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Expression of specific *var* gene subtypes is differentially associated with severe malaria syndromes

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eLife Assessment

This **valuable** study uses a large cohort of clinical malaria cases collected over 18 years to address a critical knowledge gap regarding the role of PfEMP1 variants across distinct severe malaria syndromes. The conclusions are potentially of importance and interest to those who study malaria severity, but the evidence is **incomplete**, largely due to a lack of clarity on data inclusion and the correct use of statistical tests. More up-to-date data analysis methods would further strengthen the conclusions.

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Abstract

The virulence of *Plasmodium falciparum* is closely linked to *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by a diverse *var* gene family. PfEMP1 mediates parasite immune evasion and vascular adhesion of infected red blood cells, contributing to severe disease. While expression of group A or domain cassettes (DC8 and DC13)-containing *var* genes have been associated with severe malaria, comparisons between the severe malaria syndromes remain limited. Furthermore, interactions between specific *var* gene expression and the rosetting phenotype, a known marker of severe malaria, are incompletely understood across severe malaria syndromes.

We analyzed parasites and clinical data from 712 Kenyan children with non-severe and severe malaria syndromes collected over 18 years in Kilifi, Kenya. We used RT-qPCR and DBLa-tag sequencing to quantify *var* gene expression. We show that parasites expressing *var* genes containing the cys2 MFK+REY-motif or encoding DC8 domains were associated with impaired consciousness (IC), while rosetting was associated with respiratory distress (RD) and severe malarial anaemia (SMA), but not IC. These findings demonstrate that distinct PfEMP1 variants are preferentially expressed in specific severe malaria syndromes, highlighting potential targets for variant-specific future therapeutic and diagnostic strategies.

Introduction

Plasmodium falciparum malaria remains a major global health burden, causing over half a million deaths annually, primarily in sub-Saharan Africa (WHO, 2023 [↗](#)). The clinical spectrum of malaria ranges from non-severe to severe disease, with severe cases characterized by life-threatening complications such as impaired consciousness (IC), severe malarial anaemia (SMA), and respiratory distress (RD) (Marsh et al., 1995 [↗](#)). A deeper understanding of host-parasite interactions at the molecular level that drive distinct clinical malaria syndromes is essential for

the development of targeted therapies. A key unresolved question in malaria research is whether parasites causing severe disease biologically differ from those responsible for non-severe malaria, and whether specific severe manifestations can be attributed to particular parasite features.

Central to the pathogenesis of severe malaria is the adherence of infected red blood cells (iRBCs) to endothelial cells in the microvasculature, a process known as cytoadhesion. Cytoadhesion facilitates immune evasion by preventing spleen-mediated clearance of iRBCs (Rowe et al., 2009 [\[1\]](#)). The primary parasite ligands mediating this interaction are the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family. PfEMP1 proteins mediate cytoadhesion by binding to endothelial receptors such as endothelial protein C receptor (EPCR), intercellular adhesion molecule-1 (ICAM-1), cluster of differentiation 36 (CD36), complement receptor 1 (CR1), and chondroitin sulfate A (CSA) (Bernabeu & Smith, 2016 [\[2\]](#); Smith et al., 2013 [\[3\]](#); Tuikue Ndam et al., 2017 [\[4\]](#)), with different binding specificities linked to distinct severe malaria phenotypes. For instance, the binding of iRBCs expressing VAR2CSA to CSA on placental syncytiotrophoblasts contributes significantly to placental malaria (Clausen et al., 2012 [\[5\]](#)). Additionally, rosetting, in which iRBCs bind uninfected RBCs, plays a role in severe malaria pathogenesis (Cockburn et al., 2003 [\[6\]](#); Lee et al., 2022 [\[7\]](#)).

PfEMP1 is a large 200 to 400-kDa clonally variant antigen encoded by the highly polymorphic *var* gene family (Gardner et al., 2002 [\[8\]](#); Smith et al., 1995 [\[9\]](#)). Each *P. falciparum* genome carries approximately 60 *var* genes that are transcribed in a mutually exclusive fashion. This allows the parasite to alter antigenic profiles and evade host immunity (Deitsch & Dzikowski, 2017 [\[10\]](#)). PfEMP1 typically includes an N-terminal “head structure” with an N-terminal sequence (NTS) and a Duffy Binding-Like (DBL) domain, followed by a Cysteine-rich InterDomain Region (CIDR) (Smith et al., 2000 [\[11\]](#)). *Var* genes are classified into three major groups (A, B, and C) based on their chromosomal location and promoter sequence (Smith, 2014 [\[12\]](#)). This classification is clinically relevant, as group A *var* genes are typically associated with severe malaria, whereas group B and C are more frequently expressed during mild or asymptomatic infections (Kyriacou et al., 2006 [\[13\]](#)). Additionally, *var* genes are grouped according to conserved domain arrangements known as domain cassettes (DC) (Rask et al., 2010 [\[14\]](#)), with parasites expressing DC8 and DC13 strongly correlated with severe disease presentations (Lavstsen et al., 2012 [\[15\]](#)). Further categorization is based on cysteine residue counts and conserved amino acid motifs (“MFK” and “REY”) in positions of limited sequence variation within DBL alpha domains referred to as cp grouping, with six distinct subgroups (cp1-cp6) (Table 1 [\[16\]](#)) (Bull et al., 2005 [\[17\]](#), 2007 [\[18\]](#)). Of these, cp1 has specifically been linked to IC, whereas cp2 is predominantly associated with SMA (Warimwe et al., 2009 [\[19\]](#)).

These findings suggest that parasites expressing certain PfEMP1 variants are more virulent. Despite these advances, previous studies have been limited by small sample sizes and uneven representation of clinical syndromes, which constrain statistical power and generalizability. Kilifi, a coastal region in Kenya has experienced major shifts in transmission and in the clinical spectrum of malaria in the past two decades (Mogeni et al., 2016 [\[20\]](#); Njuguna et al., 2019 [\[21\]](#)). This setting provides an opportunity to revisit these questions across well-represented syndromes using samples collected over 2 decades.

Furthermore, previous studies have predominantly relied on either classification of PfEMP1 based on segments derived through quantitative real-time polymerase chain reaction (RT-qPCR) or sequencing of expressed DBL α -tags in parasite isolates (Andisi & Abdi, 2022 [\[22\]](#); Bull et al., 2005 [\[17\]](#); Lavstsen et al., 2012 [\[15\]](#); Mkumbaye et al., 2017 [\[23\]](#)). Each approach has strengths and limitations. RT-qPCR offers quantitative resolution for predefined targets but cannot capture the full *var* repertoire. DBL α -tag sequencing on the other hand, captures *var* repertoire breadth and diversity but lacks precise quantitation of individual transcripts. Reliance on a single method has likely contributed to inconsistent associations with clinical phenotypes reported in previous studies.

To overcome these limitations, this study used clinical data, and parasite isolates from 712 children from Kilifi recruited between 1994-2012 with various clinical malaria presentations. The *var* gene expression profiles of the parasite isolates was determined using both DBL α -tag sequencing and RT-qPCR. By using these complementary datasets, our aim was to refine and robustly test associations between PfEMP1 variants and severe malaria syndromes.

Grouping	Original name	Common name	No. of cysteine residues	Unique motif
CP	Group 1 (cys2/ MFK+&REY-)	cp1	2	MFK
	Group 2 (cys2/ MFK-&REY+)	cp2	2	REY
	Group 3 (cys2/ MFK-&REY-)	cp3	2	-
	Group 4 (cys2/ MFK-&REY-)	cp4	4	-
	Group 5 (cys4/ MFK-&REY+)	cp5	4	REY
	Group 6 (cysX)	cp6	1,3,X	
BS	bs1_Cys2	group A-like	2	MFK/ REY

CP: cysteine/position of limited variation, BS: block sharing, X: any number other than 2

or 4

Table 1. DBLα-tags sequence groupings and common names used in the text.

Results

Participants and sample characteristics

Samples from 712 children (49% females) aged between 1-167 months who were slide positive for *P. falciparum* infection were included in this study (Figure 1A). 229 (32%), 252 (35%), and 231(32%) children were recruited between 1994-2002, 2003-2008 and 2009-2012 respectively corresponding to periods of high (pre-decline), rapidly declining (decline) and low malaria transmission (post-decline) respectively in Kilifi County, Kenya (Figure S1) (Mogeni et al., 2016). 340 of the participants had non-severe malaria and 372 had severe malaria that included 186 impaired consciousness (IC), 29 Respiratory distress (RD), 59 severe malarial anaemia (SMA), and 98 with overlapping severe malaria presentations (Figure 1B).

As expected, the mean age of children included in this study at the time of admission increased as malaria transmission declined (Figure S1). Among these children, those admitted with any form of severe malaria syndrome were significantly younger (median 35 vs 44 months, $p < 0.001$) and mortality was significantly higher among severe cases ($p < 0.001$) (Table S2). By syndrome, IC and SMA were significantly associated with younger age compared to NS (IC $p = 0.016$; SMA $p < 0.001$), whereas RD wasn't (Table S2). Peripheral blood parasitemia was only significantly elevated in RD/IC cases ($p=0.034$). Mortality was highest in IC ($p < 0.001$) with no significant difference in RD or SMA relative to NS (Table S2).

Impaired consciousness and severe malarial anaemia but not respiratory distress were associated with *cys2* and group A-like *var* gene expression

We fitted five independent multiple linear regression models predicting the expression of *cys2* var genes and its subtypes (bs1_*cys2*, *cys2* MFK+REY-, *cys2* MFK-REY+, *cys2* MFK-REY-) hereafter referred to as group A-like, cp1 cp2 and cp3 respectively (Table 1, Methods) as dependent variables. The explanatory variables included the three primary clinical syndromes IC, RD, and SMA, along with age and peripheral parasite density.

Compared to the non-IC clinical group, IC was significantly and positively associated with *cys2* [beta (β) (95%CI): 0.09(0.04, 0.14), $P = 0.0002$], group A-like [β (95%CI): 0.07(0.03, 0.12), $P=0.001$], and cp1 [β (95%CI): 0.09(0.05, 0.12), $P= 0.0001$] (Figure 2A, Table S3). RD did not show significant association with expression of any of the DBL α -tag-defined *var* subtypes (Figure 2B, Table S3). SMA was significantly associated with higher expression *cys2* [β (95%CI): 0.07(0.01, 0.14), $P=0.03$] and group A-like [β (95%CI): 0.07(0.01, 0.14), $P=0.02$]. Although the association of SMA with cp2 did not reach statistical significance when all samples were considered (Figure 2C, Table S3), however, it became significant when children with overlapping syndromes were excluded from the analysis (Figure S2, Table S4).

IC was differentially associated with higher DC8 transcript levels compared with SMA and RD

Next, we explored the differential association of transcript quantity obtained with primers targeting expression of general group A (gpA1, gpA2), DC8 (dc8-1 to dc8-4), group B (dc9, gpB) and group C (gpC) (Table S1) with the severe malaria syndromes. IC showed significant positive associations with the transcript levels detected by the gpA1 primer [β (95%CI): 0.36(0.09, 0.62), $P=0.008$] (Figure 2A, Table S3). Additionally, IC was positively associated with the transcript levels obtained with the DC8 targeting primers; dc8-2 [β (95%CI): 0.41(0.08, 0.74), $P=0.02$], and dc8-3 [β (95%CI): 0.35(0.04, 0.66), $P=0.03$] as well as the overall DC8 transcript quantity, as captured by the median transcript quantity of the four DC8 primers, dc8median [β (95%CI): 0.33(0.04, 0.62), $P=0.03$] (Figure 2D and Table S3). When children with overlapping syndromes were excluded, IC remained significantly associated with transcript levels of gpA1 [β (95%CI) 0.44(0.14, 0.74), $P=0.004$], as well as the transcript levels detected with the DC8 targeting primers, dc8-2, dc8-3, and

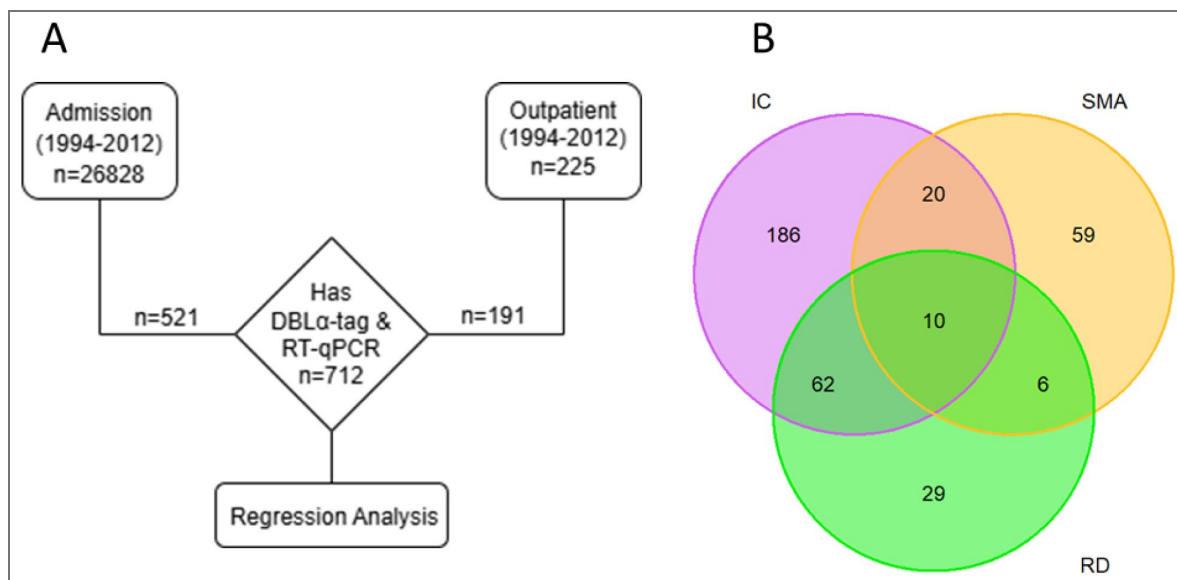


Figure 1. Sample inclusion and syndrome classification.

A). Flowchart of children whose samples were included in the study and analysis. B). Venn diagram of severe malaria syndromes among admissions included in the study. IC: impaired consciousness, RD: Respiratory distress, SMA: severe malarial anaemia.

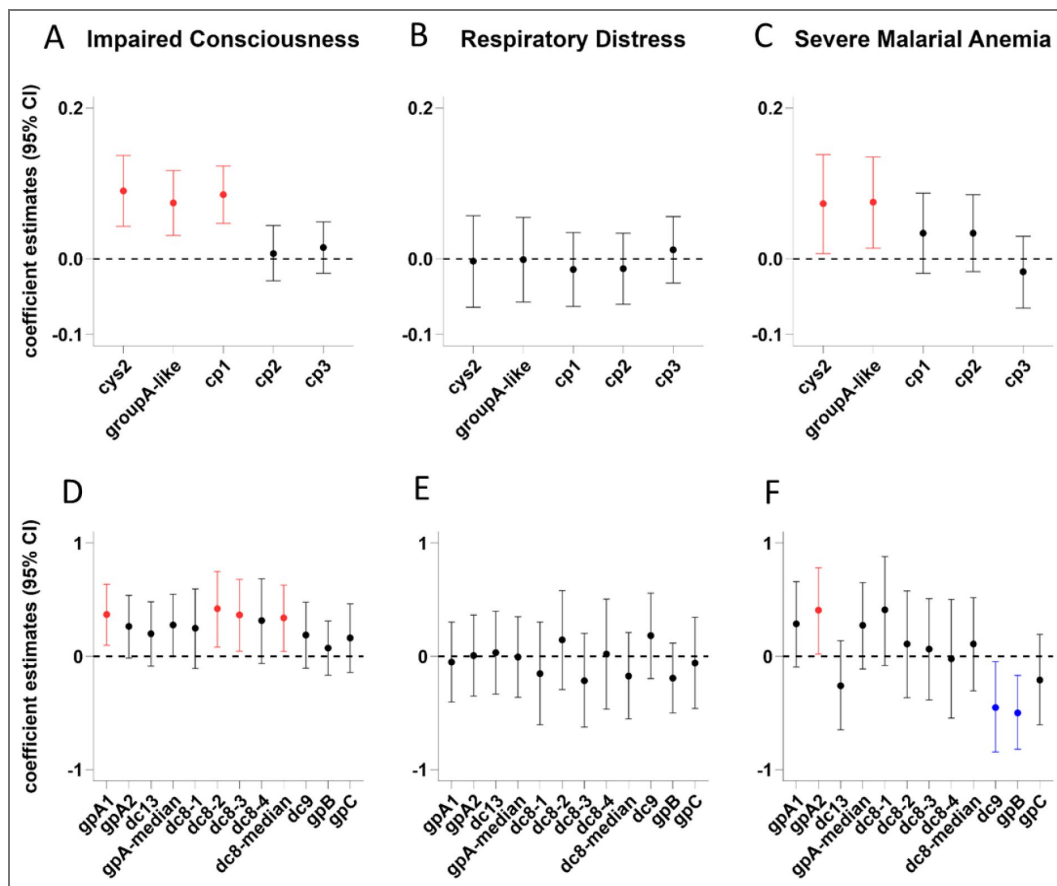


Figure 2. Associations between var gene expression and severe malaria syndromes.

A-C Results from five independent regression models in which *cys2* or its subgroups (group A-like, cp1, cp2, and cp3) were considered as single dependent variables with the severe malaria syndromes, impaired consciousness, respiratory distress and severe malaria anaemia as explanatory variables, while adjusting for age and parasitemia. **D-F** Results from twelve independent regression models considering the transcript levels for gpA1-gpC as sole dependent variables, with impaired consciousness, respiratory distress and severe malaria anaemia as explanatory variables while adjusting for age and parasitaemia. Regression coefficient (β) and corresponding 95% confidence intervals (CI) were plotted.

dc8-median (Figure S2D [↗](#) and Table S4 [↗](#)). Notably, in this restricted analysis, IC showed significant positive associations with transcript levels measured with gpA2 [β (95%CI): 0.41(0.11, 0.71), $P=0.01$], and gpA-median [β (95%CI): 0.40(0.10, 0.70), $P=0.01$] (Figure S2D [↗](#), Table S4 [↗](#)).

RD showed no significant associations with any *var* transcript quantities (Figure 2E [↗](#)). However, when children presenting with overlapping syndromes were excluded, pure RD cases were significantly associated with higher transcript levels detected by the gpA2 [β (95%CI): 0.69(0.15, 1.24), $P=0.01$], gpA-median [β (95%CI): 0.59(0.05, 1.13), $P=0.03$], and dc8-2 [β (95%CI): 0.78(0.13, 1.44), $P=0.02$] (Figure S2E [↗](#), Table S4 [↗](#)).

Conversely, SMA showed significant positive association only with the transcript levels measured by the gpA2 primer [β (95%CI): 0.39(0.02, 0.77), $P=0.04$] (Figure 2F [↗](#), Table S3 [↗](#)). Interestingly, SMA showed significant negative association with transcript quantity obtained with the primer targeting overall group B expression (gpB) and DC9 (dc9): gpB [β (95%CI): -0.49(-0.81, -0.16), $P=0.003$] and dc9 [β (95%CI): -0.44(-0.83, -0.04), $P=0.03$] (Figure 2F [↗](#), Table S3 [↗](#)). Upon exclusion of children with overlapping syndromes, SMA remained positively associated with transcript levels measured by the gpA2 primer [β (95%CI): 0.74(0.26, 1.22), $P=0.002$] but lost significance with gpB and dc9. Additional positive significant association emerged with transcripts detected by the primers gpA1 [β (95%CI): 0.51(0.03, 0.98), $P=0.04$], gpA-median [β (95%CI): 0.62(0.14, 1.09), $P=0.01$], dc8-1 [β (95%CI): 0.79(0.18, 1.39), $P=0.01$], dc8-2 [β (95%CI): 0.59(0.02, 1.17), $P=0.04$], dc8-3 [β (95%CI): 0.68(0.14, 1.22), $P=0.01$], and dc8-median [β (95%CI): 0.54(0.03, 1.04), $P=0.04$] (Figure S2F [↗](#), Table S4 [↗](#)).

SMA parasites have a higher homogenous *var* expression profile among admissions

To determine whether parasites associated with different severe malaria syndromes differ in the number of distinct *var* genes they express, we compared *var* expression homogeneity (VEH) across clinical malaria presentations. VEH was calculated from the expressed sequence tag data as previously described (Warimwe et al., 2013 [↗](#)). Parasites from children with SMA exhibited the highest VEH, with significantly higher values than those observed in IC ($p = 0.02$), moderate ($p = 0.0003$), and mild ($p = 0.03$) malaria cases (Figure 3A [↗](#)). Consistent with this, children with SMA, either alone or in combination with other severe syndromes, showed higher values than non-SMA cases ($p = 0.01$) (Figure 3B [↗](#)).

To explore factors underlying the elevated VEH observed in SMA, we examined associations with parasite and host variables. VEH was negatively associated with global *var* transcript abundance quantified by RT-qPCR. Notably, transcripts from group A *var* genes showed the weakest negative association, whereas group B and C transcripts showed the strongest negative associations (Figure 3C [↗](#)). This pattern suggests that increasingly homogeneous *var* expression profiles are more likely to be dominated by group A *var* genes, consistent with the negative association of group B and C expression with SMA and the positive association of VEH with this syndrome. In contrast, PfSir2a expression, HRP2 levels, and peripheral blood parasite density showed weak, non-significant negative correlations with VEH (Figure 3C [↗](#)), although PfSir2a expression was significantly lower in SMA compared with IC ($p = 0.03$) (Table S5 [↗](#)).

Previous work has reported higher VEH in asymptomatic infections than in clinical malaria, a pattern attributed to antibody-mediated selection against infected erythrocytes (Warimwe et al., 2013 [↗](#)). In line with this, antibody responses to infected erythrocytes were positively associated with VEH ($\rho = 0.14$, $p = 0.00006$) (Figure 3C [↗](#)). However, this mechanism is unlikely to account for the elevated VEH observed in SMA, as affected children were younger (Table S2 [↗](#)) and had the lowest antibody levels (Figure 3D [↗](#)). Fever duration prior to admission was longest in SMA and shortest in IC (Figure 3E [↗](#)), consistent with SMA reflecting a more prolonged infection, although fever duration itself was not associated with VEH ($\rho = 0.04$, $p = 0.45$) (Figure 3C [↗](#)). In contrast, febrile temperature showed a significant negative association with VEH ($\rho = -0.11$, $p = 0.01$) (Figure 3C [↗](#)) and was lowest in SMA (median [IQR] 38°C [37.3-38.8]; Table S5 [↗](#)), consistent with reduced PfSir2a expression in this group (Abdi et al., 2023 [↗](#)).

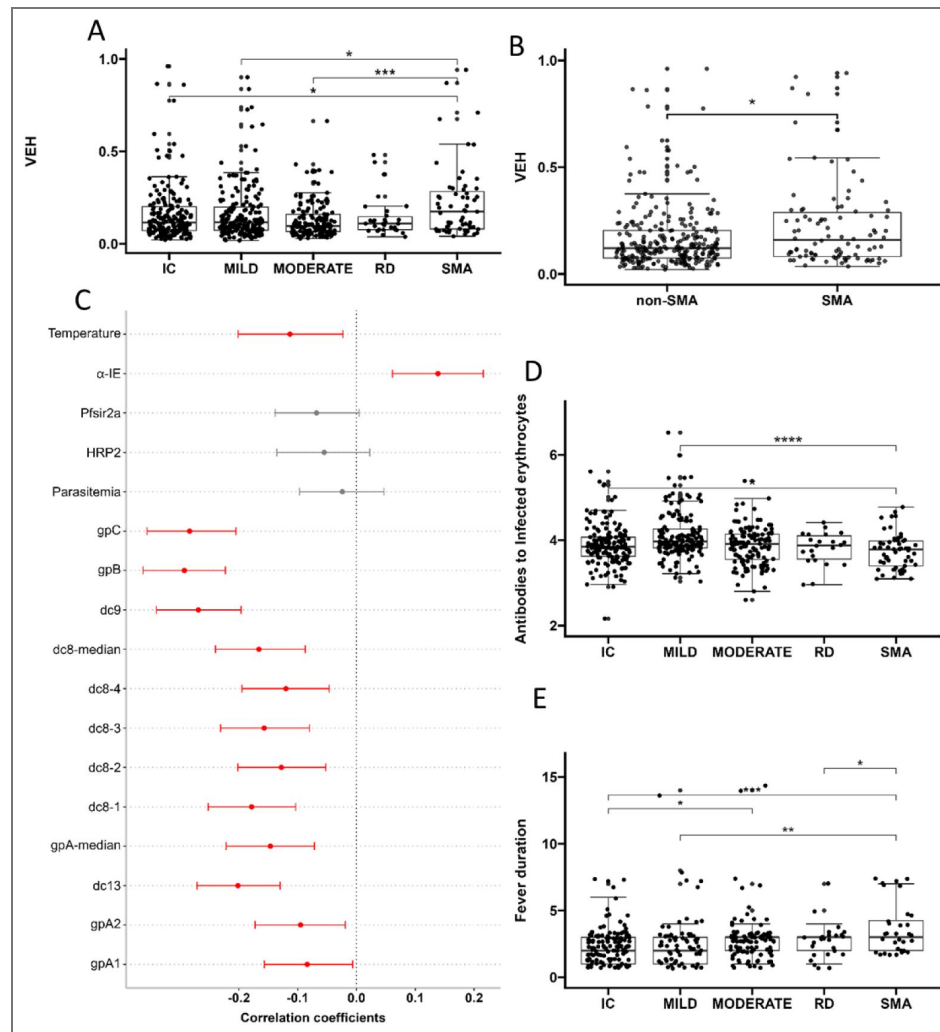


Figure 3. Relationship between VEH and parasite or host factors

A). Distribution of Variant Expression Homogeneity (VEH) values across pure clinical malaria groups in children. The y-axis shows VEH scores (higher values show more homogenous var expression); x-axis show clinical presentation. **B).** Comparison of VEH between SMA and non-SMA cases. **C).** Spearman's correlation between VEH and parasite factors, coefficient >0 indicate a positive association while coefficient <0 shows a negative association. **D).** Levels of antibodies against infected erythrocytes across clinical malaria presentation. **E).** Caregiver-reported fever duration (days) at admission. Horizontal brackets indicate significant pairwise differences (Wilcoxon rank-sum test). * <0.05 , ** <0.001 , *** p value <0.0001 , **** p value <0.0001 . α -IE; antibodies to Infected erythrocytes, ic: Impaired consciousness, rd: Respiratory distress, sma: Severe malarial anaemia.

Taken together, these findings suggest that SMA is associated with a more chronic infection state, in which sustained cytoadhesion-related selective pressures may favour parasites with a narrower, group A-dominated *var* expression profile compared with IC and respiratory distress.

Group A *var* genes and rosetting are differentially associated with severe malaria syndromes

Using 254 children with rosetting information, we fitted a binary logistic model to determine if rosetting is associated with severe malaria compared to non-severe. Rosetting frequency was significantly associated with severe malaria compared to non-severe malaria [OR: 3.16 (1.33, 7.95), $p = 0.01$] (figure 4A [↗](#)).

To evaluate the relationship between rosetting and individual syndromes while controlling for overlapping presentations, we constructed a linear regression model with rosetting frequency as the outcome, and IC, RD, and SMA as predictors while adjusting for age and parasite density. Rosetting was significantly associated with RD [β (95%CI) 0.12(0.02,0.21), $P=0.02$] and SMA [β (95%CI) 0.15(0.02,0.23), $P=0.01$], but not with IC [β (95%CI) 0.05(-0.03,0.12), $P=0.22$] (Figure 4B [↗](#)). We further show that rosetting was positively correlated with the expression of group A and DC8 *var* genes, and negatively with expression of group B *var* genes (Figure 4C [↗](#)).

To determine whether RD and SMA associations with rosetting were independent of *var* gene expression, we conducted logistic regression models for each syndrome with rosetting frequency as a predictor while adjusting for expression levels of *var* gene subtypes that showed significant association with rosetting and RD or SMA individually (Table 2 [↗](#)). Rosetting remained significantly associated with RD even after adjusting for group A-like [OR (95%CI): 3.33 (1.10, 9.79), $P=0.03$], gpA1 [OR (95%CI): 3.29 (1.06, 10.02), $P=0.04$], and gpA2 [OR (95%CI): 3.2 (1.03, 9.67), $P=0.04$] (Figure 4D [↗](#), Table 2 [↗](#) (models 5B, 6B&7B)). Similarly, Rosetting remained significantly associated with SMA after adjusting for group A-like [OR (95%CI): 3.74(1.13, 12.13), $P=0.04$] while adjusting for gpA1 and gpA2 resulted in loss of statistical significance in the association between SMA and rosetting (Figure 4E [↗](#), Table 2 [↗](#) (models 6C&7C)).

Group A-like *var* expression is independently associated with malaria mortality

Among the 712 patients included in this study, 40 died (Table S2 [↗](#)). Among the 40 patients that died, 14 had overlapping syndromes, 5 had moderate malaria, 20 had IC, and 1 had SMA while none died from either mild or RD (Figure 5B [↗](#)). We performed a logistic regression model with both group A-like *var* expression levels and parasite density as explanatory variables with death as an outcome. Parasite density was not associated with child death [OR (95%CI): 1.00 (0.99, 1.00), $p = 0.22$]. However, death was predicted by group A-like *var* gene expression levels [OR (95%CI): 5.61 (1.80, 17.54), $p=0.003$, parasite density adjusted] (Figure 5A [↗](#)). The association was maintained even when the analysis was restricted to only children with impaired consciousness [OR (95%CI): 14.40 (3.140, 74.5), $P = 0.0008$, adjusted for parasite density]. *cys2* and *cp2* were also associated with increased risk of mortality while gpB and gpC were associated with reduced risk of mortality, while the rest of *var* subtypes showed no significant association with mortality outcome (Table S6 [↗](#)).

Discussion

This study provides a comprehensive analysis of the relationship between *var* gene transcription profiles and the severe malaria syndromes IC, RD and SMA (Marsh et al., 1995 [↗](#)). By integrating *var* expression data from both DBLA-tag sequencing and RT-qPCR analysis of parasite samples collected over 18-year period of changing transmission intensity in Kilifi, Kenya, we provide important insights into the relationship between expression of *var* gene subtypes and clinical presentation of severe malaria. Our findings reveal distinct associations between expression of *var* gene subgroups and severe malaria phenotypes. Notably, transcripts belonging to the *cys2* and

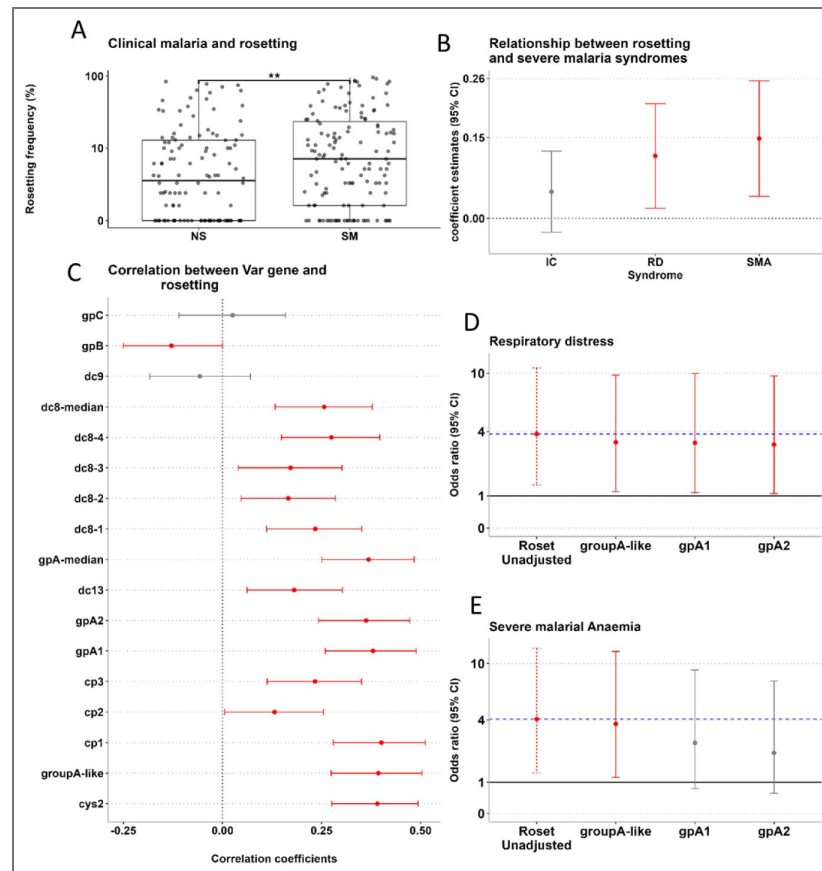


Figure 4. Relationship between rosetting, group A var gene expression and malaria

A). Mean percentage rosetting frequency between severe and non-severe malaria. NS: non-severe malaria, SM: Severe malaria. B). Association between rosetting and severe malaria syndromes. C) Spearman correlation coefficients (with 95% confidence intervals) between the frequency of rosetting and expression levels of different *var* gene subgroups. Red points indicate statistically significant correlations ($p < 0.05$). Subgroups include (cys2, groupA-like, cp1–cp3) from DBL α -tag data and gpA1, gpA2, dc8-1,–dc84, dc13, dc9, gpB and gpC from RT-qPCR data. D and E) Logistic regression models assessing the association between rosetting and malaria syndromes after adjusting for group A var genes. D) Respiratory distress, and E) severe malarial anaemia. The blue dotted line represents odds ratio for rosetting on a specific syndrome before adjusting for var gene expression. Red error bars indicate statistically significant associations ($p < 0.05$).

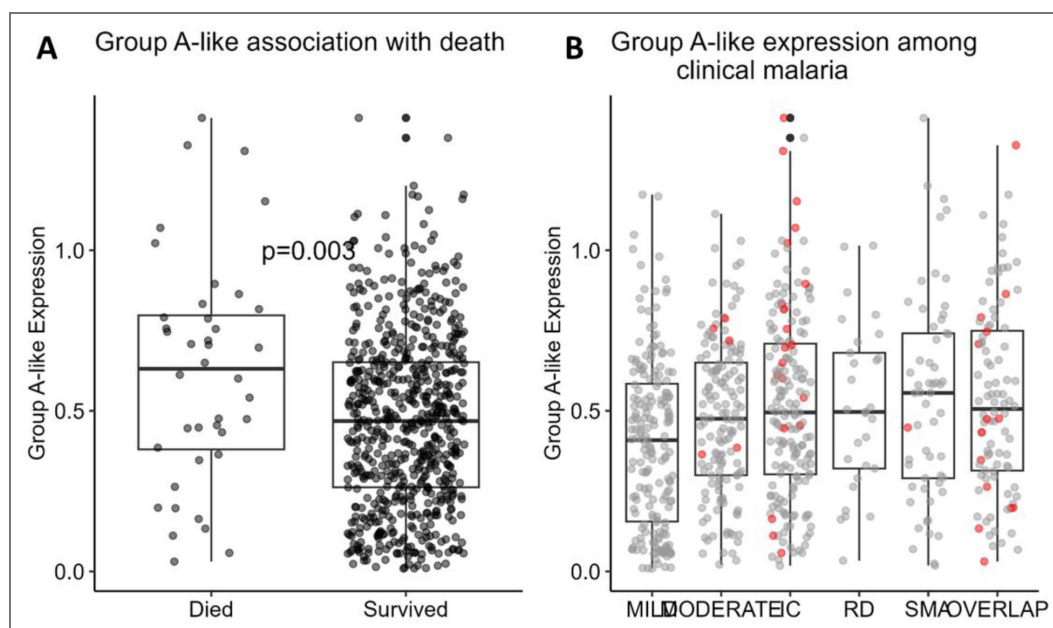
Table 2. Relationship between group A *var* genes and rosetting in different malaria syndromes using logistic regression models.

Models 1-4 are unadjusted while models 5 to 7 are adjusted for either rosetting or *var* gene expression levels. Bold pvalues are significant at alpha less than 0.05

Model	Variables	A: Impaired consciousness		B: Respiratory distress		C: Severe malaria anaemia	
		OR (95% CI)	p.value	OR (95% CI)	p.value	OR (95% CI)	p.value
1 A,B,C	Rosetting	2.37 (0.99, 5.80)	0.055	3.84(1.34, 10.91)	0.011	4.07 (1.29, 12.78)	0.015
2 A,B,C	Group A-like	3.53(1.35, 9.51)	0.011	1.96 (0.58, 6.56)	0.273	1.78 (0.48, 6.54)	0.383
3 A,B,C	gpA1	1.34 (1.12, 1.62)	0.002	1.13 (0.92, 1.46)	0.217	1.42(1.08, 1.91)	0.018
4 A,B,C	gpA2	1.20 (1.01, 1.44)	0.041	1.16 (0.93, 1.47)	0.204	1.70(1.27, 2.38)	0.001
5 A,B,C	Rosetting	1.46 (0.56, 3.76)	0.434	3.33 (1.10, 9.79)	0.030	3.74(1.13, 12.13)	0.027
	Group A-like	3.22(1.20, 8.91)	0.022	1.46 (0.41, 5.11)	0.556	1.28 (0.32, 4.99)	0.715
6 A,B,C	Rosetting	1.36(0.51, 3.61)	0.539	3.29 (1.06, 10.02)	0.036	2.64 (0.75, 9.11)	0.123
	gpA1	1.31 (1.09, 1.60)	0.005	1.05 (0.83, 1.35)	0.674	1.34(1, 1.83)	0.061
7 A,B,C	Rosetting	1.63 (0.62, 4.23)	0.322	3.2 (1.03, 9.67)	0.040	2.16 (0.59, 7.63)	0.234
	gpA2	1.17 (0.98, 1.41)	0.089	1.08 (0.85, 1.38)	0.527	1.63 (1.19, 2.30)	0.003

Figure 5. group A-like expression levels in relation to death

A) Group A-like expression in children who died (n = 40) versus those who survived (n = 614). P is a pvalue obtained from logistic regression model. B) Expression of Group A-like expression across clinical malaria syndromes; Red points indicate children who died. Number of deaths by syndrome: mild=0, moderate = 5, IC = 20, SMA = 1, overlap = 14, RD = 0. IC: impaired consciousness, RD: respiratory distress, SMA: severe malarial anaemia, overlap: mixed malaria infection with two or all the three severe malaria syndromes.



DC8 were associated with an increased risk of IC while RD showed no association with *var* expression. SMA was characterised by parasites exhibiting a more homogeneous *var* expression profile, while rosetting was associated with RD and SMA, but not with IC.

The pathogenesis of cerebral malaria, the more severe form of IC, has been closely linked to sequestration of iRBC in the brain microvasculature (MacPherson et al., 1985; Taylor et al., 2004). This process is mediated by *PfEMP1* variants capable of binding host endothelial receptors such as endothelial protein C receptor (EPCR), a characteristic feature of DC8-expressing parasites (Avril et al., 2013; Storm et al., 2019; Turner et al., 2013). Our data is consistent with previous findings that demonstrated higher expression of EPCR-binding *PfEMP1* in parasites from cerebral malaria than SMA (Shabani et al., 2017), reinforcing the role of DC8 variants in the pathogenesis of cerebral malaria. The elevated expression of group A and DC8 *var* genes in IC cases further underscores their importance in disease pathogenesis and highlights these *PfEMP1* variants as potential targets for intervention. In this context, recent a recent study has demonstrated that, despite their high sequence variability, EPCR-binding CIDR α 1 domains maintain a conserved structural motif (Rajan Raghavan et al., 2023). This structural conservation may allow recognition by broadly neutralizing monoclonal antibodies, but this remains to be tested on diverse parasite isolates (Reyes et al., 2024). Similarly, Tessema et al., reported that antibodies against cp1 recombinant proteins were significantly associated with protection against severe malaria (Tessema et al., 2019). Our findings together with previous studies (Kyriacou et al., 2006; Shabani et al., 2017; Warimwe et al., 2009), illustrate the potential value of targeting *PfEMP1* subtypes associated with cerebral malaria, which is the severe syndrome that carries the highest case fatality rate.

Our results suggest that *PfEMP1* subtypes targeted by gpA2 and dc8-1 primers, as well as those belonging to cp2 may play a central role in the pathogenesis of SMA. We also observed that parasites from SMA cases displayed a more homogeneous *var* expression profile compared to the other severe syndromes. Previously, higher VEh has been observed in asymptomatic infection compared to clinical cases (Warimwe et al., 2013), with this pattern attributed to selection pressure exerted by antibodies against iRBCs. In SMA, however, antibody levels are unlikely to drive VEh, as affected children are predominantly young with low antibody levels. Instead, the chronicity of infection, reflected by the reported long fever duration, and the lower febrile temperatures observed in SMA cases in this study may represent a shared feature underlying the observed VEh patterns in both SMA and asymptomatic infections. Overall, our data indicate that SMA is associated with parasites exhibiting a more homogeneous *var* expression pattern, dominated by group A subtypes, including those containing the cp2 motif. While *PfEMP1*-mediated cytoadhesion likely contributes to SMA pathogenesis, chronicity of the infection as previously suggested in a review by White, (2018) may also lead to loss of erythrocytes due to continuous destruction of red cells (Moss et al., 2026), and inflammation induced dyserythropoiesis (Knüttgen, 1987; Liu & Li, 2025; Lyke et al., 2004; Phillips et al., 1986).

Consistent with a previous study (Warimwe et al 2012), our data showed that rosetting was significantly associated with RD, but not with IC. Additionally, we showed that rosetting was associated with SMA. However, when we adjusted for *var* gene expression, the association between rosetting and SMA dropped and lost statistical significance while that with RD remained significant. This pattern suggests that rosetting may be a driving factor for RD pathogenesis but not for SMA. In contrast, adjusting for rosetting did not eliminate the associations between group A-like and gpA1 *var* transcripts for IC, and adjusting for gpA2 did not alter its association with SMA, supporting the hypothesis that these *var* gene subgroups play a primary role in the pathogenesis of respective syndromes.

A previous smaller study (Warimwe et al 2012) whose samples were also included in this study, showed no *var* gene group remained associated with RD after adjusting for rosetting, suggesting that rosetting may be a more dominant determinant of RD pathogenesis than *var* gene expression per se. Alternatively, the lack of association could reflect the absence of specific *var* subtypes relevant to RD pathogenesis in our dataset. Supporting this, McLean et al., (2025) identified domain cassettes DC11 and DC15 as mediators of rosetting in laboratory isolates, subtypes not

directly captured by our primers. Future studies should therefore incorporate primers targeting these domain cassettes to elucidate the molecular basis of RD in field isolates. It is also possible that other variant surface antigens (VSAs), beyond the *var* gene family contribute to RD pathogenesis. Members of the surface-associated interspersed gene family (SURFIN), repetitive interspersed family (RIFIN), and subtelomeric variable open reading frame (STEVOR) proteins have been implicated in malaria pathogenesis (Kanoi et al., 2020 [↗](#)). Future investigations of RD should therefore consider these additional VSA families to achieve a more complete understanding of RD pathogenesis.

Mortality was highest among children with IC. This finding corroborates previous work showing that mortality is highest in children with cerebral malaria, a severe form of IC (Idro et al., 2007 [↗](#)). In our analysis, group A-like *var* gene expression was significantly associated with both IC and mortality, aligning with previous reports (Warimwe et al., 2009 [↗](#), 2012 [↗](#)). These findings suggest that group A-like expressions may serve as a marker for malaria-related mortality and highlight its potential clinical utility in prognostic models.

A key strength of this study is the use of DBLα-tag sequencing and RT-qPCR approaches, which have provided complementary understanding of the *var* transcriptome. Whereas DBLα-tag sequencing captures the overall breadth of expressed *var* repertoires, RT-qPCR allows precise quantification of clinically important *PfEMP1* subsets. Considering both datasets together revealed subtype-syndrome associations that would likely have been missed using either method alone. Despite this advantage, the cross-sectional design limits causal inference, and undetermined host genetic or immunological factors may also shape *var* expression profiles. Therefore, future studies should incorporate these variables to refine the observed relationships.

In conclusion, the use of DBLα-tag sequencing and RT-qPCR has allowed us to build a clearer and more reliable picture of subtype-specific *var* gene expression across the major severe malaria syndromes. Our findings consistently point EPCR-binding observed in impaired consciousness may be driven by DC8, cp1 and group A-like *PfEMP1* variants, reinforcing the central role of sequestration in the pathogenesis of impaired consciousness. Taken together, these results highlight the importance of focusing on *PfEMP1* subsets mediating sequestration and provide a strong rationale for their consideration in the development of future therapeutics and vaccines.

Materials and Methods

Study population

This study was conducted in Kilifi County, a malaria-endemic region located along the Kenyan coast. The patient samples analyzed in this study have been previously described (Abdi et al., 2023 [↗](#)). Briefly, the cohort comprised 521 children diagnosed with malaria who were admitted to Kilifi County Hospital (KCH) and 191 treated for mild malaria at outpatient clinics in Kilifi between 1994 and 2012. This period represents three distinct phases of malaria transmission in the region: pre-decline, decline, and post-decline (Mogeni et al., 2016 [↗](#)). These children were enrolled through the hospital's admission surveillance system, as well as from children presenting with mild malaria at an outpatient clinic.

Definition of malaria syndromes

Severe malaria was defined as a hospital admission with any of the following; impaired consciousness (IC) defined using age-appropriate Blantyre coma score (BCS) thresholds (BCS of less than 5 for children over 8 months old or a BCS of less than 4 for children younger than 8 months) (Berkley et al., 2009 [↗](#)), or respiratory distress (RD) (deep breathing) (English et al., 1996 [↗](#)) or severe malarial anaemia (SMA) (hemoglobin < 5g/dl) (Marsh et al., 1995 [↗](#)) or overlapping syndromes with more than one severe malaria syndrome presentation.

Sample inclusion and size

Participants included in this study were those in the KEMRI database with complete information on clinical syndrome, age, parasite density and *var* gene expression of the iRBC determined by both DBLα-tag sequencing and RT-qPCR quantification approaches. 712 participants fulfilled these criteria including 340 with non-severe malaria and 372 children with severe malaria including pure syndromes (59 SMA, 29 RD, 186 IC) and 98 with overlapping syndromes in the form of IC/RD, IC/SMA, RD/SMA or IC/RD/SMA (Figure 1B). Non-severe cases included 191 malaria positive children managed at outpatient clinics referred to as mild and 141 children admitted to hospital who did not present with any of the three severe syndromes described above, referred to as moderate.

Peripheral Blood Parasitemia

Peripheral parasitemia was quantified from Giemsa-stained thick and thin blood films following standard microscopy procedures. Parasite density was initially recorded as the number of infected erythrocytes per 500, 200, or 100 red blood cells (RBCs) examined. Parasitemia per microlitre of blood was then calculated using World Health Organization (WHO) guidelines (WHO, 2010). Briefly, parasite density (parasites/μl) was derived using either:

$$\text{parasites}/\mu\text{l} = \frac{\text{number of parasitized RBCs} \times \text{RBCs per } \mu\text{l}}{\text{number of RBCs counted}}$$

or, for counts performed against white blood cells (WBCs):

$$\text{parasites}/\mu\text{l} = \frac{\text{number of parasites counted} \times \text{WBCs per } \mu\text{l}}{\text{number of WBCs counted}}$$

In instances where direct measurements of RBC or WBC concentrations were unavailable, standard assumptions of 5×10^6 RBCs/μl and 8,000 WBCs/μl were applied, in accordance with WHO recommendations.

PfHRP2 Assay

Plasma concentrations of *P. falciparum* histidine-rich protein 2 (PfHRP2) were measured using a sandwich ELISA as previously described (Abdi et al., 2023). In brief, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated overnight at 4°C with mouse anti-PfHRP2 monoclonal antibody (MPFM-55A; MyBioscience) diluted in PBS to a final concentration of 0.9 μg/ml. Plates were washed three times with PBS containing 0.05% Tween-20 and subsequently blocked with PBS supplemented with 3% skimmed milk for 2 hr at room temperature (RT).

After an additional wash step, plasma samples and recombinant PfHRP2 standards (MBS232321; MyBioscience), diluted in PBS with 2% BSA, were added in duplicate and incubated for 2 hr at RT. Plates were washed and incubated for 1 hr with HRP-conjugated mouse anti-PfHRP2 detection antibody (MPFG-55P; MyBioscience) diluted in PBS/2% BSA to a final concentration of 0.2 μg/ml. Following three washes, colour development was achieved using o-phenylenediamine dihydrochloride (OPD) substrate for 15 min, and reactions were stopped with 2M H₂SO₄. Optical density (OD) was read at 490 nm using a BioTek Synergy4 plate reader. The ODs were log-transformed for further analysis.

RNA and cDNA preparation

The method for RNA extraction and cDNA preparation from the samples used in this study was described previously (Abdi et al., 2016; Warimwe et al., 2012). Briefly, samples stored in Trizol (Invitrogen, Paisley, United Kingdom) at -80°C were thawed, and then total RNA was extracted by chloroform phase separation and GlycoBlue/isoamyl alcohol precipitation. Pellets were washed with 75% ethanol, briefly air-dried, and re-suspended in RNase-free water. Residual genomic DNA was removed by DNase I treatment (DNase-Free, Ambion) and inactivated with the Ambion DNase Inactivation Reagent according to the manufacturer's instructions. cDNA was prepared by reverse

transcribing RNA using reverse transcriptase (Invitrogen Superscript III). For each cDNA reaction, a negative control reaction was performed in the absence of reverse transcriptase to ensure that all contaminating DNA had been removed by DNase pre-treatment. No sample showed any amplification in these controls.

Var sequencing and classification

The expressed sequence tag (EST) sequencing approach used to generate this dataset has been previously described (Abdi et al., 2016; Warimwe et al., 2009). In summary, degenerate primers (Bull et al., 2005), were used to amplify a region within the DBL α -tag domain that is highly conserved across most var genes. Two sequencing methods were employed: capillary sequencing and the 454-pyrosequencing platform.

For the capillary sequencing approach, amplified DBL α -tags were cloned into the TOPO α -TA cloning vector and transformed into *E. coli*. From each sample, up to 96 colonies were picked and sequenced individually. While for the 454 platform, the amplicons were barcoded, multiplexed, and sequenced using high-throughput 454 sequencing (Kivisi et al., 2019).

Sequences obtained from each sample were then classified and counted following methods described previously (Bull et al., 2005; Warimwe et al., 2009). EST proportional expression scores were calculated as the proportion of sequences in each sample that belonged to specific sequence groups. These groups were defined based on the number of cysteine residues, the presence or absence of conserved amino acid sequence motifs (MFK and REY) located in positions of limited variation (PoLV) and presence of block sharing block within the DBL α -tag. Overall, according to number of cysteine residues DBL α -tag sequences were classified into three groups namely, cys2 for those containing two cysteine residues, cys4 for those with four and cysX for those containing one, three, five, or six cysteine residues. Combining the number of cysteine residues and PoLV (cp grouping) resulted into six groups (cp1–cp6) as shown in Table 1. All known cp1 sequences fell in group A var genes; however, not all group A sequences can be grouped as CP1 (Bull et al., 2007). A network of recombining sequence that consisted of sequence blocks that tent to recombine with each other was used to determine block sharing groups (BS). The BS groups were made up of polymorphic blocks together with the number of cysteine residues in the DBL α -tag. Sequences that fall into block-sharing Group 1 and have two cysteine residues (BS1_cys2) belong to group A-like var genes. Only cp1, cp2, cp3 and group A-like are used in the analysis because of the previous association with severe malaria (Warimwe et al., 2009).

RT-PCR var transcription profiles

The qRT-PCR primers used to determine var transcript profile data in this study was published by Abdi et al. (Abdi et al., 2016) and was originally designed by (Lavstsen et al., 2012; Rask et al., 2010; Rottmann et al., 2006). Data was generated using primers that showed differential expression between severe and non-severe malaria in previous studies (Lavstsen et al., 2012). These included four primer sets targeting DC8 (named dc8-1, dc8-2, dc8-3, dc8-4), one primer set for each of DC13 (dc13), DC4 (dc4), and DC9 (dc9), two primer sets targeting the majority of group A var genes (gpA1 and gpA2). Group B and C var gene transcription was quantified using primer sets b1 and c2 respectively. The names and sequences of the original primers are shown in Table S1. In addition, we included a primer targeting *Pfsir2a*, which plays a role in epigenetic control (Abdi et al., 2016).

Levels of var gene transcription were normalized relative to the housekeeping gene *seryl tRNA synthetase* and *fructose biphosphate aldolase*. The arbitrary transcript unit (Tus) was calculated using the formula ($Tus = 2^{(5-\Delta ct)}$) where Δct is the averaged change in cycle threshold of var gene relative to housekeeping genes (Abdi et al., 2016; Lavstsen et al., 2012).

Variant Expression Homogeneity (VEH) Calculation

To quantify the degree of dominance among *var* gene transcripts expressed by each parasite isolate in a sample, we assessed Variant Expression Homogeneity (VEH). VEH reflects the extent to which a small number of *var* gene sequences dominate an isolate's expression profile (Warimwe et al., 2013 [\[1\]](#)).

We employed Simpson's Index of Diversity to capture the overall homogeneity of the *var* expression repertoire (Simpson, 1949 [\[2\]](#); Warimwe et al., 2013 [\[1\]](#)). For each sample, we calculated Simpson's Index (λ) as the sum of the squares of proportions of all distinct *var* gene expressed in each sample:

$$\lambda = \sum_{i=1}^N \pi_i^2$$

Where; π_i is the proportion of *var* gene *i* among *N* total *var* genes counted in a sample. The index ranges from $\frac{1}{N}$ (indicating maximal diversity and minimal homogeneity) to 1 (indicating complete dominance by a single sequence and thus maximal VEH).

Rosetting Assay

Cryopreserved parasite isolates were thawed following previously described methods (Kinyanjui et al., 2004 [\[3\]](#)). A suspension containing trophozoite-infected erythrocytes at 4% hematocrit was prepared in RPMI 1640 medium (Gibco) supplemented with 5 μ g/ml acridine orange (Sigma-Aldrich). For each isolate, 9.5 μ l of this suspension was mixed with 2.5 μ l of non-immune AB serum in a U-bottomed 96-well plate. The mixture was rotated vertically at room temperature for 30 minutes. Subsequently, the sample was transferred onto a microscope slide, covered with an 18mm x 18mm coverslip, and examined under a fluorescence microscope (Nikon Eclipse 80i, Japan) at 400x magnification. Rosetting frequency was determined as the percentage of 200 trophozoite-infected erythrocytes binding to two or more uninfected erythrocytes. Scoring was conducted without knowledge of the clinical category of the patient from whom each isolate originated.

Antibody Recognition of trophozoite-infected erythrocytes (α -IE Assay)

Total IgG binding to the surface of trophozoite-infected erythrocytes (IEs) was quantified by flow cytometry, as described previously (Kivisi et al., 2019 [\[4\]](#)). Briefly, plasma samples were incubated with IEs from four heterologous clinical isolates, and antibody binding was detected using a fluorescein isothiocyanate (FITC)-conjugated anti-Fc γ antibody (AF004, The Binding Site). Infected and uninfected erythrocytes were differentiated by ethidium bromide staining, enabling correction for nonspecific IgG binding by subtracting the MFI of uninfected erythrocytes from that of matched IEs for each sample. To account for isolate-specific background reactivity, the highest MFI observed among plasma from four malaria-unexposed European donors was subtracted from the corresponding values obtained with each child's plasma. The resulting background-adjusted MFI values were used in all downstream analyses.

Statistical analysis

We selected samples with both RT-qPCR and DBL α -tag sequence transcript data for analysis. RT-qPCR-derived *var* transcript expression values were log-transformed, while proportions of DBL α -tag-defined *var* subtypes were arcsine square root transformed (Warimwe et al., 2009 [\[5\]](#)). To identify associations between *var* gene expression and severe malaria syndromes (IC, RD, and SMA), binary variables of the severe syndromes were created and non-severe cases were considered as controls. We fitted multiple linear regression models using stats R core package (R Core Team, 2021 [\[6\]](#)). The model followed the general structure below in equation 1 [\[7\]](#):

$$y \sim b + c + d + e + f \quad \dots \dots \dots \text{equation 1}$$

Where:

y represents specific *var* gene defined by RT-qPCR primer sets or DBL α -tag subtype, b represents binary representation where all children with IC syndrome were assigned 1 and those not containing it were assigned 0, c represents binary representation where children with RD syndrome were assigned 1 and those not containing were assigned 0; d ; represents binary representation where all children with SMA syndrome were assigned 1 and those not containing were assigned 0 e ; represents age of the participant in months while f represent log transformed values of peripheral blood parasite density. This analysis was repeated where children with overlapping syndromes were excluded with the aim of identifying syndrome-specific *var* gene variants.

The output of the model are p-values and beta coefficient of estimate with 95% confidence interval (β (95%CI). Positive and negative beta (β) values with p-values less than 0.05 show positive and negative significant associations with specific malaria syndrome respectively in comparison to the control group. The results are presented as forest plots or tables.

We compared VEh scores across pure clinical presentations using two-sided Wilcoxon rank-sum tests to determine the syndrome with more homogenous *var* expression. To explore determinants of VEh, we assessed associations with host factors (α -IE, fever duration before admission and febrile temperature) and parasite factors (Pfsir2a, peripheral blood parasite density and RT-qPCR *var* gene transcripts expression levels) using Spearman's rank correlation. We report correlation coefficients (ρ) and p values, and visualize group differences and associations using ggplot2 in R.

To dissect the relationship between *var* expression, severe malaria syndromes and rosetting, the frequency of rosetting was arcsine square root transformed. Binary logistic regression model on 253 children with rosetting data including 133 severe and 120 non-severe cases was fitted to determine the association between rosetting and severe malaria using logistic regression. Correlation between rosetting frequency and *var* gene expression was assessed using Spearman's rank correlation coefficient. To estimate 95% confidence intervals (95% CIs) for the correlation coefficients, we applied nonparametric bootstrapping with 5000 replicates using boot R package (Canty & Ripley, 2024 [\[1\]](#)). This was followed by evaluating the relationship between rosetting and individual severe malaria syndromes. We constructed a linear regression model as in equation 1 [\[1\]](#) and replaced y with rosetting frequency. To test whether the relationship between rosetting and the severe syndromes is independent of *var* expression, we conducted logistic regression models considering each of syndromes as outcome with rosetting frequency as a predictor while adjusting for expression levels of *var* gene subtypes.

To identify parasite factors associated with mortality, we fitted binary logistic regression models with death (yes/no) as the outcome. We first ran univariable models for each *var* transcript measure and for parasite density separately and then fitted multivariable models including the *var* measure and parasite density together to estimate the effect of each on death while adjusting for age in all models. Odds ratios (ORs) with 95% CIs are reported.

All analyses were conducted in R (version 4.4.1) (R Core Team, 2021 [\[2\]](#)). Forest plots of effect sizes from linear and logistic regression models were generated using ggplot2 (Wickham, 2016 [\[3\]](#)). Data manipulation was performed using dplyr and tidyverse packages (Wickham et al., 2023 [\[4\]](#)), and model tidying was implemented with broom.

Supplementary Figures

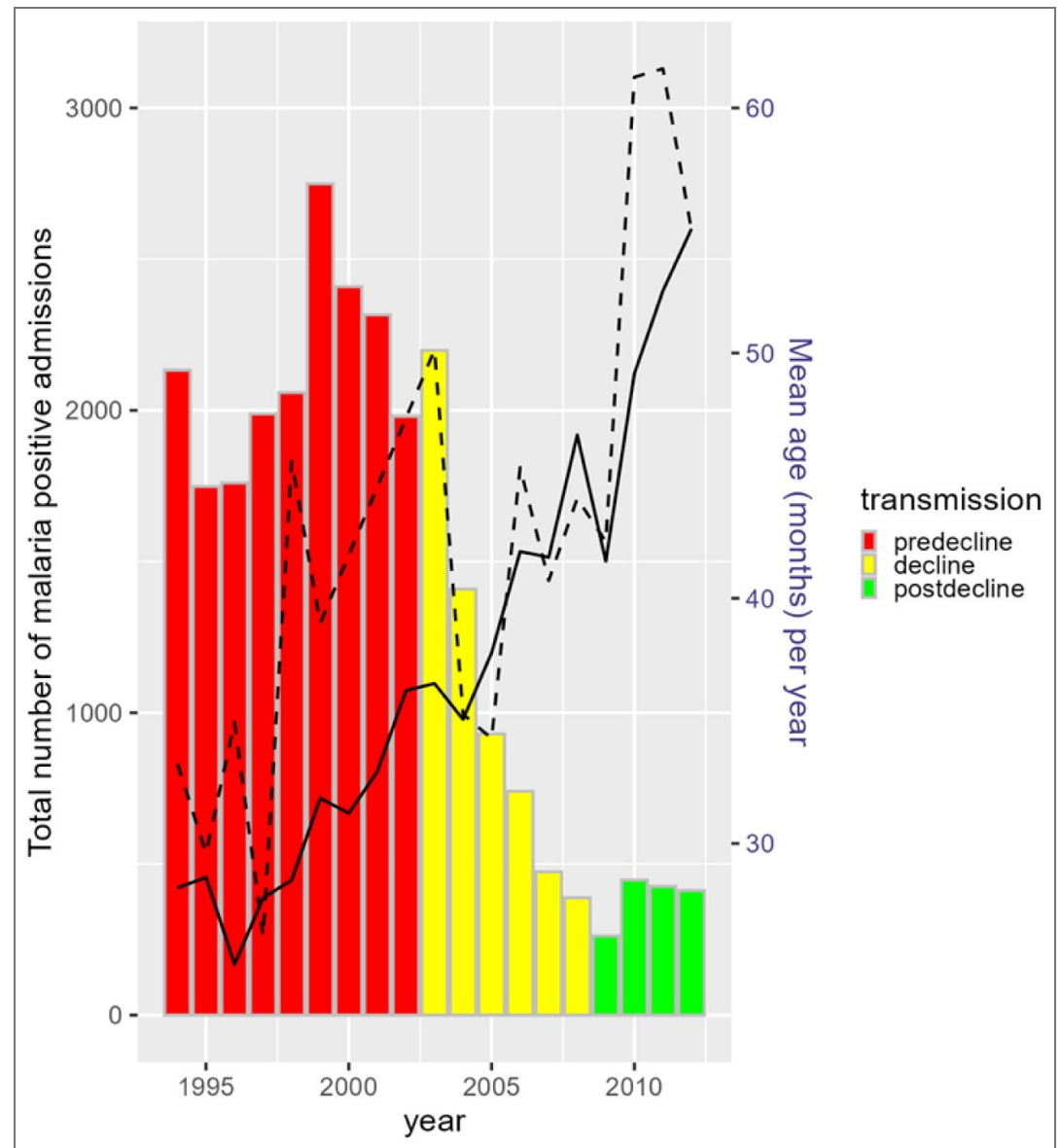


Figure S1. Total malaria admissions and mean patient age per year. Number of patients per year (left axis), mean patient age per year (right axis). The solid black line is the average patient age of total admission, the dashed line is the average patient age in this study

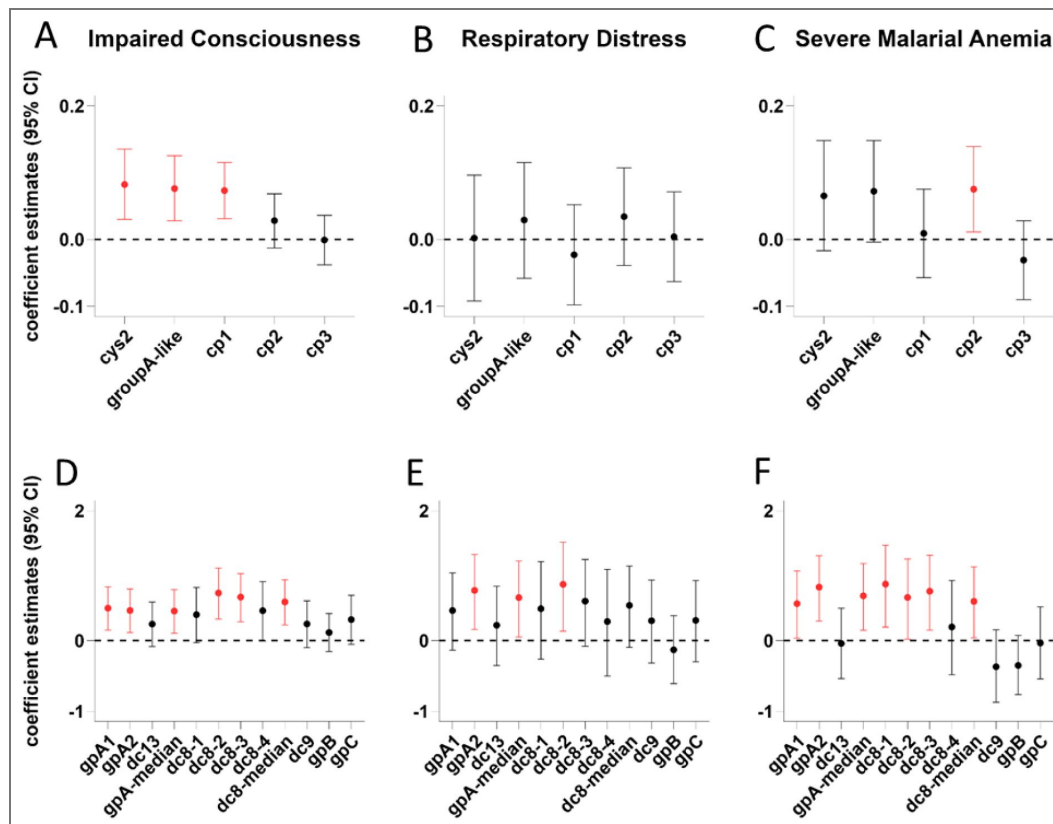


Figure S2. Associations between var gene expression and pure severe malaria syndromes.

A-C Results from five regression models in which *cys2* or its subgroups (group A-like, cp1, cp2, and cp3) were considered as single dependent variables with the pure severe malaria syndromes, impaired consciousness, respiratory distress and severe malaria anaemia as explanatory variables, while adjusting for age and parasitemia. D-E Results from twelve regression models considering the transcript levels for gpA1-gpC as sole dependent variables, with and impaired consciousness, respiratory distress and severe malaria anaemia as explanatory variables while adjusting for age and parasitemia. Regression coefficient (β) and corresponding 95% confidence intervals (CI) were plotted.

Supplementary Tables

Primer name	Common name	Description	Primer sequences
dbla_not_var3	gpA1	Majority of group A vars	Forward: TTGGGRAATBTGTTAGTTAYRGCAA Reverse: CTGCAAACTKCGWGCAAG
dbla2/a1.1/2/4/7	gpA2	Mainly group A but also DC8	Forward: TCCTACCAATTTAGATTACGTCCCTCA Reverse: CGTACAATCATATCCATTAAGACTAC
cidra1.4	DC13	group A subset containing domain cassette 13	Forward: AACAAATGGAAATGCTATTAYRA Reverse: TCGKTTTCCCACTTTATASTGT
cidra1.1	DC8-1	Group B subset containing domain cassette 8	Forward: TAARGTGGGAACATCAACTTAAGAA Reverse: CTAAATCTTYCGTAAATTGATCCCAT
dbla_cidra	DC8-2	Group B subset containing domain cassette 8	Forward: GYTCARAWTATTGCCAACCC Reverse: ACCSTYACAWWBAACTACAC
dblb12&dblb3&5	DC8-3	Group B subset containing domain cassette 8	Forward: CATCAAGTKTGGAGAGCTATGAAATGTG Reverse: TAATCTTCTATKGGGATACCATTACA
dblg4/6	DC8-4	Group B subset containing domain cassette 8	Forward: AGAAACAHATTTTGCKTGG Reverse: CCATGTTTCWTTCCACCAGTCDTCAAG
DBLg	DC9	Group B subset containing domain cassette 9	Forward: ATTTACACGAWSAATTAVAAAACTTTG Reverse: CTTTAGGTGGATWATCSAAAGCTTCAGG
b1	gpB	5' sequence of majority of group B	Forward: CATCCGCCATGCAAGTATAA Reverse: CGTGCACGATTTGATTTTT
c2	gpc	5' sequence of majority of group C	Forward: GTAGCGACAACCACGRYATCATGG Reverse: CATTGTTAACATAGTCTACCATTA

Table S1. Description of Primers used in quantifying var genes.

Syndrome	Age (Median[IQR])	Pvalue	Parasitemia [Median[IQR]]	Pvalue	Mortality (%)	Pvalue
NS (n=340)	44[23–71]	ref	168.60[36.81–350]	ref	5 (1%)	ref
SEVERE (n=372)	35[21–49]	<0.001	198.20[30.28–439.5]	0.351	35 (10%)	<0.001
IC (n=186)	39[25–56]	0.016	150[19.19–395.8]	0.603	20 (11%)	<0.001
RD (n=29)	48[33–59]	0.985	272[23.54–500]	0.173	0 (0%)	1
SMA (n=59)	26[12–38]	<0.001	190[67–407.40]	0.373	1 (2%)	1
IC/SMA (n=20)	26[18–36]	<0.001	126.60[35.78–363]	0.949	2 (10%)	0.052
RD/IC (n=62)	34[23–44]	0.007	263.00[57.50–529.40]	0.034	11 (18%)	<0.001
SMA/RD (n=6)	32[28–38]	0.24	252.40[171.9–351.2]	0.391	0 (0%)	1
IC/SMA/RD (n=10)	15[7–28]	<0.001	167[29.87–385.9]	0.953	1 (12%)	0.161

Table S2. Clinical Severity Comparison vs Non-Severe

model	<i>PfEMP1</i>	A: Impaired consciousness (n=277)		B: Respiratory distress (n=119)		C: Severe malaria anaemia (n=95)	
		β (95% CI)	p.value	β (95% CI)	p.value	β (95% CI)	p.value
1(A,B,C)	cys2	0.09(0.04, 0.14)	0.0002*	-0.003(-0.07, 0.06)	0.912	0.07(0.01, 0.14)	0.029
2(A,B,C)	groupA-like	0.07(0.03, 0.12)	0.001*	-0.003(-0.06, 0.06)	0.966	0.07(0.01, 0.14)	0.016
3(A,B,C)	cp1	0.09(0.05, 0.12)	0.0001*	-0.01(-0.06, 0.04)	0.57	0.03(-0.02, 0.09)	0.205
4(A,B,C)	Cp2	0.01(-0.03, 0.04)	0.691	-0.01(-0.06, 0.03)	0.587	0.03(-0.02, 0.09)	0.189
5(A,B,C)	Cp3	0.02(-0.02, 0.05)	0.396	0.01(-0.03, 0.07)	0.602	-0.02(-0.07, 0.03)	0.475
6(A,B,C)	gpA1	0.34(0.09, 0.62)	0.008	-0.05(-0.39, 0.29)	0.777	0.28(-0.09, 0.65)	0.14
7(A,B,C)	gpA2	0.26(-0.01, 0.52)	0.063	0.001(-0.34, 0.35)	0.972	0.39(0.02, 0.77)	0.04
8(A,B,C)	dc13	0.19(-0.08, 0.47)	0.172	0.03(-0.32, 0.39)	0.838	-0.25(-0.63, 0.13)	0.199
9(A,B,C)	gpA-median	0.27(0.00, 0.53)	0.051	-0.01(-0.35, 0.34)	0.972	0.26(-0.11, 0.64)	0.165
10(A,B,C)	dc8-1	0.24(-0.10, 0.58)	0.172	-0.15(-0.59, 0.29)	0.509	0.40(-0.08, 0.87)	0.103
11(A,B,C)	dc8-2	0.41(0.08, 0.74)	0.016	0.14(-0.28, 0.57)	0.517	0.11(-0.35, 0.56)	0.655
12(A,B,C)	dc8-3	0.35(0.04, 0.67)	0.027	-0.21(-0.61, 0.19)	0.31	0.06(-0.37, 0.50)	0.782
13(A,B,C)	dc8-4	0.31(-0.06, 0.67)	0.103	0.02(-0.45, 0.49)	0.938	-0.02(-0.53, 0.49)	0.937
14(A,B,C)	dc8-median	0.33(0.04, 0.62)	0.025	-0.17(-0.54, 0.20)	0.374	0.11(-0.30, 0.50)	0.607
15(A,B,C)	dc9	0.18(-0.10, 0.47)	0.211	0.18(-0.19, 0.54)	0.345	-0.44(-0.83, -0.04)	0.03
16(A,B,C)	gpB	0.07(-0.16, 0.30)	0.557	-0.19(-0.49, 0.11)	0.233	-0.49(-0.81, -0.16)	0.003
17(A,B,C)	gpC	0.16(-0.14, 0.45)	0.3	0.06(-0.45, 0.33)	0.776	-0.20(-0.59, 0.19)	0.308

Table S3. Associations between var gene expression and severe malaria syndromes from linear model.

Each regression model (1-17) included; Impaired consciousness, Respiratory distress and severe malaria anaemia as explanatory variables while adjusting for age and parasitemia. This model allowed for adjusting for overlapping syndromes.

*shows significant adjusted p-value

model	<i>PfEMP1</i>	A: Impaired consciousness(n=186)		B: Respiratory distress(n=29)		C: Severe malaria anaemia(n=59)	
		β (95% CI)	p.value	β (95% CI)	p.value	β (95% CI)	p.value
1(A,B,C)	cys2	0.08(0.03, 0.14)	0.002*	0.002(-0.09, 0.10)	0.967	0.07(-0.02, 0.15)	0.123
2(A,B,C)	groupA-like	0.08(0.03, 0.13)	0.002*	0.03(-0.06, 0.21)	0.517	0.07(-0.00, 0.15)	0.063
3(A,B,C)	cp1	0.07(0.03, 0.12)	0.001*	-0.02(-0.10, 0.05)	0.547	0.01(-0.06, 0.08)	0.789
4(A,B,C)	Cp2	0.03(-0.01, 0.07)	0.183	0.03(-0.04, 0.11)	0.363	0.08(0.01, 0.14)	0.022
5(A,B,C)	Cp3	-0.001(-0.04, 0.04)	0.96	0.004(-0.06, 0.07)	0.911	-0.03(-0.09, 0.03)	0.304
6(A,B,C)	gpA1	0.44(0.14, 0.75)	0.004*	0.41(-0.13, 0.95)	0.138	0.51(0.03, 0.98)	0.037
7(A,B,C)	gpA2	0.41(0.11, 0.71)	0.009*	0.69(0.15, 1.23)	0.013*	0.74(0.27, 1.22)	0.002*
8(A,B,C)	dc13	0.22(-0.08, 0.53)	0.152	0.21(-0.34, 0.76)	0.458	-0.054(-0.52, 0.44)	0.87
9(A,B,C)	gpA-median	0.40(0.10, 0.71)	0.009*	0.59(0.05, 1.13)	0.033	0.62(0.14, 1.09)	0.012*
10(A,B,C)	dc8-1	0.31(-0.03, 0.74)	0.071	0.44(-0.25, 1.12)	0.216	0.79(0.18, 1.39)	0.011*
11(A,B,C)	dc8-2	0.66(0.29, 1.02)	0*	0.78(0.13, 1.44)	0.02	0.59(0.02, 1.17)	0.044
12(A,B,C)	dc8-3	0.60(0.25, 0.85)	0.001*	0.54(-0.08, 1.16)	0.087	0.68(0.14, 1.22)	0.014*
13(A,B,C)	dc8-4	0.51(0.01, 0.82)	0.054	0.26(-0.49, 1.00)	0.497	0.18(-0.47, 0.84)	0.582
14(A,B,C)	dc8-median	0.53(0.21, 0.85)	0.001*	0.48(-0.09, 1.06)	0.101	0.54(0.04, 1.04)	0.037
15(A,B,C)	dc9	0.23(-0.10, 0.55)	0.168	0.27(-0.31, 0.85)	0.361	-0.36(-0.86, 0.15)	0.164
16(A,B,C)	gpB	0.11(-0.15, 0.37)	0.413	-0.13(-0.59, 0.34)	0.596	-0.34(-0.75, 0.07)	0.103
17(A,B,C)	gpC	0.29(-0.05, 0.62)	0.098	0.27(-0.29, 0.84)	0.342	-0.03(-0.53, 0.46)	0.893

Table S4. Associations between var gene expression and non-overlapping severe malaria syndromes from linear model.

Each regression model (1-17) included; Impaired consciousness, Respiratory distress and severe malaria anaemia as explanatory variables while adjusting for age and parasitemia. *shows significant adjusted p-value

Table S5. Expression of pfsir2a among clinical syndromes.

Pvalues are derived from Wilcoxon rank-sum test

group	pValue
IC vs SMA	0.026
Mild vs IC	0.088
RD vs SMA	0.088
Moderate vs IC	0.166
Mild vs RD	0.226
Moderate vs RD	0.257
Moderate vs SMA	0.257
Mild vs SMA	0.337
IC vs RD	0.658
Mild vs Moderate	0.817

Table S6. The median body temperature within each syndrome at admission

Syndrome	median[IQR]
SMA	38[37.3-38.8]
RD	38.2[37.7-39.4]
IC	38.5[37.5-39.1]
Moderate	38.7[37.8-39.5]
Mild	38.8[37.7-39.4]

Var genes	OR(95%CI)	p.value
cys2	4.92 (1.64 , 15.06)	0.005
group A-like	5.61 (1.8 , 17.54)	0.003
cp1	1.1 (0.28 , 3.98)	0.892
cp2	5.32 (1.59 , 16.65)	0.005
cp3	1.96 (0.43 , 8.11)	0.365
gpA1	1.18 (0.96 , 1.46)	0.123
gpA2	1.1 (0.91 , 1.35)	0.345
dc13	0.98(0.80-1.17)	0.823
gpAmedian	1.08(0.89-1.33)	0.437
dc8_1	1.1(0.95-1.29)	0.201
dc8_2	1.07(0.92-1.25)	0.39
dc8_3	1.1(0.93-1.32)	0.265
dc8_4	1.07(0.94-1.21)	0.287
dc8median	1.08 (0.90 , 1.31)	0.401
dc9	0.96 (0.80 , 1.15)	0.625
gpB	0.72 (0.58 , 0.89)	0.003
gpC	0.79 (0.62 , 0.99)	0.043

Table S7. Association of var gene expression, and mortality.

p-value<0.05 are highlighted

Data availability

The code for the analyses presented in this paper and data is deposited at <https://doi.org/10.7910/DVN/W4FFT9>

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Additional information

Ethical approval

This study obtained ethical approval from the Scientific Ethics Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/3149) and written informed consent was obtained from parents or guardians of the study participants.

Author contribution

Henry Ndugwa Conceptualization, Investigation, methodology, Formal analysis, Visualization, Writing – original draft, review and editing,

Michelle Muthui methodology, Writing–review and editing,

J. Alexandra Rowe Writing –review and editing

Kinyanjui SM, Project administration, Supervision, Conceptualization, analysis, Writing-original draft, review and editing

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Abdirahman I. Abdi Conceptualization, Supervision, formal analysis, Writing – original draft, review and editing, All authors contributed to the interpretation of the analyses and revised the draft manuscript.

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Wickham H., François R., Henry L., Müller K., Vaughan D. (2023) dplyr: A Grammar of Data Manipulation. CRAN. <https://CRAN.R-project.org/package=dplyr>

Peer reviews

Reviewer #1 (Public review):

Summary:

Severe childhood malaria is associated with three main overlapping syndromes: impaired consciousness (IC), respiratory distress (RD), and severe malaria anaemia (SMA). One central feature of severe malaria, driven by host and parasite factors, is the sequestration of parasitized red blood cells in vascular beds, leading to impaired tissue perfusion and lactic acidosis. The causing agent, the parasite ligand PfEMP1, is expressed on the surface of infected red blood cells, where it binds to a broad range of different endothelial receptors. Accumulation of parasite-infected erythrocytes in the host's microvasculature has been repeatedly confirmed for cerebral malaria, but there are scarce data on the extent of sequestration in the other severe malaria syndromes. However, the absence of effective adjunctive therapies for severe malaria implies that our understanding of its pathogenesis remains incomplete. Thus, by comparing var gene expression from a large Kenyan cohort (n=372 severe cases; n=340 non-severe cases), this study addresses a critical knowledge gap regarding the role of PfEMP1 across distinct severe malaria syndromes. The substantial sample size, phenotypic stratification, and use of two complementary methods (DBLa-tag

sequencing and RT-qPCR), along with data about the parasite's ability to form rosettes and antibody level assessments, provide a strong setup. Var gene expression data - either proportions of different DBLa-tags classified by the number of cysteine residues and presence of particular motifs or relative expression RT-qPCR data from a set of primer pairs targeting conserved regions of var groups or particular domains - is associated with (a) severe malaria syndromes, (b) variant expression homogeneity, (c) rosetting ability, and (d) mortality using independent linear regression models, spearman ranks correlations, or logistic regression models. In summary, the study confirms that A-type and DC8-containing gene expression correlate with IC, that RD is associated with rosetting, and that SMA is linked to a high variant expression homogeneity (VEH) of var-A expression, which may indicate a longer infection duration. However, some findings remain inconclusive. For example, when analyzing pure syndromes, several associations changed: DC8 expression was also found to be significantly enriched in SMA (with multiple primer pairs) and RD, not exclusively with IC. Additionally, rosetting was associated with DC8 expression but not with IC, even though IC itself is linked to DC8 expression. Overall, the findings are significant and supported by a large dataset, though the reported evidence remains largely associative rather than mechanistic.

Strengths:

As the authors stated themselves, one of the key unresolved questions is whether severity-causing parasites are biologically different from parasites responsible for asymptomatic infections. This study is among the first to address this question using data from a large, phenotypically stratified cohort. The use of two complementary methods (DBLa-tag sequencing and RT-qPCR), together with data on the parasites' ability to form rosettes and assessments of antibody levels, provides an excellent experimental framework.

Weaknesses:

Even when assessing var gene expression using two different approaches - DBLa-tag sequencing and RT-qPCR targeting pre-defined variants - only a glimpse of the parasites' actual biology is captured. Moreover, a well-known confounder in gene expression studies of *P. falciparum* field isolates is variation in parasite age (hours post-invasion) or synchronicity, both of which significantly influence var gene expression. The methods employed in this study, unfortunately, do not allow for controlling or correcting for these factors. Then, the old classification system of DBLa-tag data developed by Bull et al is certainly still valid; however, more recent advances in bioinformatic tool development now allow for a more in-depth exploration of DBLa-tag datasets. Tools such as *Varia* (doi: 10.1186/s12859-022-04573-6), *cUPS* (<https://doi.org/10.1371/journal.ppat.1012813>), and *upsML* (doi: <https://doi.org/10.1101/2025.05.19.654848>) enable the prediction of DBLa-tag-connected PfEMP1 domains and the var group affiliations.

As A-type var gene expression has already been associated with severity, most expression studies (including this one) have a selection bias towards A- and B/A-type var genes. Here, A- and B/A-types are covered by 8 primer pairs (gpA1, gpA2, 4x DC8, DC13, DC4), whereas high polymorphic B-types are targeted by only 2 primer pairs (b1, DC9) and C-types only by a single primer (c2). Thus, any association with A-type expression is more likely to be observed, although evidence is accumulating that parasites are preferably expressing B-type var genes at the onset of blood stage infection in naïve/less immune individuals; this is also consistent with the observation of the authors that VEH is positively associated with immunity (measured as anti-IE) and negatively associated with temperature.

I am not an expert in biostatistics, but to my understanding, independently performed regressions should be corrected for multiple testing.

Overall, the authors largely achieved their aims, identifying specific var groups associated with different severity syndromes. However, due to the complexity of var gene data and the

interdependence of parameters, the resulting picture is not entirely clear. Some opposite results between different analyses may also be difficult for the reader to interpret. Nevertheless, this study can be considered a pioneering effort, providing valuable insights into the complex interplay of var gene expression across different severity syndromes and offering useful data for the field. Follow-up studies will be important to validate these findings and further dissect the mechanisms linking parasites gene expression to clinical outcomes.

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Reviewer #2 (Public review):

Summary:

The manuscript presents results of a study using two complementary approaches (RT-qPCR and DBL) to analyze the putative relationship between var gene transcription (and hence, PfEMP1 expression) and clinical presentation among Kenyan children with *Plasmodium falciparum* malaria. Binary rosetting (yes/no) data are used in a similar way. The study includes samples collected over a period of almost 20 years from about 700 children presenting with either severe (impaired consciousness [IC], respiratory distress [RD], severe anemia [SA]) or non-severe malaria. During the study period, the study area experienced a remarkable drop in *P. falciparum* transmission intensity.

Strengths:

The study stands on the shoulders of many similar studies of this kind, both by the authors and by other research teams, and the inferences made largely confirm those made previously. The current study has analytical rigor and a large sample size. Disentangling the multiple parameters of the above-mentioned relationship is of obvious and crucial importance to an improved understanding of *P. falciparum* malaria pathogenesis and of the targets and mechanisms of protective immunity to the disease. The present study is a valuable effort towards that. The study is well-structured, and the figures are clear.

Weaknesses:

It is somewhat unclear to this reviewer to what extent the samples and data analyzed and reported here are new (i.e., not used/analyzed in previous studies). If there is substantial overlap with earlier studies, this is a weakness because of the risk of circular inferences. The Discussion section would benefit from less repetition of the results section and a more in-depth discussion of the findings obtained relative to the existing literature. Better inclusion of key primary references is recommended.

<https://doi.org/10.7554/eLife.110810.1.sa1>

Reviewer #3 (Public review):

Summary:

In this manuscript, Ndugwa et al. attempt to link specific severe malaria manifestations with particular var gene expression patterns. This is an important question, and the dataset the authors have assembled over decades is impressive. However, greater clarity in the descriptions and statistics would, in my view, help this reviewers, and readers in general develop a more precise understanding of the significance of the findings.

Strengths:

The study addresses a critically important question in malaria pathogenesis, and the dataset is extensive and represents a significant long-term effort by the authors.

Weaknesses:

The Results section often lacks clarity: clinical group definitions (NS, non-IC, non-SMA, mild vs. moderate) are sometimes ambiguous, and key methodological details, including the VEH index calculation, RT-qPCR quantification, antibody detection methods, and rosetting assays, are either missing from the results text or poorly explained in the figure legends. Additionally, figure presentation requires improvement, with inconsistent reporting of sample sizes, undefined colors, and p-values that overlap with data points rather than being clearly displayed above them.

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