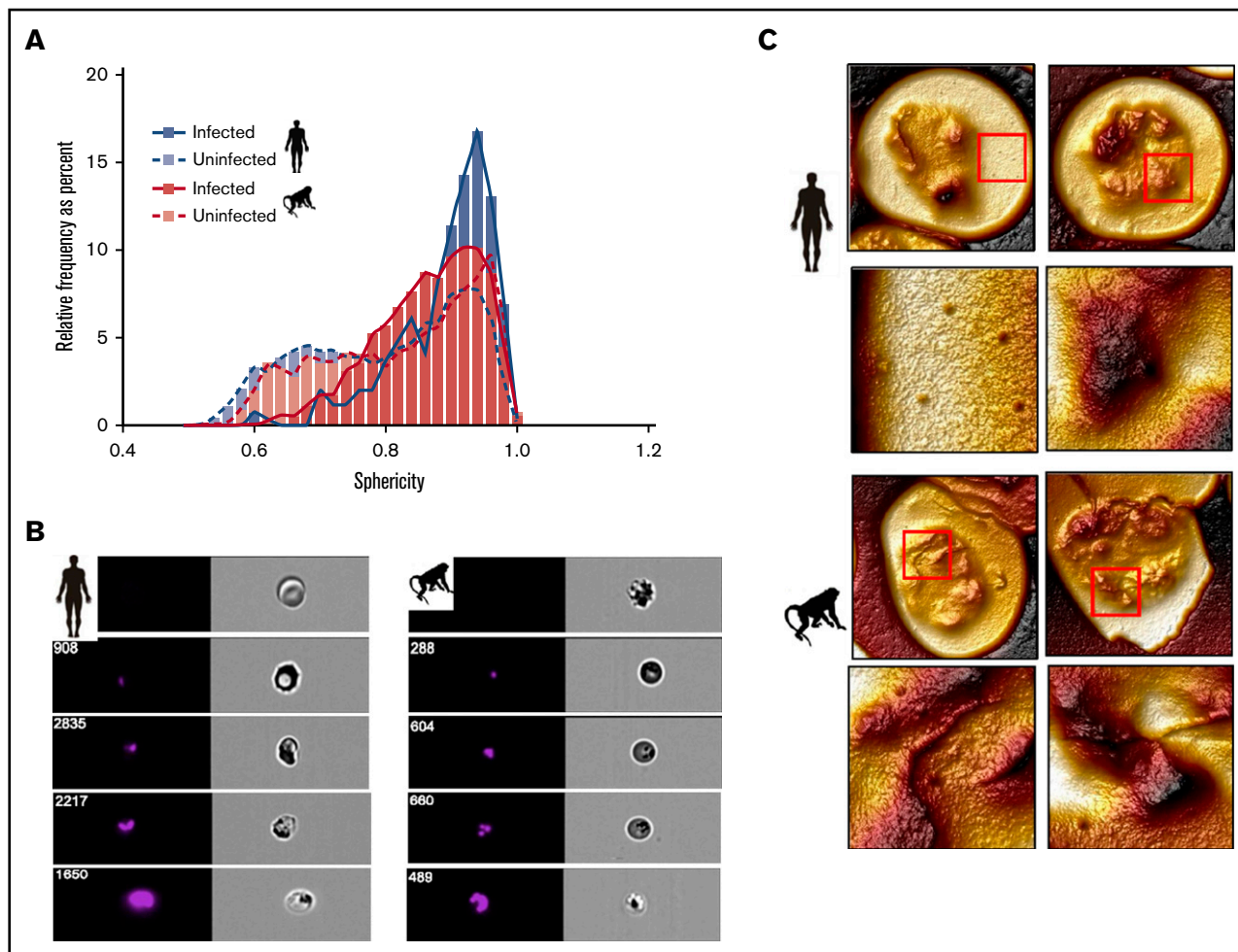


Figure 4. Ultrastructural and geometrical comparison of iRBCs and uRBCs from humans and *M. fascicularis* monkeys with knowlesi malaria. (A) The sphericity (approximate aspect ratio) of iRBCs and uRBCs from 3 humans and 3 *M. fascicularis* monkeys with knowlesi malaria, as determined by single-event flow cytometry (Amnis). In both species, sphericity was increased in iRBCs vs uRBCs. (In 3 humans, the median aspect ratio of iRBCs was 0.91, 0.87, and 0.93, respectively, vs 0.77, 0.84, and 0.84 for uRBCs, respectively; $P < .0001$. In 3 monkeys, the median aspect ratio of iRBCs was 0.87, 0.85, and 0.87, respectively, vs 0.83, 0.81, and 0.84 for uRBCs, respectively; $P < .0001$). Sphericity was increased in human iRBCs compared with monkey iRBCs ($P < .0001$). (B) Examples of images captured from the flow cytometer of *P. knowlesi* iRBCs stained with Hoechst stain, ordered from earliest asexual forms (ring stage) to mature forms (schizonts). (C) Atomic force scans of *P. knowlesi* iRBCs from humans (upper 4 images) and *M. fascicularis* monkeys (lower 4 images) demonstrating increased sphericity of the human iRBCs. In each group of 4 images, the bottom 2 images (side = $2 \mu\text{m}$) represent the magnified area in the image directly above (side = $8 \mu\text{m}$) indicated by a red square, and show caveola pits.

have not been assessed in human or monkey knowlesi malaria and require further investigation.

The LORCA results in this study demonstrate an overall reduction in the deformability of RBCs in knowlesi malaria. With the vast majority of RBCs in knowlesi malaria being uRBCs, this suggests, as in falciparum malaria, reduced deformability of uRBCs as well as iRBCs. The LORCA, however, does not distinguish the deformability of iRBCs and uRBCs or the effects of parasite stage on RBC-D.³⁶ Thus, a strength of our study was the use of micropipette aspiration to demonstrate increased stiffness of both iRBCs and uRBCs in patients with knowlesi malaria. Furthermore, we found that RBC stiffness was influenced by the presence of echinocytosis, which we have shown to be a major feature of human knowlesi malaria. In the case of uRBCs, echinocytes were stiffer than uninfected normocytes and stiffer than RBCs of healthy controls.

This finding is consistent with previous non-malarial micropipette aspiration studies that demonstrate increasing resistance to membrane extension in echinocytes.³⁷ In this study, the echinocyte finding is of particular importance, given that it is the uRBCs in knowlesi malaria that make up the large majority of the total RBC pool. Thus, the reduced deformability of the echinocytic uRBCs is likely a major contributor to the overall reduction in RBC-D demonstrated by using the LORCA.

Interestingly, we found that at the ring and trophozoite stages, the echinocytic cells were less rigid than uninfected echinocytes and less rigid than ring- and trophozoite-infected normocytes. This increase in flexibility of *P. knowlesi* ring-stage echinocytes (compared with *P. knowlesi* uRBCs) is similar to previous findings in *P. vivax*, in which invasion of immature reticulocytes leads to a rapid increase in RBC deformability, which can be partly attributed

to increased RBC surface area.^{38,39} In *P knowlesi*, the reduction in stiffness observed in ring- and trophozoite-infected echinocytes may reflect an ability of the younger and more metabolically active parasites to modulate the echinocytic surface membrane, with this ability lost as the parasite matures to the schizont stage. Similarly in *P vivax*, despite the increased flexibility of the trophozoite RBCs, rigidity of schizont RBCs is markedly increased.⁴⁰

The rigidity of *P knowlesi* schizonts in humans with knowlesi malaria was particularly notable, and is consistent with the early studies in rhesus macaques mentioned above.¹⁵ Importantly, although schizonts account for only a small proportion of circulating RBCs, the proportion of schizonts observed in peripheral blood films is likely to underestimate the total number of schizonts present, probably because of the accumulation of rigid schizonts in the microvasculature. Of note, the percentage of schizonts in the peripheral blood film is an independent risk factor for severe disease in knowlesi malaria.⁵

An important finding from our study was that in *P knowlesi*-infected *M fascicularis* (the natural host of *P knowlesi*), echinocytes were not observed, and the deformability of uRBCs was not significantly affected. In addition, the increase in RBC stiffness associated with late schizont-stage parasites was not as marked in *M fascicularis* as it was in humans. This may represent one mechanism by which *P knowlesi* is better adapted to parasitizing the RBCs of *M fascicularis*, a coadaptive process for at least the last 40 000 years.⁴¹

Our study had limitations. First, most patients were enrolled after receiving antimalarial treatment, and it is possible that drug treatment may have affected RBC-D. However, this effect has primarily been described in iRBCs,⁴² whereas the LORCA predominantly measures deformability of uRBCs. Furthermore, in patients with knowlesi malaria, we found a trend toward increased RBC-D in those measured after treatment compared with those measured before treatment, suggesting that treatment does not explain the reduced RBC-D seen in this study. Second, measurements of RBC sphericity obtained from the imaging flow cytometer did not include uninfected human and monkey controls; thus, we are unable to comment on whether there is an increase in sphericity of uRBCs from infected participants compared with RBCs from uninfected controls.

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In conclusion, this study demonstrated that RBC-D is reduced among patients with knowlesi malaria in proportion to disease severity, with impairment in severe disease at least as great as in severe falciparum malaria. We also demonstrate that echinocytes are prevalent in knowlesi malaria, and that this is associated with increased membrane stiffness of both schizont-infected RBCs and uninfected RBCs. With the reduction in deformability associated with blood lactate, this may contribute to microvascular sludging, microvascular accumulation of iRBCs and uRBCs, and impaired organ perfusion in severe knowlesi malaria in humans.

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Authorship

Contribution: N.M.A., T.W.Y., B.E.B., B.R., T.W., and A.M.D. designed the research; B.E.B., M.J.G., R.Z., A.A., Y.L.L., and B.R. conducted the study; M.D.C. assisted with statistical analysis; and all authors reviewed and approved the final manuscript.

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