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Prognostic Indicators of Life-Threatening Malaria are Associated with Distinct Parasite Variant Antigen Profiles

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Abstract

PfEMP1 is a family of cytoadhesive surface antigens expressed on erythrocytes infected with *Plasmodium falciparum*, the parasite that causes the most severe form of malaria. These surface antigens play a role in immune evasion and are thought to contribute to the pathogenesis of the malaria parasite. Previous studies have suggested a role for a specific subset of PfEMP1 called “group A” in severe malaria. To explore the role of group A PfEMP1 in disease, we measured the expression of the “*var*” genes that encode them in parasites from clinical isolates collected from children suffering from malaria. We also looked at the ability of these clinical isolates to induce rosetting of erythrocytes, which indicates a cytoadhesion phenotype that is thought to be important in pathogenesis. These two sets of data were correlated with the presence of two life-threatening manifestations of severe malaria in the children: impaired consciousness and respiratory distress. Using regression analysis, we show that marked rosetting was associated with respiratory distress, whereas elevated expression of group A-like *var* genes without elevated rosetting was associated with impaired consciousness. The results suggest that manifestations of malarial disease may reflect the distribution of cytoadhesion phenotypes expressed by the infecting parasite population.

INTRODUCTION

Severe malaria caused by *Plasmodium falciparum* in African children encompasses a wide clinical spectrum. The clinical symptoms can be grouped into three frequently overlapping syndromes suggesting multiple pathophysiological mechanisms: impaired consciousness, severe malarial anemia and respiratory distress. Of these, impaired consciousness and respiratory distress are the major prognostic indicators for death of African children in a hospital setting (1). Determinants of the course of disease are still poorly understood, partly because the clinical manifestations of malaria can have multiple underlying causes (2).

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Finding clear pathophysiological correlates of specific disease manifestations may be important for the development of effective anti-malarial interventions (3).

The feasibility of developing interventions against severe malaria is supported by the fact that children acquire natural immunity to severe disease relatively rapidly in comparison to immunity to mild disease and asymptomatic infection. In addition, different manifestations of severe malaria have distinct age patterns. Severe malarial anemia and malaria with respiratory distress tend to occur in younger children than malaria with impaired consciousness (2, 4). One explanation for this finding is that the molecular targets of immunity to each of these syndromes are distinct.

Clinical isolates of parasites obtained from individuals with malaria adhere to a wide range of host molecules *in vitro* (5-7). These are expressed on various cell types including capillary endothelial cells, erythrocytes and platelets [reviewed in (8)]. Differences in the cytoadhesion phenotypes of parasite-infected erythrocytes (IE) and the resulting variation in the pattern of infected erythrocyte sequestration in different host tissues are believed to reflect the multiple approaches used by the parasite to avoid clearance by the host's spleen (9, 10). Adhesion to endothelial cells in tissue capillaries provides a direct method of sequestration, whereas adhesion to uninfected erythrocytes (a phenotype called "rosetting") has been suggested to lead to sequestration through mechanical obstruction of the capillaries (11, 12). Cytoadhesion is believed to play a role in the pathogenesis of malaria (5-7, 11, 13, 14), but the role of specific parasite ligands as targets of immunity and their correlation to the major clinical syndromes of life-threatening malaria still needs to be established.

The large family of multi-domain proteins called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates several cytoadhesion properties of parasite-infected erythrocytes. PfEMP1 is encoded by about 60 *var* genes per parasite genome and exported to the infected erythrocyte surface (15). They appear to be important targets of naturally acquired immunity to malaria (16-19). Transcriptional switching between *var* genes results in expression of PfEMP1 variants with distinct antigenic and cytoadhesion properties (20, 21). PfEMP1 has a modular structure, and each variant contains different combinations of cytoadherent domains that can re-assort through recombination (15, 22). Despite this diversity, there is evidence for genetic structuring of *var* genes within the repertoire carried by each parasite genome. Group A PfEMP1 are a subset of variants shown to form a genetically distinct subgroup within the genomic PfEMP1 repertoire (22, 23).

Several studies have presented evidence that severe malaria is associated with only specific subsets of the vast diversity of PfEMP1 variants that are present within the parasite population (19, 24-27). Previously, we classified and counted 14,516 expressed *var* gene sequence tags from 217 clinical isolates from Kenyan children. These tags were sampled from within the DBL α domain of the molecules. We showed that expression of PfEMP1 carrying two cysteine residues ("cys2") within the tag region was associated with the severe syndromes of impaired consciousness and severe malarial anemia, though not respiratory distress (19). Further classification of the sequence tags suggested that the association with impaired consciousness was strongest in a subset of cys2 sequences related to known group A PfEMP1 (henceforth termed "group A-like" *var* genes) (19, 28).

To help differentiate parasites obtained from children with different clinical syndromes due to severe malaria, we use existing *var* gene expression data (19) together with rosetting (spontaneous binding of infected erythrocytes to un-infected erythrocytes), a cytoadhesion phenotype that has previously been associated with disease severity (5, 6, 29-32). Rosetting can be mediated by the DBL α domain of PfEMP1 (29). Thus, the sequence tags used in the expressed sequence tag analysis of *var* gene expression (29, 33) were sampled from the

same domain thought to mediate rosetting. Furthermore, various studies suggest an association between group A-like PfEMP1 and the rosetting phenotype in clinical parasite isolates, raising the possibility that it is the rosetting phenotype that drives the association between group A *var* expression and both low host immunity and severe disease (26, 27, 34). Here, by comparing *var* gene expression patterns in 131 clinical isolates with different rosetting frequencies (table S1), we sought to test this possibility.

RESULTS

Rosetting and group A-like *var* expression are differentially associated with malaria syndromes

Consistent with previous studies group A-like *var* expression and rosetting showed a significant overall correlation (Spearman's $\rho = 0.45$, $P < 0.0001$) (26-29, 34) (Fig. 1A and fig. S2). In addition, rosetting was predictive of severe malaria when used as the only explanatory variable in a logistic regression model [odds ratio (OR), 3.7; 95% confidence interval (CI), 1.04 to 13.45; $P = 0.04$, age-adjusted]. However, this positive association dropped out [OR, 1.8; 95% CI, 0.47 to 6.97; $P = 0.4$, age-adjusted] and an accompanying improvement to the model fit was observed [Likelihood ratio (LR) $\chi^2 = 12.04$; $P = 0.0005$] when group A-like *var* expression levels were added to the model. This suggests that the association between group A-like *var* gene expression and severe malaria cannot be explained by the rosetting phenotype.

One possible explanation is that the rosetting phenotype merely acts as a marker for group A-like *var* gene expression but has no independent association with severe malaria. To test this, we examined whether group A-like *var* expression and rosetting exhibit different patterns of association with respiratory distress and impaired consciousness (Table 1, A and B, respectively). Logistic regression was used to predict each syndrome in parasites from 131 patients using rosetting and group A-like *var* expression as explanatory variables while allowing for the age of the patients. First, rosetting and group A-like *var* expression were tested in turn as the only explanatory variables predicting either syndrome (Table 1, models 1, A and B and 2, A and B, respectively). Then, to test the independence of the observed associations, the analysis was repeated with both rosetting and group A-like *var* expression used together in a model predicting respiratory distress (Table 1, model 4A) and impaired consciousness (Table 1, model 4B), respectively.

Despite no evidence for a specific association between group A-like *var* expression and respiratory distress (19) (Table 1, models 2A and 4A) that would distinguish this manifestation of disease, there was evidence for a positive association between rosetting and respiratory distress (Table 1, models 1A and 4A). Conversely, despite the association between group A-like *var* expression and impaired consciousness described previously (19), there was no evidence for an association between rosetting and impaired consciousness (Table 1, models 1B and 4B). Using cerebral malaria (severely impaired consciousness, Blantyre coma score < 3) rather than any level of impaired consciousness as the dependent variable did not alter these relationships (Table 2). Thus, group A-like *var* expression and the rosetting phenotype appear to be independent in relation to their associations with manifestation of disease.

These distinct associations were observed despite the overall correlation between rosetting and group A-like *var* expression (Fig. 1A). One explanation may be that, because of the ability of *var* genes on nonhomologous chromosomes to recombine, a subset of group A-like *var* genes exists that is associated with both rosetting and respiratory distress but not impaired consciousness. To test the idea that distinct subsets of *var* genes may explain the relationship between rosetting and respiratory distress, we adjusted the association between

rosetting and respiratory distress (Table 1, model 1A) for expression of various previously defined subsets of *var* tags that show varying degrees of association with rosetting (fig. S2). None of the *cys2* *var* subsets showed evidence of being able to explain the relationship between rosetting and respiratory distress through an observed reduction in OR (Fig. 1B). Two additional *var* tag subsets were considered, both of which exhibited an association with rosetting both in the present and in previous studies (fig. S2). One was of the “H3 subset” defined as a rosetting subset by Normark *et al.* (27). The other was the “block-sharing group 1-cys/PoLV group 6” (BS1-CP6) subset previously reported to be associated with rosetting in isolates from Kilifi (28) (fig. S2). The H3 subset showed no evidence for being able to explain the association between rosetting and respiratory distress (Fig. 1B). The only subset that showed any suggestion of meeting these criteria was the BS1-CP6 subset. Although rarely expressed overall, this subset reduced the OR more than any other (Fig. 1B) and exhibited no evidence for an association with impaired consciousness (Fig. 1C).

As well as being compatible with recombination between *var* genes, the association between rosetting and respiratory distress is also compatible with heterogeneity of the rosetting phenotype and evidence that this phenotype can be encoded by non-group A *var* genes (22, 28, 35).

To explore further the contrasting relationships with severe malaria, we used two biochemical features that frequently occur in children with respiratory distress: metabolic acidosis (36) and hypoglycemia (37). Respiratory distress, and more specifically, deep breathing, considered here, is a clinical manifestation of metabolic acidosis (36), and children presenting with metabolic acidosis also tend to be those at most risk for hypoglycemia (37). As shown in Table 3, no association was evident between hypoglycemia (blood glucose levels used as the dependent variable, available for 78 children) and either rosetting ($B = -3.1$; 95% CI, -7.01 to 0.73 ; $P = 0.1$, adjusted for group A-like *var* expression levels and age) or group A-like *var* expression levels ($B = 0.8$; 95% CI, -1.51 to 3.09 ; $P = 0.5$, adjusted for rosetting and age). However, metabolic acidosis (base excess levels used as the dependent variable, available for 62 children) was independently associated with rosetting ($B = -9.4$; 95% CI, -16.87 to -1.83 ; $P = 0.02$, adjusted for group A-like *var* expression levels and age) but not group A-like *var* expression levels ($B = -2.4$; 95% CI, -7.04 to 2.22 ; $P = 0.3$, adjusted for rosetting and age). The contrasting relationships with metabolic acidosis were again confirmed by analyzing the effect of adding each variable in turn to the models (Table 3).

Group A-like *var* expression is an independent marker for life-threatening malaria

Despite treatment, seven of the 217 patients on which this study is nested died (Fig. 2; (19)). In a logistic regression model with both parasite density and group A-like *var* expression levels as explanatory variables, parasite density, as observed previously (1), was not associated with host death (OR, 1.2; 95% CI, 0.70 to 2.17; $P = 0.5$, adjusted for group A-like *var* expression). However, death was predicted by group A-like *var* expression levels (OR, 20.4; 95% CI, 2.13 to 195.79; $P = 0.009$, adjusted for parasite density). Although all of these deaths occurred in children with impaired consciousness (two of whom also had respiratory distress), the association was maintained even when the analysis was restricted to only children with impaired consciousness (OR, 13.8; 95% CI, 1.16 to 164.58; $P = 0.038$, adjusted for parasite density). These observations further support a potential role for group A PfEMP1 in the pathophysiology of life-threatening malaria.

Rosetting and group A-like *var* expression are differentially associated with patient antibodies to infected erythrocytes

We previously observed that group A-like *var* expression levels tend to be high in isolates from young children (19) and those with low levels of heterologous infected erythrocyte surface antibodies carried at the time of disease (19, 34). If rosetting and group A-like *var* gene expression can be considered as measures of independent methods of parasite sequestration, we might expect them to exhibit differential associations with host immunity. Previous studies show that children make highly specific antibody responses to the parasites causing a disease episode, reflecting the high antigenic diversity of the infected erythrocyte surface. For this reason, heterologous infected erythrocyte surface antibodies carried at the time of disease most likely reflect the endogenous repertoire of antibodies accumulated by the child before the disease episode. Heterologous infected erythrocyte surface antibodies are here defined as those tested against the infected erythrocyte surface of parasites sampled from other children as opposed to homologous parasites that are actually causing the current episode of disease. The lower expression of group A-like *var* genes in children carrying such infected erythrocyte surface antibodies suggests that protective antibodies to parasites expressing group A-like *var* genes tend to be acquired relatively rapidly as children gain exposure. This could occur if group A-like PfEMP1 carry relatively restricted sets of epitopes compared to other PfEMP1 variants (38-40).

We asked whether the association between group A-like *var* expression and infected erythrocyte surface antibodies carried at the time of disease is driven primarily by antibodies that select against parasites expressing the rosetting phenotype. If this was the case, we would expect the association between infected erythrocyte surface antibodies and group A-like *var* expression to be weakened by statistical correction for parasite rosetting. We considered antibody responses against eight individual parasite isolates. To capture each child's breadth of infected erythrocyte surface antibodies carried at the time of disease, we also derived the median of their immunoglobulin G (IgG) response to all eight clinical isolates [acquired as mean fluorescence intensity (MFI)]. As expected, the infected erythrocyte surface antibody breadth measure showed a positive correlation with host age ($\rho = 0.34$, $P < 0.0001$).

As shown in Table 1 and Fig. 3A, the negative relationship between group A-like *var* expression and host antibodies to the infected erythrocyte surface antigens of eight clinical isolates was independent of rosetting (Fig. 3A). In addition, despite the association with respiratory distress, the rosetting phenotype *per se* (after adjusting for group A-like *var* expression, as above) showed no independent association with host infected erythrocyte surface antibodies (Fig. 3B). Thus, between-isolate variation in rosetting may be influenced by specific anti-rosetting antibody responses (30) or other host factors not captured by the infected erythrocyte surface antibody assay at the time of disease (41).

Putative causal immune pathways involving group A-like *var* genes and infected erythrocyte surface antibodies

To what extent are these relationships observed at the parasite level maintained in clinical disease? We would expect, on the basis of the observations above, an association between impaired consciousness and carriage of infected erythrocyte surface antibodies. Table 1, model 3B shows evidence for such a negative association between the breadth of infected erythrocyte surface antibodies and impaired consciousness. Given the negative relationship between these antibodies and group A-like *var* expression, we considered the possibility that there may be a simple causal pathway between these variables. For example, infected erythrocyte surface antibodies carried at the time of disease may reduce group A-like *var* expression, which in turn reduces the risk of impaired consciousness. Given the known

diversity of both *var* genes and infected erythrocyte surface antibodies, we cannot assume that the observed relationships between group A-like *var* expression, infected erythrocyte surface antibodies, and disease involve the same subsets of *var* sequences or antibodies. One way of exploring this is by testing for the effects of statistical adjustment on the relationships between these variables in regression models. If group A-like *var* expression and infected erythrocyte surface antibodies did belong to a single causal pathway, we would expect the relationships between them and severe disease to show some lack of independence. Thus, addition of group A-like *var* expression as a variable in a regression model of the association between infected erythrocyte surface antibodies and impaired consciousness may diminish the relationship between these latter two variables. Alternatively, addition of infected erythrocyte surface antibodies as a variable in a regression model of the association between group A-like *var* expression and impaired consciousness may diminish the association between group A-like *var* expression and impaired consciousness.

Consistent with this, addition of the infected erythrocyte antibody breadth measure to logistic regression model 4B (Table 1, to create model 5B) lowered the estimate of the association between group A-like *var* expression and impaired consciousness (OR reduced from 11.5 to 7.0; Table 1, model 5B). No such effect of correction for infected erythrocyte surface antibody breadth was observed on the association between rosetting and respiratory distress [OR 5.8 (before adjustment) and 5.6 (after adjustment); Table 1, model 5A). A similar effect on the relationship between group A-like *var* expression and impaired consciousness was observed using the complete set of 217 isolates (Fig. 3C) (19).

The effect of adjustment for group A-like *var* expression on the relationship between host antibodies and impaired consciousness was only marginally evident (Fig. 3D). The fact that infected erythrocyte surface antibodies and *var* expression levels maintain a degree of independence in these simple models suggests that our assays and models for group A *var* expression or infected erythrocyte surface antibodies have not fully captured the complexity of the host–parasite interaction. There is a need for more realistic approaches for testing causal pathways such as structural equation modelling.

What are the biological roles of rosetting and group A-like PfEMP1?

Aside from the proposed role for PfEMP1 in parasite sequestration in host tissues, little is known about the biological function of rosetting and group A PfEMP1. Rosetting has previously been proposed to promote re-invasion of uninfected erythrocytes (12). Consistent with this, a significant positive correlation was observed both in the present and in previous studies between rosetting and peripheral parasite density at the time of sampling (32, 42) (fig. S1). This supports a possible role for rosetting in increasing parasite growth rate or survival *in vivo* (12). In contrast, group A-like *var* expression levels show no relationship with peripheral parasite density (19). To test whether parasite density may explain the relationship between rosetting and respiratory distress, we assessed the effect of adjusting for parasite density in a logistic regression model predicting respiratory distress using rosetting and group A-like *var* expression as explanatory variables (Table 1, models 6A and 7A). Although rosetting was predictive of respiratory distress, this association dropped out and a significant improvement to the model fit was observed when parasite density was added to the model as a variable (Table 1, models 6A and 7A). No such effect was observed for the relationship between group A-like *var* expression and impaired consciousness (compare models 6A and 7A with models 6B and 7B). Thus, the parasite-related variables associated with severe life-threatening malaria can be reduced to peripheral parasite density, and group A-like *var* expression levels.

The apparent susceptibility of group A-like *var* genes to host immunity (19) (Fig. 3A), their lack of an independent association with peripheral parasite density (19), and their consistent presence in parasites sampled worldwide (15) raises the question: What maintains group A-like PfEMP1 in the genomic PfEMP1 repertoire if they are associated with severe disease in the host?

Because clinical malaria tends to be seasonal, whereas asymptomatic infections are prevalent all year round in areas of stable *P. falciparum* transmission (43), we explored whether group A-like *var* genes are expressed in asymptomatic infections. We examined group A-like *var* expression patterns in isolates from 33 children with asymptomatic infection sampled during a single cross-sectional survey that had no lower parasitemia cutoff and thus sampled parasites that were being effectively controlled by the host (see Materials and Methods). Because the survey was within the low transmission season, it is likely that these parasites induced chronic infections that had been carried since the last transmission season. These infections generally had much lower parasite densities than the cut-off used in the study of clinical malaria (table S1). Nevertheless, a considerable number of the infections carried high expression levels of group A-like *var* genes (Fig. 4; eight infections had expression levels of >65%). This suggests that, rather than being exclusively associated with parasite virulence, group A-like PfEMP1 may play a role along with other PfEMP1 in maintaining chronic infections.

DISCUSSION

Our current understanding of the pathogenesis of severe malaria is hampered by the difficulty in defining distinct disease states that reflect specific underlying pathophysiology. Here, we compared parasite markers of the host-parasite relationship in two severe complications of childhood malaria, impaired consciousness and respiratory distress, and propose that these two syndromes are associated with different methods of parasite sequestration in tissues. The results suggest a model in which (i) rosette-independent adhesion through non-rosetting group A PfEMP1 variants causes tissue-specific sequestration that can lead to impaired consciousness in the absence of a high overall parasite burden and (ii) rosetting, because it does not rely on endothelial adhesion, supports tissue independent sequestration and high parasite burden (12) leading to metabolic acidosis and respiratory distress.

All models of severe malaria are complicated by the potential heterogeneity of the underlying cause of clinical syndromes. Malaria with impaired consciousness is a particularly difficult condition to treat. A possible reason for this is that impaired consciousness can be caused by either metabolic acidosis or sequestration of parasites in the brain (2, 13). Thus, impaired consciousness accompanied by respiratory distress may have a different cause compared to impaired consciousness when it occurs in the absence of respiratory distress. This has important implications because treatment for metabolic acidosis by, for example, intravenous fluid therapy would be predicted to have a negative impact on impaired consciousness if it is caused by intracranial hypertension associated with parasite sequestration in the brain (44). The heterogeneity of impaired consciousness is supported by autopsy studies. These studies support a role for cerebral sequestration of parasitized erythrocytes in the pathogenesis of cerebral malaria (3, 45, 46). However, not all patients who died after severely impaired consciousness show evidence of cerebral sequestration (3).

Assays that focus more directly on the host parasite interaction would potentially benefit the clinical management of malaria. In support of this, examination of the deep structures of the eye in malaria patients has been successfully used to show that retinal pathology, caused by

parasite sequestration in the retinal vasculature, correlates well with disease outcome (47) (48). In autopsy studies, retinopathy was shown to provide the best prediction of whether there was sequestration in the brain (3). Autopsy studies have also shown that parasites sequestered in different tissues tend to express different subsets of *var* genes (49), supporting the idea that patterns of sequestration may be determined by the distribution of cytoadherence characteristics expressed by the infecting parasite population.

The data presented here are consistent with the heterogeneity in the underlying cause of impaired consciousness. Although overall, impaired consciousness was associated with elevated group A-like *var* expression, several isolates, in particular those from children presenting with both impaired consciousness and respiratory distress together, had very low levels of group A-like *var* expression (Fig. 2). Although such patients with overlapping syndromes have been reported to have higher mortality (1), impaired consciousness in the absence of signs of metabolic acidosis, is associated with neurological sequelae (50). The observations that group A-like gene expression was associated with mortality even within the group of children with impaired consciousness support the use of this as an independent marker of severe malaria. However, a combination of approaches is clearly necessary to develop a full picture of the pathological consequences of tissue-specific parasite sequestration. Although we would not expect this information to have an immediate impact on clinical management of malaria, correlating parasite features with clinical observation may prove valuable in the refinement and standardization of these clinical observations. Studies that aim to correlate retinopathy data with *var* expression data could potentially lead to the development of rapid and inexpensive diagnostic methods.

In the future, improved understanding of the heterogeneity of severe malaria will be potentially useful in the development of new treatments (11). The parasite rosetting phenotype can be reversed by sulfated glycoconjugates such as heparin. This has led to studies of compounds that may potentially be used as anti-rosetting therapies (51) (52). However, contrasting associations have been observed between rosetting and different manifestations of severe malaria in different geographical settings in Africa (5, 30-32, 53) [reviewed in ref 32]. Overall the data support an association between rosetting and all forms of severe malaria as has been found by Doumbo *et al.* (32). However, previous studies have not fully accounted for respiratory distress or metabolic acidosis. Because metabolic acidosis can contribute to the development of impaired consciousness (2), accounting for respiratory distress or metabolic acidosis may help resolve the differences observed between the studies. The effectiveness of anti-rosetting therapies might be underestimated if the heterogeneity of severe malaria is not taken into account in clinical trials and, on the basis of the evidence presented here, we would predict that these therapies may not be effective against all forms of malaria with impaired consciousness. In clinical trials, assessment of rosetting and group A *var* expression of clinical isolates may be necessary to accurately assess the effectiveness of such therapies.

The approach used in our study was based on testing relative expression levels of previously defined groups of *var* sequences sampled from a single region of the *var* genes. We have used various sequence features within these sequences as only as makers to provide information about broad classes of sequence. These sequence groups were identified in the absence of clinical data and are present within the repertoire of *var* genes in all parasite genomes (28,34). Normark *et al.* (27) previously used a complementary approach in which a clinical data set was used to identify putative disease-associated motifs among the dominantly expressed *var* sequences. In doing so they made an assumption that dominantly expressed variants are most likely to be the ones causing severe malaria. Cross-validation between different data sets and approaches is now a priority, and will require agreement about methods of disease phenotyping and *var* gene sequence sampling. Studies need to be

performed at different time points and a different locations to determine the stability of different associations. We should not necessarily expect completely stable associations with disease especially for a parasite that is so capable of phenotypic and antigenic variability.

A major limitation of our study was the use of capillary sequencing to profile parasite isolates. This is an expensive and labor-intensive approach that only provides sequence information on a short stretch of sequence encoding an N-terminal portion of the PfEMP1 molecule. New sequencing technologies will potentially provide whole-transcriptome information that would enable rapid, full-length *var* gene expression profiling (54). This is likely to be necessary for the identification of specific molecular signatures that can reliably differentiate rosetting and non-rosetting group A PfEMP1. Progress has already been made in laboratory isolates. Clinical isolates that form rosettes vary in their tendency to bind nonspecifically to IgM. Rosetting associated with IgM binding is the most common form of rosetting associated with severe malaria (55). In a well characterized, rosetting laboratory isolate, the IgM binding was found to be mediated by a specific PfEMP1 domain DBL4 β , distant from the DBL α domain (56), which, owing to recombination, may not be associated with a clear signature within the DBL α tag region.

In summary, despite the relatively few children with each clinical manifestation in our data set, we find evidence for associations between clinical manifestations of life-threatening malaria and two parasite features, rosetting and group A-like *var* expression. To date none of the established cytoadhesion phenotypes apart from rosetting exhibit a clear association with group A PfEMP1. Future studies are therefore needed to determine the role of group A PfEMP1 in parasite survival, their correlation with sequestration in the brain, and whether their appearance in clinical infections is adaptive or part of a dead-end process that benefits neither host nor parasite. A first step will be to identify the binding specificities that are specific to group A-like PfEMP1. This could potentially lead to new therapies against specific molecular targets associated with malaria with impaired consciousness.

MATERIALS AND METHODS

Study site

The study was carried out at Kilifi District Hospital, situated at the coast of Kenya. Ethical approval for this study was granted by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee and informed consent was obtained from the parents/guardians of all study participants.

Parasite sampling and clinical classification of patients

The samples used for the rosetting assays were a subset of 217 clinical isolates used in a recently published study (19). Severe malaria was defined as hospital admission with impaired consciousness (Blantyre coma score <4 in patients under 8 months old; <5 in patients aged 8 months) (57), severe malarial anemia (Hemoglobin <5g/dl, see table S1 for these data) (1), or respiratory distress (deep “Kussmaul” pattern of breathing) (36). Of the 217 children from the previous study, 55 presented with impaired consciousness, 15 with respiratory distress and 33 with both respiratory distress and impaired consciousness (19). Isolates sampled from a subset of 131 children (31 with impaired consciousness, 11 with respiratory distress and 19 with both clinical manifestations; Table S1) were used in the rosetting assays as described below. Of these 36 had cerebral malaria (defined as Blantyre coma score <3). We also sampled isolates from 33 children with asymptomatic infection of any parasite density during a cross-sectional survey in May 2007 (table S1). For each child, expressed *var* sequences were generated from ring-stage parasites as described previously (34). An aliquot of each isolate used in the rosetting analysis was cultured *in vitro* to the

mature trophozoite stages and cryopreserved in liquid nitrogen using published methods (19, 34, 58).

Rosetting assay

Rosetting frequency was assessed in 131 isolates that successfully grew to mature trophozoites in culture (table S1). Each cryopreserved parasite isolate was thawed as previously described (59). A 4% hematocrit suspension of the trophozoite-infected erythrocytes was then prepared in RPMI 1640 medium (Gibco) containing 5µg/ml acridine orange (Sigma-Aldrich). For every isolate 9.5µl of the 4% hematocrit suspension of trophozoite-infected erythrocytes was added to 2.5µl of nonimmune AB serum in a 96-well U bottomed plate. The plate was then rotated for 30 min on a vertical rotor at room temperature, after which the suspension was placed on a microscope slide, covered with an 18mm X 18mm coverslip and scored at x40 on a fluorescence microscope (Nikon eclipse 80i, Japan). Each parasite isolate's rosetting frequency was assessed by determining the percentage of 200 trophozoite-infected erythrocytes that were bound to two or more uninfected erythrocytes. The clinical category of the patient from whom each isolate was obtained was blinded at the time scoring the rosetting frequency.

Infected erythrocyte surface antibody data

We measured each child's IgG antibody levels (acquired as mean fluorescence intensity, MFI) to the infected erythrocyte surface of eight clinical isolates using flow cytometry as described elsewhere (60). IgG binding to the surface of erythrocytes was detected with a fluorescein isothiocyanate (FITC)-conjugated anti-Fcγ antibody (AF004, The Binding Site). Analysis was performed with FlowJo v7.2 software (TreeStar Inc., USA). To account for nonspecific IgG binding to each isolate, we first distinguished infected erythrocytes from uninfected erythrocytes on the basis of their ethidium bromide staining. Next, the MFI of uninfected erythrocytes was subtracted from that of trophozoite-infected erythrocytes for all plasma including that from seven nonexposed European donors. Finally, for each of the eight heterologous isolates, the highest MFI obtained with any of the seven plasma from the European donors was then subtracted from the MFIs obtained with each child's plasma before use in the analyses. As previously reported (19), the heterologous isolates were obtained from eight children with clinical malaria and were selected on the basis of their *var* expression patterns such that *cys2* *var* expression levels were high in isolates P6921, P7063, P7671 and P7148, and low in isolates P7860, P8073, P7237 and P7542. Of these P7148 had high *cys2* *var* expression (19) but these were not classified as group A-like (Fig. 3E). As with isolates used for the rosetting assays, the eight isolates were each cultured for ≈20 hours to allow development of the sampled ring stages into mature pigmented trophozoites and cryopreserved in liquid nitrogen. When required for the flow cytometry assay, each isolate was thawed and tested against all the plasma in a single run.

Sequence classification

Sequence assembly, classification and counting were done with two previously described analysis pipelines (table S2 (19)). The sampled DBLα tag sequences can be classified with two different approaches. First, they can be classified according to whether they contain two (*cys2*), rather than the usual four (*cys4*) cysteine residues (34). Group A *var* genes are never *cys4*. Second, they can be classified according to whether they fall into groups that tend not to share polymorphic regions (28) as would be expected of genetically distinct, nonrecombining sets of genes. We have previously identified a distinct group of tag sequences sampled from Kilifi, Kenya, termed block-sharing group 1, that are defined by a set of 573 polymorphic sequence blocks (table S3). Together with the classification based on cysteine counts, this collection of blocks can be used to identify known group A PfEMP1 with high sensitivity and specificity (19, 28). Therefore, we classified *cys2* sequences as

“group A-like” if they carried one or more of these sequence blocks and used this group A-like subgroup as markers for group A *var* genes in this analysis. The *cys2 var* sequence subclassification yielding groups based on the presence of mutually exclusive MFK and REY motifs has been described (34). The MFK motif is only found only in group A sequences (28, 34). Additional minor groups of sequences were considered (fig. S2). One, falling in block sharing group 1, but carrying only one cysteine (*cys1*) was also found to be associated with rosetting (28). A Perl script that can be used to perform these classifications is included in the supplementary information of (28) (Folder S2). The H3 subset was defined as those sequences containing the following motifs: DDKVQK, DKVEKG, EDKVQK, HDAVEK, KDAVQK, KDAVQN, KDDVEK, KDEVKE and NDEVWK (27).

The number of individual clones carrying each *var* sequence type was expressed as a percentage of the total number of clones sequenced from each parasite isolate and used for the analysis (see below and tables S1 and S2).

Statistical analysis

The statistical software Stata™ version 11 was used for all the analysis and *P* value <0.05 was considered significant for all tests.

Univariate analyses—Spearman’s rank correlation coefficient was used to assess the relationship between: rosetting and both the percentage sequence expression scores and parasite density (Fig. 1A and figs. S1 and S2) and, host age and the infected erythrocyte surface antibody breadth (see text).

Regression analyses—Before use in regression analyses, the parasite density data were log transformed. In addition, both the percentage rosetting and the percentage sequence expression data were arcsine transformed with the formula ($\sin^{-1}\sqrt{P}$), where *P* represents the percentage rosetting or sequence expression data, respectively. Weighting for 0% and 100%

values in the arcsine transformation was done by substituting *P* with $\frac{1}{4n}$ for 0% values and $(100 - \frac{1}{4n})$ for 100% values, where *n* represents the number of trophozoite-infected erythrocytes counted for the percentage rosetting data (that is 200; see assay details above) and the number of clones sequenced for the *var* expression data (see table S1).

Host age, infected erythrocyte surface antibody levels, parasite density, rosetting frequency, *var* expression levels, base excess and blood glucose levels were all used as continuous variables in the modeling whereas severe malaria, impaired consciousness, respiratory distress and cerebral malaria were used as binary variables. Linear regression modeling was used in all analyses where the dependent variables were continuous: base excess (see text and Table 3), blood glucose levels (see text and Table 3), group A-like *var* expression (Fig. 3A) or rosetting (Fig. 3B). Logistic regression modeling was used where the dependent variable was binary: severe malaria (see text), impaired consciousness (Table 1 and Figs. 1C and 3, C and D), respiratory distress (Table 1 and Fig. 1B) and cerebral malaria (Table 2). All models were adjusted for host age.

Evaluating the fit of regression models—Normality of residuals is necessary for validity of hypothesis testing in linear regression models. We confirmed the validity of the observed associations by examining the interquartile ranges of the standardized residuals from linear regression models. None of the models had severe outliers and thus providing no evidence to reject normality of the residuals at a 5% α level. The goodness-of-fit of all logistic regression models was assessed with the Hosmer-Lemeshow goodness-of-fit test.

Again, all logistic regression models had a good fit as indicated by P values of >0.05 in this test. Finally, where mentioned (see text, Tables 1 to 3), the effect of adding each explanatory variable on the fit of the respective regression models was assessed with the LR χ^2 improvement test and a P value <0.05 considered as a significant improvement to the model fit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A Relationship between rosetting and group A-like expression

B Relationship between rosetting and respiratory distress

C Relationship between var expression and impaired consciousness

Fig. 1. Rosetting and group A-like *var* expression in relation to respiratory distress and impaired consciousness in malaria patients

(A) The Spearman's rank correlation coefficient and *P* value for the association between rosetting and group A-like *var* expression. (B) Age-adjusted ORs and 95% CIs for the relationship between rosetting and respiratory distress before and after adjustment for expression levels of previously defined subgroups of *var* genes (Materials and Methods). The dashed line represents the unadjusted results. The BS1-CP6 subgroup has previously been associated with rosetting and represents sequences falling into the cys/PoLV group 6 subset that maps onto the same network region as the group A-like subgroup (see Materials and Methods) (28). The H3 subset has also been associated with rosetting and represents sequences containing the following motifs: DDKVQK, DKVEKG, EDKVQK, HDAVEK, KDAVQK, KDAVQN, KDDVEK, KDEVKE and NDEVWK (27). (C) The relationship between each *cys2* *var* subgroup and impaired consciousness is shown before and after adjustment for rosetting. The analysis is based on 131 children, 30 of whom had respiratory distress and 50 with impaired consciousness (Materials and Methods). * *P* < 0.05, logistic regression.

Fig. 2. Rosetting frequency, group A-like expression levels and parasite density in relation to death

Presented is the distribution of rosetting frequency (A; $n = 131$), group A-like *var* expression (B; $n = 217$) and parasite density (C; $n = 217$) among children included in the study. Arrows indicate children who died.

Fig. 3. Rosetting and group A-like *var* expression in relation to host infected erythrocyte surface antibodies and impaired consciousness

(A) and (B) Results from age-adjusted linear regression models predicting group A-like *var* expression (A) and rosetting (B) using host infected erythrocyte surface antibodies to each of eight clinical isolates (P7671 to P8073) or the infected erythrocyte surface antibody breadth measure (“median antibodies”) as an explanatory variable. Results from models predicting group A-like *var* expression (A) are shown before and after adjustment for rosetting, and vice versa (B). This analysis is based on 131 children, 30 of whom had respiratory distress and 50 with impaired consciousness (Materials and Methods). (C and D) Age-adjusted ORs and 95% CIs for the relationship between impaired consciousness and both group A-like *var* expression (C) and host infected erythrocyte surface antibodies to each of the eight clinical isolates (D). In (C), the effect of adjustment for host antibodies is compared to the model with just group A-like *var* expression as an explanatory variable. The dashed line represents the OR for this latter model. In (D), the effect of adjustment for group A-like *var* expression in models predicting impaired consciousness using host antibodies is shown. The analysis in (C) and (D) is based on 217 children, 88 of whom had impaired consciousness (19). (E) Group A-like *var* expression levels of the isolates used for the infected erythrocyte (IE) surface antibody assay and the clinical manifestation of the respective child each was obtained from. * $P < 0.05$, linear regression in (A) and (B) and logistic regression in (C) and (D). IE, infected erythrocyte.

Fig. 4. Group A-like *var* expression levels in asymptomatic infections

Presented is the distribution of *cys2* *var* expression levels in isolates from 33 children with asymptomatic infection. From left to right, the expression levels of overall *cys2* *var* genes and the four previously described *cys2* subgroups (MFK+REY–, MFK–REY+, MFK–REY– and group A-like) are shown.

Table 1

Correlation of rosetting and group A-like *var* expression with prognostic indicators of malaria. Presented are age-adjusted statistics from 14 logistic regression models, 7 predicting respiratory distress and 7 predicting impaired consciousness using rosetting frequency only (model 1), group A-like *var* expression only (model 2), host infected erythrocyte (IE) surface antibody breadth only (model 3) or different combinations of the three explanatory variables (models 4-7). Models 6 and 7 present the results following inclusion of parasite density as an explanatory variable to model 4 and 5, respectively. The effect of dropping each explanatory variable on the fit of models 4 to 7 was assessed using the likelihood ratio χ^2 improvement test and a *P* value <0.05 considered as a significant improvement to the model fit. The analysis is based on 131 children (Materials and Methods).

Model s	Explanatory variables	A. Respiratory Distress		B. Impaired Consciousness	
		OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
1 A, B	Rosetting frequency	5.8 (1.59, 20.80)	0.008	1.4 (0.44, 4.59)	0.6
2 A, B	Group A-like <i>var</i> genes	1.7 (0.48, 6.29)	0.4	9.7 (2.69, 35.00)	0.0005
3 A, B	IE surface antibodies	0.1 (0.01, 1.46)	0.1	0.01 (0.001, 0.17)	0.002
4 A, B	Rosetting frequency	5.8 (1.49, 22.81)	0.01 *	0.6 (0.15, 2.39)	0.5
	Group A-like <i>var</i> genes	1.0 (0.23, 3.97)	0.95	11.5 (2.92, 45.71)	0.0005 *
5 A, B	Rosetting frequency	5.6 (1.42, 22.33)	0.01 *	0.6 (0.14, 2.27)	0.4
	Group A-like <i>var</i> genes	0.7 (0.16, 3.12)	0.7	7.0 (1.68, 28.90)	0.008 *
	IE surface antibodies	0.2 (0.01, 1.81)	0.1	0.02 (0.001, 0.31)	0.006 *
6 A, B	Rosetting frequency	2.3 (0.52, 9.84)	0.3	0.2 (0.05, 1.19)	0.08
	Group A-like <i>var</i> genes	0.8 (0.17, 3.55)	0.7	13.8 (3.26, 58.32)	0.0004 *
	Parasite density	2.4 (1.39, 4.13)	0.002 *	1.6 (1.15, 2.37)	0.007 *
7 A, B	Rosetting frequency	2.2 (0.49, 9.46)	0.3	0.2 (0.04, 1.15)	0.07
	Group A-like <i>var</i> genes	0.6 (0.12, 2.85)	0.5	8.0 (1.85, 34.91)	0.005 *
	Parasite density	2.4 (1.39, 4.19)	0.002 *	1.7 (1.14, 2.44)	0.008 *
	IE surface antibodies	0.2 (0.01, 1.96)	0.2	0.02 (0.001, 0.32)	0.007 *

* Variables that significantly reduced the fit of the model when removed as explanatory variables.

Table 2

Relationship between cerebral malaria and group A-like *var* expression levels. Presented are age-adjusted statistics from 6 logistic regression models predicting cerebral malaria (defined as Blantyre coma score <3; n = 36) using rosetting frequency only (model 1), group A-like *var* expression only (model 2) or both explanatory variables (model 3). Models 4-6 present the results following inclusion of infected erythrocyte (IE) surface antibodies, parasite density or both as explanatory variables to model 3, respectively.

Models	Explanatory variables	OR (95% CI)	P
1	Rosetting frequency	2.2 (0.64, 7.34)	0.2
2	Group A-like <i>var</i> genes	9.7 (2.54, 37.19)	0.0009
3	Rosetting frequency	1.0 (0.26, 4.29)	0.9
	Group A-like <i>var</i> genes	9.6 (2.32, 39.42)	0.002 *
4	Rosetting frequency	1.0 (0.26, 4.22)	0.96
	Group A-like <i>var</i> genes	6.7 (1.57, 28.34)	0.01 *
	IE surface antibodies	0.06 (0.003, 1.09)	0.06 *
5	Rosetting frequency	0.5 (0.11, 2.68)	0.4
	Group A-like <i>var</i> genes	10.2 (2.40, 43.24)	0.002 *
	Parasite density	1.5 (1.00, 2.13)	0.05 *
6	Rosetting frequency	0.5 (0.11, 2.69)	0.5
	Group A-like <i>var</i> genes	7.1 (1.63, 30.73)	0.009 *
	Parasite density	1.5 (0.98, 2.14)	0.06
	IE surface antibodies	0.06 (0.003, 1.17)	0.06 *

* Variables that improved the fit of the model as assessed using the likelihood ratio χ^2 improvement test (Materials and Methods).

Table 3

Relationship between metabolic acidosis and rosetting frequency. Presented are age-adjusted statistics from linear regression models predicting metabolic acidosis (base excess levels used as dependent variable) and hypoglycaemia (blood glucose levels used as dependent variable) using either rosetting frequency only (model 1), group-A like *var* expression only (model 2), parasite density only (model 3) or different combinations of these explanatory variables (models 4 and 5). The more negative the base excess measure the greater the deficit in bases in host tissues and hence the worse the degree of metabolic acidosis (2).

Model s	Explanatory variables	Metabolic Acidosis		Hypoglycemia	
		Regression coefficient (95% CI)	P	Regression coefficient (95% CI)	P
1	Rosetting frequency	-6.6 (-10.95, -2.24)	0.004	-1.6 (-3.76, 0.55)	0.1
2	Group A-like <i>var</i> genes	-4.4 (-8.95, -0.09)	0.06	-0.02 (-2.11, 2.07)	0.98
3	Parasite density	-1.1 (-2.32, 0.06)	0.06	-0.5 (-1.01, 0.03)	0.06
4	Rosetting frequency	-5.7 (-10.37, -1.12)	0.02 *	-1.9 (-4.32, 0.44)	0.1
	Group A-like <i>var</i> genes	-2.5 (-7.10, 2.12)	0.3	0.8 (-1.52, 3.04)	0.5
5	Rosetting frequency	-4.5 (-9.78, 0.73)	0.09	-1.2 (-3.83, 1.46)	0.4
	Group A-like <i>var</i> genes	-3.0 (-7.70, 1.74)	0.2	0.5 (-1.83, 2.80)	0.7
	Parasite density	-0.6 (-1.94, 0.66)	0.3	-0.4 (-0.96, 0.21)	0.2

* Variables that improved the fit of the model as assessed using the likelihood ratio χ^2 improvement test (Materials and Methods).