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High Levels of *Plasmodium falciparum* Rosetting in All Clinical Forms of Severe Malaria in African Children

Ogobara K. Doumbo, Mahamadou A. Thera, Abdoulaye K. Koné, Ahmed Raza, Louisa J. Tempest, Kirsten E. Lyke, Christopher V. Plowe, and J. Alexandra Rowe*

Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Bamako, Mali; Centre for Immunity, Infection and Evolution, Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, UK; Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, UK; Center for Vaccine Development and Howard Hughes Medical Institute, University of Maryland School of Medicine, Baltimore, Maryland

Abstract. *Plasmodium falciparum* rosetting (the spontaneous binding of infected erythrocytes to uninfected erythrocytes) is a well-recognized parasite virulence factor. However, it is currently unclear whether rosetting is associated with all clinical forms of severe malaria, or only with specific syndromes such as cerebral malaria. We investigated the relationship between rosetting and clinical malaria in 209 Malian children enrolled in a case-control study of severe malaria. Rosetting was significantly higher in parasite isolates from severe malaria cases compared with non-severe hyperparasitemia and uncomplicated malaria controls ($F_{2,117} = 8.15$, $P < 0.001$). Analysis of sub-categories of severe malaria (unrousable coma, severe anemia, non-comatose neurological impairment, repeated seizures or a small heterogeneous group with signs of renal failure or jaundice) showed high levels of rosetting in all sub-categories, and no statistically significant differences in rosetting between sub-categories ($F_{4,67} = 1.28$, $P = 0.28$). Thus rosetting may contribute to the pathogenesis of all severe malaria syndromes in African children, and interventions to disrupt rosetting could be potential adjunctive therapies for all forms of severe malaria in Africa.

INTRODUCTION

The binding of *P. falciparum* infected erythrocytes to uninfected erythrocytes to form clusters of cells called rosettes is a parasite adhesion property that varies between isolates and is associated with malaria severity in numerous sub-Saharan African studies.[1–9] Experimental models suggest that rosetting enhances microvascular obstruction,[10] which is thought to be a key pathological process in severe malaria.[11,12] Genetic epidemiological studies show that human erythrocyte polymorphisms that reduce the ability of *P. falciparum* to form rosettes (such as complement receptor 1 deficiency[13] and blood group O)[4,14] confer protection against severe malaria, reducing the odds ratio for severe disease by about two-thirds.[15,16] Taken together, these data support the hypothesis that rosetting contributes to the pathogenesis of severe malaria and suggest that rosette-disrupting therapies could have clinical benefit.[17]

A number of sulfated glycoconjugate drugs have been identified that disrupt rosettes[18,19] and are plausible candidates for therapy.[20,21] However, before clinical trials of rosette-disrupting therapy can be considered, it is important to know which patients would be appropriate targets for treatment. Currently it is unclear whether all severe malaria patients might benefit from rosette-disrupting adjunctive therapies, or whether rosetting only occurs at high levels in specific sub-categories of severe disease. Previous work on the association between rosetting and severe malaria (Table 1) has tended to focus either on specific syndromes such as cerebral malaria (unrousable coma) or on mixed groups of severe malaria cases incorporating a variety of different clinical syndromes including cerebral malaria, severe anemia, respiratory distress (difficulty breathing), and prostration (inability to sit, or in infants, to breast feed).

To clarify the association of rosetting with different clinical forms of severe disease, we carried out a detailed study of the factors affecting rosetting in a case-control study of severe falciparum malaria in a moderately high malaria transmission area in sub-Saharan Africa, carefully characterizing distinct clinical syndromes of uncomplicated and severe malaria.

MATERIALS AND METHODS

Study site and field isolates. Parasite isolates were collected as part of the Bandiagara Malaria Project case-control study.[22–24] Bandiagara, Mali, is an area with intense seasonal transmission of *P. falciparum* (up to 20–60 infected bites per person per month at the peak of the July–December transmission season).[25] Community permission and individual written informed consent from the patients' parents or guardians were obtained as described by Diallo et al.[26] Protocols were approved by the institutional review boards of the University of Mali (now University of Bamako) Faculty of Medicine and the University of Maryland. Blood samples were collected from children with malaria as described,[22–24] and severe malaria was managed following standardized protocols to initiate rapid treatment and prevent and treat complications. Severe malaria syndromes were defined following World Health Organization criteria,[27] although patients with hyperparasitemia (> 500,000 parasites per microlitre of blood) and no other symptoms or signs of severe disease were analyzed as a separate group. Previous studies indicate negligible mortality for children with non-severe hyperparasitemia,[22,28] and this category can therefore be considered as a form of uncomplicated malaria with particularly high parasite densities. Uncomplicated malaria cases were children with *P. falciparum* infection and fever but with no symptoms or signs of severe malaria and no hyperparasitemia. Sub-categories of severe malaria were cerebral malaria (unrousable coma with a Blantyre Coma Score (BCS) of ≤ 2, with other obvious causes of coma excluded), severe anemia (Hb < 5 g/dL), neurological impairment (impaired consciousness or prostration but with a BCS of > 2), repeated seizures but no lasting neurological impairment, and a small

* Address correspondence to J. Alexandra Rowe, Centre for Immunity, Infection and Evolution, Institute of Immunology and Infection Research, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT, UK. E-mail: Alex.Rowe@ed.ac.uk

Table 1
Rosetting and severe malaria in sub-Saharan Africa

Site and reference	Mean or median rosette frequency* (range) *Number of isolates* Cerebral malaria†	Severe malaria‡	Uncomplicated malaria§	*P* value
The Gambia[1]	35 (6–85) *24*	ND¶	17 (0–71) *57*	< 0.001
The Gambia[2]	28.3 (0.5–70) *24*	ND	8.5 (0–55) *106*	< 0.000001
Madagascar[3]	19.5 (5–28) *6*	30.5 (20–43) *6*	5 (0–19) *9*	< 0.05 (CM vs. UM), < 0.002 (SM vs. UM)
Kenya[4]	6 (0–94) *21*	7 (0–97) *15*	1 (0–82) *54*	< 0.003 (SM and CM vs. UM)
Kenya[5]	5.0 (0–37) *45*	9.5‖ (0–46) *49*	4.5 (0–26) *50*	< 0.05 (SM vs. UM) NS# (CM vs. UM)
Gabon[6]	ND	16 *47*	8 *47*	< 0.05
Malawi[32]	13.9 (0–57) *46*	16.4‖ (1–49) *18*	15.0 (0–61) *62*	NS
Kenya[7]	ND	6.5** (2.0–34.9)†† *57*	4.1 (0.9–10.8)†† *64*	0.02/0.054‡‡
Kenya[8]	17.2 *11*	29.7‖ *21*	12.9 *45*	0.001 (SM vs. UM), NS (CM vs. UM)
Kenya[9]	ND	14.0** (4–32)†† *25*	3.5 (1–10)†† *49*	< 0.001

* Rosette frequency = percentage of mature-infected erythrocytes binding two or more uninfected erythrocytes.
† CM, cerebral malaria is defined as unrousable coma (Blantyre coma score of two or less).
‡ SM, severe malaria includes a variety of syndromes such as severe malarial anemia (Hb < 5 g/dL), respiratory distress, prostration, and hypoglycemia.
§ UM, uncomplicated malaria is defined as acute falciparum malaria with no complications of severe or cerebral disease.
¶ ND = not determined.
‖ Severe malarial anemia only.
NS = not significant.
** Includes cerebral malaria cases.
†† Interquartile range.
‡‡ Mann Whitney *U* test/logistic regression.

heterogeneous group with no neurological abnormalities or anemia but with evidence of renal failure (anuria or hematuria) or jaundice. Cerebral malaria and severe anemia were taken as primary defining criteria when they co-existed with other criteria, as described previously.[22] Respiratory distress did occur, but always co-existed with other severe criteria such as coma, anemia, or neurological impairment, therefore it is not shown as a separate category.

Parasite culture. Blood samples were depleted of lymphocytes via density centrifugation, suspended in Glycerolyte, and frozen to –70°C. Frozen samples were shipped to Edinburgh where they were thawed by standard methods. Briefly, the isolates were diluted in a gradient of salt solutions and washed in RPMI 1640 medium containing 2 mM glutamine, 25 mM Hepes, 20 mM glucose, and 25 μg/mL gentamicin (incomplete RPMI) before culturing in complete RPMI (incomplete RPMI supplemented with 10% human AB serum) at 2% hematocrit. The parasites were incubated at 37°C in 3% CO_2, 1% O_2, 96% N_2. Cultures were monitored by Giemsa-stained thin smears for 18–36 hours, and only those with normal morphology that matured to the pigmented-trophozoite stage were included in the study. Two hundred and seventy-two out of a possible 378 *P. falciparum*-infected samples collected as part of the Bandiagara case-control study[22] were put into culture, the remainder having been lost in a freezer breakdown. The rosette frequency of 209 of these isolates was assessed, the remainder being excluded either because the parasites failed to mature beyond ring stage *in vitro* or because the parasitemia was too low for reliable assessment (< 0.5%).

Rosetting assays. The rosetting of each isolate was assessed in the first cycle of *in vitro* growth when the majority of the parasites had reached the pigmented-trophozoite stage. A 100 μL aliquot of culture suspension was stained with 25 μg/mL of ethidium bromide for 5 minutes. A wet preparation was made by placing a 10 μL drop of culture suspension (2% hematocrit) on a microscope slide and covering it with a 22 × 22 mm coverslip. Wet preparation slides were blinded so that the microscopist did not know the clinical category of each sample. The wet preparation was viewed with a fluorescence microscope (40× objective) using both white light and fluorescence simultaneously to visualize both infected and uninfected erythrocytes. Mature-infected erythrocytes were counted and assessed for rosetting, with a rosette being defined as an infected erythrocyte binding two or more uninfected erythrocytes. The rosette frequency is the percentage of infected erythrocytes in rosettes out of 200 infected erythrocytes counted.

Platelet-mediated clumping assays. Platelet-mediated clumping was assessed when the parasites reached the mature pigmented-trophozoite stage as described previously.[29] Briefly, parasite cultures were suspended at 2% hematocrit in 10% platelet-rich plasma from an AB+ malaria-naive donor (to avoid ABO compatibility problems) in incomplete RPMI medium (final concentration 1×10^7 platelets per mL). Twenty-five μg/mL of ethidium bromide was added and the mixture was gently rotated for 30 minutes at room temperature. A wet preparation was viewed on a fluorescence microscope and 500 infected red cells were counted and scored for clumping, with three or more infected erythrocytes adherent to each other constituting a clump. The clumping frequency is the percentage of infected erythrocytes in clumps out of 500 infected erythrocytes counted.

Statistical analysis. The original matched design of the Bandiagara case-control study[22] was not used in the rosette

frequency analysis because technical problems (see parasite culture) meant that it was not possible to obtain rosette frequency data from all samples. The χ^2 test was calculated using a 3×2 contingency table at http://www.physics.csbsju.edu/cgi-bin/stats/contingency.html. Univariate analysis was carried out using Statview (version 5, SAS Institute, Inc., Cary, NC), and multivariate analysis was carried out using the statistical package S-PLUS 6.0 (Release 1, Insightful Corp., Palo Alto, CA), using Generalized Linear Models (GLM). Because the response variables were proportions, and therefore bound between 0 and 1, they were analyzed using binomial errors with a logit linear predictor.[30,31] The rosette frequency percentages were analyzed as counts with binomial errors. Explanatory variables in the statistical model included category of disease (severe malaria, non-severe hyperparasitemia, and uncomplicated malaria, as defined above), patient parasitemia on admission to hospital (% of erythrocytes parasitized), parasite platelet-mediated clumping frequency (%), patient ABO blood group, age, and hemoglobin level. The analyses were repeated using only cases in the severe disease category. In these analyses, rosetting was examined between subcategories of severe disease (which includes coma, neurological impairment, seizures, anemia, and other), and sub-category replaced category of disease as an explanatory term in the model. Models were fitted by initially including all explanatory terms and allowing interactions up to second order. Interactions including more than two terms were not permitted due to small sample sizes. The statistical significance of a term in a GLM with binomial errors was assessed by the change in deviance of the model when the term was dropped from it. To correct for overdispersion, significance of a term was assessed using an F ratio. Minimal models were obtained by step-wise deletion of non-significant terms, using an α-value of 0.05. Data on the relationship between rosetting and ABO blood group using this dataset have been reported previously,[16] and rosetting in relation to invasion properties of a subset of the samples has also been described.[24] However, the analysis presented here on the whole dataset and the relationship between rosetting and sub-categories of severe malaria has not previously been reported.

RESULTS

Rosetting was assessed in the first cycle of *in vitro* growth in 209 *P. falciparum* isolates from Malian children (91 from children with uncomplicated malaria, 40 from children with non-severe hyperparasitemia, and 78 from children with severe malaria). The characteristics of the patients from which the parasite isolates were derived are summarized in Table 2. The children in the three disease categories did not differ significantly in age. The children with severe malaria had lower hemoglobin levels than the other two groups, as expected because severe anemia is a criterion for severe disease. The highest mean parasitemia was seen in children with non-severe hyperparasitemia and the lowest in children with uncomplicated malaria (Table 2), which is also as expected, because parasitemia was a defining feature of these categories.

Rosetting was most common in parasite isolates from severe malaria patients (severe malaria, rosettes in 70/78 (90%), non-severe hyperparasitemia 31/40 (77%), and uncomplicated malaria 50/91 (55%) isolates, $\chi^2 = 26.0$, two degrees of freedom, $P < 0.0001$). Furthermore, examination of the distribution of rosette frequencies within each disease category showed that the median rosette frequency in the severe disease category (median 20%, interquartile range (IQR) 10–40%) was significantly higher than in the two non-severe categories (hyperparasitemia: median 8.5%, IQR 2–20%; uncomplicated malaria: median 1%, IQR 0–12%, Kruskal-Wallis test $P < 0.0001$, Figure 1).

TABLE 2
Summary of patient characteristics

Disease category	N	Age* (months)	Hb* (g/dL)	Pt*† (%)
Uncomplicated malaria	91	42.9 (27.3)	9.8 (1.7)	2.0 (1.8)
Non-severe hyperparasitemia	40	47.2 (27.8)	10.2 (1.5)	12.6 (6.5)
Severe malaria	78	38.5 (25.3)	8.0 (2.5)	7.0 (6.5)
P value‡		0.232	< 0.0001	< 0.0001

* Mean (standard deviation).
† Pt = parasitemia.
‡ ANOVA.

Our previous work on platelet-mediated clumping (a parasite adhesion phenotype whose relationship with disease severity remains controversial) showed that univariate analysis can be misleading, and that apparent associations with disease category can arise if confounding factors such as parasitemia differ between disease categories.[29] To determine if other variables influence the association between rosetting and severe malaria, we performed multivariate analysis of factors that affect rosetting, including the disease category (severe, non-severe hyperparasitemia, and uncomplicated), patient's admission parasitemia, patient age, hemoglobin level, and ABO blood group type. The platelet-mediated clumping frequencies of these clinical isolates had been measured previously,[29] therefore parasite clumping frequency was also included in the multivariate analysis, to investigate the relationship between clumping and rosetting. Multivariate analysis suggested that disease category explained most of the deviance in rosetting, with highly significant differences in rosetting demonstrated between categories ($F_{2,117} = 8.15$, $P < 0.001$). The other variable that had a major effect on rosetting was ABO blood

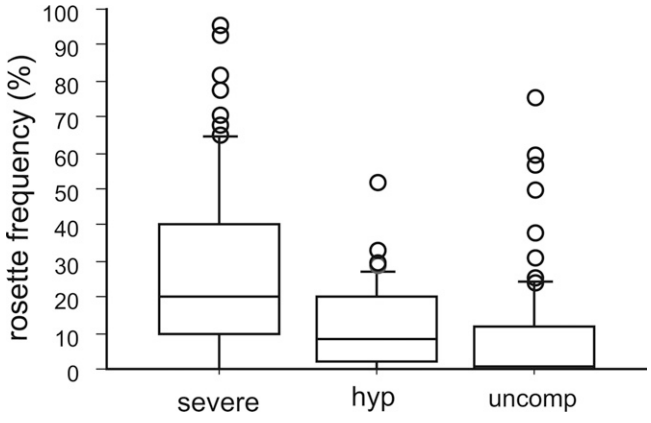

FIGURE 1. Distribution of *P. falciparum* field isolate rosette frequencies in relation to malaria severity. Box plots showing significantly higher rosette frequencies in parasite isolates collected from children with severe malaria (severe, $N = 78$) compared with isolates from children with non-severe hyperparasitemia (hyp, $N = 40$) or uncomplicated malaria (uncomp, $N = 91$), Kruskal-Wallis test, $P < 0.0001$. Boxes indicate the median (central line) and the interquartile range. The error bar indicates the 90th percentile, and points beyond the 90th percentile are shown as circles.

group ($F_{3,117} = 3.54$, $P = 0.017$), with lower rosetting in isolates from group O patients compared with those from groups A, AB, and B as reported previously.[16] There was also an interaction between blood group and hemoglobin, such that in blood group A and AB, rosetting frequency was negatively associated with hemoglobin level, whereas this negative association was not apparent in blood groups B and O ($F_{3,117} = 4.88$, $P = 0.003$). There was also evidence for a negative association between rosetting and platelet-mediated clumping ($F_{1,117} = 5.09$, $P = 0.026$), supporting the clear distinction between these two phenotypes.[29] Patient age and percentage parasitemia on admission were non-significant terms in the model. In summary, this multivariate analysis shows that disease category is the major factor affecting rosetting, and indicates that the relationship between rosetting and severe disease is not due to confounding factors such as host age or parasitemia.

To address whether rosetting is associated with distinct clinical forms of severe malaria, rosette frequencies in the subcategories of severe disease were examined. Within the severe malaria category there were 26 children with unrousable coma (Blantyre coma score ≤ 2), 23 non-comatose children with impaired consciousness or prostration, 15 children with repeated seizures, eight children with severe malarial anemia (Hb < 5 g/dL) and six children with assorted other symptoms and signs suggestive of renal failure (anuria, hematuria) or jaundice. High levels of rosetting were noted in each subcategory (Figure 2), and univariate analysis demonstrated no significant differences between sub-categories (Kruskal-Wallis test $P = 0.63$). There were five isolates from children who had

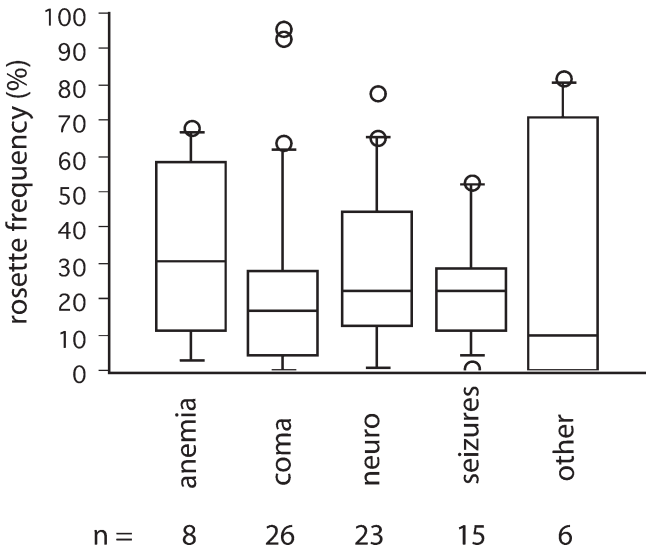

FIGURE 2. Distribution of *P. falciparum* field isolate rosette frequencies in relation to severe malaria sub-categories. Box plots (as in Figure 1) showing no significant difference in rosette frequencies between various severe malaria sub-categories including severe malarial anemia (anemia, median 30.5%, interquartile range (IQR) 11–58%), cerebral malaria (coma, median 16.5%, IQR 4–28%), non-comatose neurological impairment (neuro, median 22.0%, IQR 13–45%), repeated seizures (seizures, median 22.0%, IQR 11–29%), and renal failure/jaundice (other, median 9.5%, IQR 0–71%), Kruskal-Wallis test, $P = 0.63$. A full definition of each sub-category is given in the methods. The number of isolates (N) in each subcategory is shown. For comparison, the non-severe hyperparasitemia patients had a median rosette frequency of 8.5%, IQR 2–20%, and the uncomplicated malaria patients had a median rosette frequency of 1%, IQR 0–12%.

both cerebral malaria and severe malarial anemia, and these isolates were included in the cerebral malaria category for analysis (Figure 2). However, inclusion of these five isolates in the severe anemia category (giving a median rosette frequency 25%), or analyzing them as a separate group (median rosette frequency 19%) did not materially alter the results shown here. The cerebral malaria group had a median rosette frequency of 16% if the five isolates with concurrent severe anemia were removed. Of the eight children in the severe anemia category, two also had respiratory distress and one had hematuria. Nine children from the severe group died (11.5% mortality), with the highest mortality rate occurring in children with coma plus severe anemia (3/5, 60%). The median rosette frequency of the severe malaria patients who died (20%, IQR 9.5–46.8%) was not significantly different from the median rosette frequency of the severe malaria patients who survived (20%, IQR 10.0–37.8).

The multivariate analysis was repeated using the smaller dataset containing only the severe malaria cases. As described in the full dataset, patient age and percentage parasitemia on admission were non-significant terms in the model, whereas ABO blood group and platelet-mediated clumping were significant factors. Importantly however, the multivariate analysis confirmed that there were no significant differences in rosette frequency between the sub-categories of severe disease ($F_{4,67} = 1.28$, $P = 0.28$).

DISCUSSION

This study confirms that high levels of rosetting are associated with severe malaria and reveals that the association between rosetting and severe malaria remains highly statistically significant after multivariate analysis that allows other factors such as patient parasitemia, hemoglobin (Hb) level, and ABO blood group to be taken into consideration. In addition, this study demonstrates, for the first time, that subcategories of severe malaria all show high levels of rosetting, with no statistically significant difference in rosette frequencies between sub-categories (Figure 2).

Early studies on rosetting and severe malaria focused on patients with strictly defined cerebral malaria and showed markedly higher rosette frequencies in *P. falciparum* isolates from comatose patients compared with isolates from patients with uncomplicated malaria in West Africa (Table 1).[1,2] Studies in East Africa showed an association between rosetting and cerebral malaria in some,[3,4] but not all studies.[5,8,32] Furthermore, an association between rosetting and disease severity in mixed groups of severe malaria patients[3,6,7,9] or patients with severe malarial anemia[5,8] was noted. Considerable uncertainty remains regarding whether rosetting is associated with all clinical forms of severe malaria or only some specific syndromes. The current study aimed to clarify the relationship between rosetting and sub-categories of severe disease. We found high levels of rosetting in all sub-categories of severe malaria in Mali, including cerebral malaria (coma), severe malarial anemia, non-comatose neurological impairment (including prostration), repeated seizures without long-lasting neurological impairment, and in a mixed group of patients with symptoms or signs of renal failure or jaundice (Figure 2).

Despite the fact that this is one of the largest studies of rosetting and severe malaria carried out to date, the sample size is still small in each sub-category of severe disease. This is

particularly true for the heterogeneous renal failure/jaundice sub-category, as these clinical features are rare in African children with severe malaria.[28] Severe anemia is also relatively uncommon in this study, due to the age distribution and epidemiology of severe disease in the study area.[33] Neurological abnormalities are frequently seen in children with severe malaria, and all manifestations (unrousable coma, non-comatose impaired consciousness, and repeated seizures) were associated with high rosetting levels (Figure 2). Ideally, larger studies should be carried out to confirm these results and to clarify the associations between rosetting and severe malaria syndromes under different transmission intensities. However, studies of rosetting and malaria severity are likely to remain problematic due to the logistical difficulties of collecting and culturing large numbers of *P. falciparum* isolates from severely ill children. Parasite isolates collected from patients have to be cultured *in vitro* for 12–36 hours before assessment of rosetting to allow development of ring-stage parasites (the only form found in peripheral blood) to the pigmented-trophozoite stage at which rosetting occurs. This process is time-consuming and requires facilities for cell culture and microscopy that limit the sites at which studies can be undertaken.

Despite the logistic difficulties and small sample sizes affecting many rosetting studies, a clear pattern emerges across sub-Saharan Africa. Children with uncomplicated malaria are infected with parasite isolates that form few rosettes (Table 1 and Figure 1). In contrast, most (although not all) children with severe malaria are infected with parasite isolates showing high rosetting levels. In the current study, more than half of the severe malaria cases had parasite isolates with rosette frequencies of 20% or higher. The rosette frequency threshold at which pathogenic effects occur *in vivo* is unknown, and could be influenced by numerous factors such as the patient's overall parasite burden, and the size and strength of the rosettes.[34,35]

Although the association between rosetting and severe malaria in sub-Saharan Africa is well-established, direct evidence that rosetting plays a causal role in malaria pathogenesis in humans is lacking. This is because experiments addressing the role of rosetting in pathogenesis cannot be performed in humans for ethical reasons, and there is no animal model that fully mimics the pathological and clinical features of falciparum malaria. There is, however, strong indirect evidence from human genetic epidemiological studies that supports a pathogenic role for rosetting. Human erythrocyte polymorphisms of rosetting receptors that reduce the ability of *P. falciparum* to form rosettes, such as blood group O[4,14] and complement receptor 1 deficiency,[13] confer protection against severe malaria.[15,16,36,37] These polymorphisms have a specific effect on rosetting and do not influence total parasite burdens,[15,16] therefore their protective effect is compelling evidence that rosetting plays a causal role in pathogenesis. In addition, a plausible mechanism for a pathogenic effect of rosetting has been demonstrated. In an *ex vivo* microvasculature model, rosetting parasites cause significantly greater obstruction to flow in small blood vessels that cytoadherent non-rosetting parasites.[10] In this model, rosettes were disrupted by high shear forces in the arterial side of the circulation, but in capillaries and post-capillary venules, rosetting parasitized erythrocytes bound to endothelial cells and uninfected erythrocytes simultaneously to occlude vessels and impair blood flow. Impairment of microvascular blood flow leading to hypoxia, ischemia, and metabolic disturbances is thought to be the fundamental cause of tissue damage and death in severe malaria.[11,12] Taking all the above data together, current evidence supports a direct role for rosetting in the pathogenesis of severe malaria.

One unexpected result that emerged from the multivariate analysis of rosetting was an interaction between ABO blood group and hemoglobin. Rosette frequency was negatively associated with hemoglobin level in isolates from patients with blood groups A and AB, whereas this negative association was not apparent in blood groups B and O ($F_{3,117} = 4.88$, $P = 0.003$). Further investigation will be required to determine if this is a reproducible finding and to examine its biological significance. Previous work has shown that rosetting parasites show a preference for erythrocytes bearing either A or B blood group antigens, and form larger, stronger rosettes with cells of the preferred type compared with group O cells.[14,38] The preference for the A antigen is particularly common,[39] and direct binding of the parasite rosetting ligand PfEMP1 to the A antigen has been demonstrated.[40] The mechanism through which binding to A antigen (and rosetting in general) might lead to lower hemoglobin levels is unclear. One possibility is that parasite-induced damage of the uninfected erythrocytes in rosettes could occur, including the formation of oxidative products such as 4-hydroxynonenal,[41] that could lead to the phagocytic clearance of uninfected cells and so contribute to anemia. If rosettes in group A patients are larger (i.e., contain more uninfected erythrocytes per rosette than in B and O patients) this could lead to greater clearance of uninfected erythrocytes and account for the relationship with hemoglobin level seen here.

Recent research has identified compounds that reverse rosetting *in vitro* and may have potential as adjunctive therapies for severe malaria.[20,21] The mortality rate of severe malaria is as high as 15–20%, even in patients who reach hospital and are treated with effective antimalarial drugs. Approximately 85% of severe malaria-related deaths in hospital occur in the first 24 hours after admission, before the parasite-killing effects of antimalarial drugs have time to act.[28] Therefore adjunctive therapies for severe malaria that target the underlying disease process are urgently needed.[17] Rosette-disrupting therapies that relieve or prevent microvascular obstruction have potential to ameliorate the symptoms of severe malaria. Heparin has been shown to reverse rosetting in a subset (one-third to one-half) of rosetting isolates,[18,19,42] and a heparin derivative reverses sequestration in an animal model.[20] Curdlan sulfate, a glycoconjugate drug that was initially developed as a possible AIDS therapy,[43] was shown to be an effective rosette-reversing agent against a range of clinical isolates[21] and is another potential candidate for severe malaria adjunctive therapy. Curdlan sulfate was shown to be safe for use in Thai adult patients with severe malaria,[44] however, rosetting is not associated with severe disease in this region (possibly due to differing pathogenic mechanisms related to low levels of malaria transmission and immunity, reviewed in reference[17]). Curdlan sulfate has not yet been tested for its effectiveness as an adjunctive therapy in the most appropriate patient population, i.e., children in sub-Saharan Africa. We have demonstrated that all clinical forms of severe malaria are associated with high levels of rosetting in a sub-Saharan African study, which suggests that all severe malaria syndromes in this region might benefit from rosette-disrupting therapies. Clinical trials in well-defined patient populations in parallel with further studies

on parasite rosetting properties will be required to determine whether rosette-reversing therapies can reduce the high mortality rate of severe malaria.

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Authors' addresses: Ogobara K. Doumbo, Mahamadou A. Thera, and Abdoulaye K. Koné, Malaria Research and Training Center, Département d'Epidémiologie des Affections Parasitaires, Faculté de Médecine, de Pharmacie et d'Odonto-Stomatologie, Bamako, Mali, E-mails: okd@MRTCBKO.org, mthera@MRTCBKO.org, and fankone@icermali.org. Ahmed Raza and J. Alexandra Rowe, Centre for Immunity, Infection and Evolution, Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, UK, E-mails: Ahmed.Raza@ed.ac.uk and Alex.Rowe@ed.ac.uk. Louisa J. Tempest, Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK, E-mail: Louisa.Tempest@ed.ac.uk. Kirsten E. Lyke and Christopher V. Plowe, Malaria Section, Center for Vaccine Development/Howard Hughes Medical Institute, University of Maryland School of Medicine, Baltimore, MD, E-mails: klyke@medicine.umaryland.edu and cplowe@medicine.umaryland.edu.

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