

# Virulence of Malaria Is Associated with Differential Expression of *Plasmodium falciparum* var Gene Subgroups in a Case-Control Study

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*Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is a major pathogenicity factor in falciparum malaria that mediates cytoadherence. PfEMP1 is encoded by ~60 var genes per haploid genome. Most var genes are grouped into 3 subgroups: A, B, and C. Evidence is emerging that the specific expression of these subgroups has clinical significance. Using field samples from children from Papua New Guinea with severe, mild, and asymptomatic malaria, we compared proportions of transcripts of var groups, as determined by quantitative polymerase chain reaction. We found a significantly higher proportion of var group B transcripts in children with clinical malaria (mild and severe), whereas a large proportion of var group C transcripts was found in asymptomatic children. These data from naturally infected children clearly show that major differences exist in var gene expression between parasites causing clinical disease and those causing asymptomatic infections. Furthermore, parasites forming rosettes showed a significant up-regulation of var group A transcripts.

Various factors contribute to the pathologic characteristics of severe malaria, including cytoadherence (reviewed in [1]). This is the adhesion of late-stage-infected erythrocytes to various receptors—such as CD36 or intercellular adhesion molecule-1 on the vascular endothelium, chondroitin sulfate A in the placenta, and complement receptor (CR)-1 on red blood cells (RBCs; also called “rosetting”)—that leads to microvascular obstructions in various organs (reviewed in [2]). Cytoadherence is mainly mediated by a family of parasite-derived polymorphic *Plasmodium falciparum* antigens on the surface of late-stage-infected erythrocytes [3].

These *P. falciparum* erythrocyte membrane proteins (PfEMP1) are large (200–350 kDa) and antigenically variant. PfEMP1 is structured into different semiconserved, adhesive domains: Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (reviewed in [4]).

In the *P. falciparum* line 3D7, PfEMP1 is encoded by 1 of 59 var genes [5–8]. Most var genes can be assigned to 1 of 3 types (var groups A, B, and C), mainly according to their conserved 5' upstream sequences [6, 9]. In 3D7, the majority of var genes belongs to the subtelomerically located var group B, whereas 13 var group C genes are arranged in chromosome internal clusters. Ten mostly larger, subtelomerically located var genes with a distinct domain structure belong to var group A [10]. var genes are exclusively expressed but undergo switching within parasite lineages [11, 12]. Transcripts of several var genes can be detected within a single host at any time [13, 14].

Various studies have analyzed the association between disease outcome and the binding of field isolates to endothelial receptors [15–18]. Using agglutination assays, Bull et al. [19] identified a restricted subset of variant surface antigens (VSAs) involved in severe disease. This raises the question of whether var group A,

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B, or C would represent such subsets. We conducted a case-control study of malaria in Papua New Guinea (PNG) involving 65 children, to study associations between the expression of *var* group A, B, or C genes and clinical presentation. We compared the relative contribution of transcripts of each *var* group, as determined by quantitative real-time polymerase chain reaction (qPCR), in children with severe, mild, and asymptomatic malaria. To our knowledge, this is the first study to have compared the expression of *var* subgroups and clinical outcome in vivo. We also analyzed the rosetting capacity of infected erythrocytes, to test whether a certain *var* gene group mediates this trait in PNG.

## SUBJECTS, MATERIALS, AND METHODS

**Population and study design.** The study was conducted in the Madang General Hospital, PNG, during the wet season (February–May) of 2003. *P. falciparum* malaria is holoendemic in Madang and has perennial transmission. Infections with *P. vivax* are also common. Malaria accounts for 15.3% of deaths in children in this hospital, with a case-fatality rate of 3.6% [20]. After written, informed consent was obtained from parents, venous blood (1 mL) was taken from 14 children (age range, 0.5–6 years) with severe malaria, as defined in accordance with World Health Organization criteria (2000) [1]. All children had asexual *P. falciparum* parasites present, with cerebral malaria, prostration, several convulsions within the preceding 24 h, or severe anemia (hemoglobin level, <5 g/dL) (table 1). None of the children died. In addition, 26 children of similar age ( $\pm 20\%$  of the age of the children with severe malaria) with mild malaria were enrolled at the hospital and the town clinic of Madang. Mild malaria was defined as the presence of asexual *P. falciparum* parasites and an axillary temperature  $>37.5^\circ\text{C}$  or symptoms of headache, fever, or myalgia. For children with severe malaria, age- and location-matched samples were col-

lected from 25 asymptomatic children with a positive *P. falciparum* OptiMAL test (DiaMed) and subsequent positive microscopic results. Exclusion criteria for all children were a confirmed diagnosis of a coinfection, malnutrition (mid-upper arm circumference, <12 cm), or having received antimalarial treatment within the preceding 2 weeks. The study was approved and ethical clearance was given by the PNG Medical Research Advisory Committee.

**Assessment of *P. falciparum* infections.** Malaria parasites were counted per 200 white blood cells on Giemsa-stained blood films. Parasite densities were multiplied by 40, to convert values to parasites per microliter [21]. MOIs were determined in *P. falciparum*-positive samples by *msp2* genotyping, as described elsewhere [22]. Briefly, 30  $\mu\text{L}$  of whole blood was spotted on filter papers (Isocode Stix; Schleicher & Schuell) and dried for 20 min at  $80^\circ\text{C}$ . After washing, *msp2* PCR was performed directly on the filter papers, and nested PCR products were analyzed by restriction fragment-length polymorphism, to record the number of infecting strains.

**Parasite culture and assessment of the frequency of rosetting.** If the parasitemia level was  $>5000$  parasites/ $\mu\text{L}$ , parasites from children with severe or mild malaria were cultured according to standard methods in 10% heterologous AB serum, to quantify rosetting [23]. Rosettes were defined as the binding of infected RBCs to at least 2 uninfected RBCs and were counted when most parasites were at the late trophozoite/early schizont stage. An aliquot of culture at 2% hematocrit was stained with ethidium bromide, and rosettes were counted as a proportion of 200 mature-stage parasites, by use of a fluorescence microscope. The frequency of rosetting is presented as the percentage of mature parasite-infected cells found in rosettes.

**Table 1. Clinical assessment of case patients and control subjects.**

Characteristic	Type of malaria		
	Asymptomatic (n = 25)	Mild (n = 26)	Severe (n = 14)
Sex (female/male)	9/16	10/16	7/7
Age, mean (range), months	41.3 (14–65)	42.2 (12–84)	35.8 (8–60)
Parasitemia, geometric mean (range), parasites/ $\mu\text{L}$	3715 (40–108,000)	27,241 (160–310,200)	32,928 (240–480,000)
MOI, mean (range)	1.3 (1–3)	1.5 (1–3)	1.2 (1–2)
>1 convulsion/24 h	0	0	5 (36)
Prostration	0	0	7 (50)
Impaired consciousness	0	0	1 (7)
Coma	0	0	4 (29)
Alterations in neurological function	0	0	4 (29)
Severe anemia (hemoglobin level <5 g/dL)	0	0	3 (21)
Hyperlactatemia (lactate level >5 mmol/L)	0	0	0

**NOTE.** Data are no. (%) of children, unless otherwise indicated.

### Isolation of full-length var transcripts and cDNA synthesis.

The isolation of full-length var mRNA and reverse transcription (RT) was performed as described elsewhere [14]. Briefly, total RNA of mainly late ring-stage parasites was extracted using TRIzol (Invitrogen), in accordance with the manufacturer's instructions. RNA was treated with 3 U of RQ1 DNase (Promega). To obtain only full-length var transcripts, RNA was incubated with 1 pmol of biotinylated oligonucleotides complementary to the acidic terminal sequence domain. Then, 200 µg of Dynal beads with M-280 streptavidin was added to the RNA. After washing, RT was performed on the captured hybrids, which had been primed using 500 ng of hexamers (Invitrogen) and Sensiscript (Qiagen) reverse transcriptase, in accordance with the manufacturer's protocol, in a final volume of 20 µL. An aliquot without reverse transcriptase was used as negative control. After RT, cDNA was treated with RNase A.

**Isolation of genomic DNA.** DNA was extracted as described elsewhere [24]. Briefly, 30 µL of full blood was spotted on filter papers (Isocode Stix; Schleicher & Schuell) and dried for 20 min at 80°C. After washing, PCR was performed directly on the filter papers.

**qPCR.** Before qPCR, 1 µL of DNA was amplified in a primary PCR, to increase sensitivity. DNA was amplified over the var 5' untranslated region (UTR)–DBL1α in 50-µL volumes with Advantage cDNA polymerase (Clontech) using 400 nmol/L var group-specific 5' UTR forward primers and a degenerated DBL1α reverse primer (table 2). PCR conditions were 94°C for 5 min and 16 cycles (for cDNA) or 14 cycles (for gDNA) of 95°C for 30 s, 52°C for 1 min, and 64°C for 70 s. Electrophoresis of the primary PCR product showed no visible band, indicating that the subsequent qPCR did not exceed the linear range.

qPCR was performed over the var group A, B, and C 5' UTR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Reactions were done with 5 µL of primary PCR product in 25-µL volumes with Advantage cDNA polymerase (Clontech) using 250 nmol/L minor groove binder probes labeled with FAM (Applied Biosystems) and 900 nmol/L primers for the respective sequences (see table 2). Oligonucleotides were designed according to alignments of 5' UTR var gene sequences from the 3D7 genome project (available at: <http://www.plasmodb.org>; Joe Smith, Seattle Biomedical Research Institute, Seattle, WA; personal communication) and var gene sequences from PNG (Genbank accession numbers AY462581–AY462851). PCR conditions were 94°C for 5 min and 40 cycles of 95°C for 30 s, 54°C for 1 min, and 65°C for 70 s. Electrophoresis of real-time PCR products was performed to control for single bands and equal size. All cDNA samples were run in triplicate. All cycle-threshold ( $C_T$ ) values were in the linear range between 15 and 31. If all  $C_T$  values of var group A, B, and C were >31, the sample was discarded and RT-PCR was repeated. Negative cDNA controls (no reverse transcriptase) of all samples and no-template controls (NTCs; per 96-well plate) were amplified in parallel. If the NTC was positive, the plate was discarded. If the negative cDNA control was positive, the sample was discarded and RT-PCR was repeated. Then, 2.5 ng of gDNA from *P. falciparum* 3D7 was amplified in parallel per plate and var group as a positive control and plate calibrator. Quantification was done using ABI Prism 7000 SDS software (version 1.1; Applied Biosystems).

**Standard curve and relative quantification.** Standard curves were linear over a dilution series of 6 log of 10–14 different dilutions, each in triplicate. The PCR efficiency ( $E$ )

**Table 2. Oligonucleotide primers for amplification of var gene regions.**

var gene region (length of amplified product), name	Primer sequence (5'→3')
DBL1αrev, DBL_FADall_rev <sup>a</sup>	CC(A/T)AT(A/G)(G/T)C(A/G/T)GCAAAACT(C/G/T)C(G/T)(A/T)GC
var group A, 5' UTR (150 bp)	
upsA1_for <sup>b</sup>	AACTTACCATAAATTATCATCAAA
upsA3_probe	6FAM-AAACCTTTGGTATAGAAAAAATATT-MGB
upsAj_rev <sup>c</sup>	TCACCTACAACAAT(A/G)TAATAAA
var group B, 5' UTR (360 bp)	
17deg_for <sup>b</sup>	CTCAT(A/T)TATAATTTT(C/G)AAAATA(A/T)A(A/T)AAAAC
upsB1_probe	6FAM-TCTAACAAAAAAAAAAAAACAACAATTAC-MGB
RT-17.2_rev	TTA(A/T)GGGAGTAT(A/T)GT(A/G/T)ATATGGTAGAAT
var group C, 5' UTR (240 bp)	
RT-5B1.1_for <sup>b</sup>	AATATTCATATCCCACATT(A/G)TCATATAT
upsC_probe	6FAM-ACATATAATACGACTAAGAAAC-MGB
5B1.4_rev	ATTATGTGGTAATATCATGTAATGG

**NOTE.** MGB, minor groove binder; RT, reverse transcription; UTR, untranslated region.

<sup>a</sup> Reverse primer used in the primary polymerase chain reaction (PCR).

<sup>b</sup> Forward primers were identical in primary and real-time PCR.

<sup>c</sup> J. Smith, personal communication.

**Table 3. Clinical assessment of children with severe malaria, with reference to *var* group A transcription.**

Patient	Age, months	Alterations in neurological function <sup>a</sup>	Several convulsions/24 h	Prostration	Severe anemia (hemoglobin level <5 g/dL)	Parasitemia, parasites/ $\mu$ L	Rosetting frequency >10%	Amplification efficiency of group A <sup>b</sup>	Transcription of group A <sup>c</sup>
84	44	Yes	No	Yes	No	6400	No	++	--
55	60	No	No	Yes	Yes	240	ND	-	--
24	8	No	Yes	No	No	300,000	No	+	-
116	60	No	Yes	Yes	No	560	ND	+	-
23	40	No	No	Yes	No	177,600	No	--	-
96	36	No	Yes	No	No	480,000	No	++	-
25	29	No	No	Yes	Yes	200,000	No	+	+
83	56	No	Yes	No	No	124,800	No	++	+
61	12	No	Yes	No	No	97,600	No	--	+
44	42	Yes	No	Yes	Yes	8480	No	-	+
22	36	Yes	No	Yes	No	52,400	Yes	+	+
14	45	No	Yes	No	No	13,240	No	-	+
58	12	No	Yes	No	No	40,000	No	ND	++
27	21	Yes	No	No	No	139,680	Yes	++	++

<sup>a</sup> Includes Blantyre coma score <4 or the presence of neurological disorders, such as bruxism or decerebrate rigidity.

<sup>b</sup> --, lowest quartile of *var* group A proportions of all genomic DNA samples (reflecting potential primer bias); -, second quartile; +, third quartile above the median; ++, fourth quartile (high amplification efficiency of *var* group A genes); ND, not done.

<sup>c</sup> --, lowest quartile of *var* group A proportions of all cDNA samples (reflecting low transcription vs. *var* group B and C); -, second quartile; +, third quartile above the median; ++, fourth quartile (reflecting the increased presence of *var* group A transcripts vs. *var* group B and C transcripts).

was calculated using the formula  $E = 10^{(1/\text{slope})} - 1$ . The mean efficiencies of 3 independent standard curves with high reproducibility were 100% for *var* group A, 86% for *var* group B, and 95% for *var* group C. The lower limit of detection of the system was <50 copies/mL (data not shown). Relative quantification was done using the  $\Delta\Delta C_T$  method (Application Guide; Qiagen) with the following modifications:  $C_T$  values were converted to approximate copy numbers using the formula  $C/E^{\Delta C_T}$ , where  $C$  is the number of *var* gene copies in the corresponding *var* groups A, B, or C of the plate calibrator (2.5 ng of 3D7 gDNA);  $E$  is the real-time PCR efficiency of the corresponding *var* group (*var* group A, 2; group B, 1.86; group C, 1.95); and  $\Delta C_T$  is the difference in average  $C_T$  values between the sample and the corresponding *var* group plate calibrator. The numbers of *var* copies in the plate calibrator ( $C$ ) were estimated by comparing the real-time PCR oligonucleotide sequences (table 2) and 5' UTR *var* gene alignments (see above). According to these alignments, *var* group A oligonucleotides showed <2 mismatches with 6 of 10 *var* group A genes; *var* group B oligonucleotides showed <2 mismatches with 20 of 22 *var* group B genes, 2 of 4 *var* group B/A genes, and 4 of 9 *var* group B/C genes; and *var* group C oligonucleotides showed <2 mismatches with 7 of 13 *var* group C genes (*var* grouping according to Lavstsen et al. [10]). Because of the *var*-specific mRNA isolation, no endogenous reference gene could be used. For statistical analysis, we evaluated proportions of *var* group transcripts.

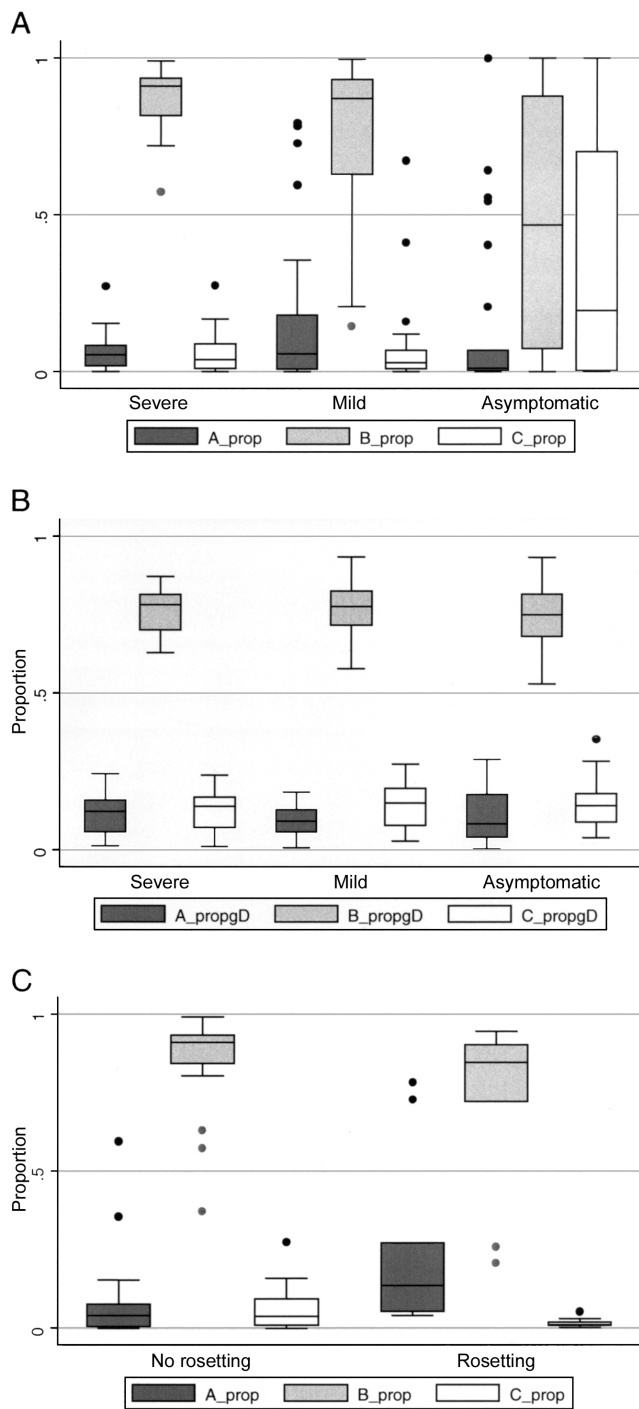
To validate our qPCR method, we used RNA from a previous study of *var* transcription of parasites selected in vitro for severe malaria and unselected control subjects [25]. This RNA was

quantified by qPCR, and our results agreed with the previous findings—that is, in these selected parasites, we found a 3.2-fold increase in proportions of *var* group A transcripts, a 3.6-fold increase in proportions of *var* group B transcripts, and a 1.6-fold decrease in proportions of *var* group C transcripts.

**Statistical analysis.** Statistical analysis was performed using Stata software (Intercooled Stata, version 8.2; available at: <http://www.stata.com>). Levels of *var* gene transcription were expressed as transcript proportions of *var* groups—that is, the number of transcripts of 1 *var* group as a proportion of the total transcript numbers for all 3 *var* groups A, B, and C. Associations between *var* group proportions and clinical outcome were analyzed using the Mann-Whitney  $U$  test or the Kruskal-Wallis test. Logistic-regression analyses were performed to calculate the odds ratio (OR) for disease. ORs were calculated and compared unadjusted or, in multivariate logistic-regression analyses, with adjustment for parasitemia. Statistical significance was evaluated using 2-tailed likelihood-ratio tests.

## RESULTS

A total of 65 children were enrolled in the study: 14 with severe malaria, 26 with mild malaria, and 25 who were asymptomatic (table 1). There was no significant difference in the age and sex distribution of case patients and control subjects (age and clinical outcome,  $P = .6$ , Kruskal-Wallis test; sex and clinical outcome,  $P = .72$ , Fisher's exact test). Parasite density showed a significant relationship with clinical outcome ( $P < .001$ , Mann-Whitney  $U$  test), but there was no association between clinical



**Figure 1.** Box plots of proportions of *var* groups A, B, and C transcripts. Boxes indicate the median and quartiles. Vertical lines represent the data range extending to a maximum of 1.5 times the interquartile range and are not SE bars. Dots indicate the remaining data points. *A*, Box plots of clinical outcome (severe, mild, and asymptomatic malaria) and proportions of *var* group-specific transcripts (A<sub>prop</sub>, B<sub>prop</sub>, and C<sub>prop</sub>, respectively). *B*, Box plots of clinical outcome and proportions of *var* group-specific genomic DNA (A<sub>propgD</sub>, B<sub>propgD</sub>, and C<sub>propgD</sub>, respectively). *C*, Box plots of parasites mediating rosetting and proportions of *var* group-specific transcripts.

outcome and the number of infecting *P. falciparum* strains, as determined by *msp2* genotyping (table 3).

To test whether the genomic composition of *var* subgroups in case patients and control subjects was similar and to exclude primer bias, we quantitatively analyzed *var* groups A, B, and C by PCR of genomic DNA. The total amplified *var* group templates correlated well with parasite loads of the corresponding children (Spearman's  $\rho$ : *var* group A, 0.73; *var* group B, 0.81; *var* group C, 0.74; all  $P < .001$ ). The genomic distribution of the 3 *var* subgroups was similar among different clinical outcomes (figure 1*B*), with 10% overall of amplified genes belonging to *var* group A, 76% to *var* group B, and 14% to *var* group C. However, in 14 of 59 samples, fewer *var* group A genes were amplified, as indicated by proportions of  $<5\%$ . This was also the case for *var* group C genes in 9 of 59 samples. Furthermore, proportions of *var* group B genes were only between 50% and 60% in 4 samples. All of these samples were equally distributed among case patients and control subjects. The corresponding proportions of *var* group transcripts were also mostly low in these samples.

Of the 65 children, *var* group A transcripts were found in 55, *var* group B transcripts were found in 62, and *var* group C transcripts were found in 56. In children with mild and severe clinical malaria, a significantly higher proportion of *var* group B transcripts was found, compared with that in children who were asymptomatic (ORs in table 4 and figure 1*A*). Both fever and headache were also significantly correlated with increased *var* group B transcription. Proportions of *var* group B transcripts were not significantly higher in children with severe malaria, compared with those who had mild malaria.

In 60% of asymptomatic children (15/25), proportions of *var* group C transcripts were  $>10\%$ , and, in 10 of 25 asymptomatic children, more *var* group C than B transcripts were found. This is in agreement with observations from culture lines 3D7, NF54, and FCR3S1.2, in which we also found high proportions of *var* group C transcripts. Of children with clinical malaria, only 18% (7/40) had proportions of *var* group C transcripts  $>10\%$ . This difference between asymptomatic children and those with clinical malaria was highly statistically significant (table 4 and figure 1*A*). Absolute levels of *var* group C transcripts were divided by corresponding genomic DNA levels and resulted in a 15.6-fold increase in the number of normalized *var* group C transcripts in asymptomatic children, compared with that in children with clinical malaria ( $P = .015$ , Mann-Whitney *U* test). There was no significant difference in numbers of *var* group C-specific transcripts between children with mild versus those with severe malaria.

Overall, there was no association between parasite density and the proportion of *var* groups on the transcript level (Spearman's  $\rho$  testing the correlation in all 65 children: *var* group A, 0.06 [ $P = .62$ ]; *var* group B, 0.14 [ $P = .27$ ]; *var* group C,  $-0.06$

**Table 4. Odds ratios (ORs) for disease with increased proportions of *var* group-specific transcripts.**

Type of malaria, comparison	<i>var</i> group A		<i>var</i> group B		<i>var</i> group C	
	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Asymptomatic vs. clinical						
Unadjusted	0.94 (0.11–8.0)	.96	18.95 (3.21–111.87)	<.001 <sup>a</sup>	0.01 (0.01–0.22)	<.001 <sup>a</sup>
Adjusted	0.76 (0.07–8.00)	.82	31.63 (3.68–271.71)	<.001 <sup>a</sup>	0.02 (0.01–0.24)	<.001 <sup>a</sup>
Asymptomatic vs. mild						
Unadjusted	1.56 (0.18–13.35)	.69	10.53 (1.74–63.64)	.006 <sup>a</sup>	0.02 (0.01–0.37)	<.001 <sup>a</sup>
Adjusted	1.11 (0.11–11.75)	.93	21.71 (2.45–192.67)	.002 <sup>a</sup>	0.02 (0.01–0.33)	<.001 <sup>a</sup>
Asymptomatic vs. severe						
Unadjusted	0.17 (0.01–8.90)	.33	111.47 (2.43–5108.28)	<.001 <sup>a</sup>	0.01 (0.01–1.20)	.004 <sup>a</sup>
Adjusted	0.28 (0.01–43.73)	.59	302.16 (1.44–63248)	.002 <sup>a</sup>	0.01 (0.01–1.13)	.006 <sup>a</sup>
Mild vs. severe						
Unadjusted	0.04 (0.01–6.67)	.13	12.61 (0.26–612.36)	.15	0.64 (0.01–159.1)	.87
Adjusted	0.05 (0.01–7.18)	.15	9.89 (0.20–494)	.20	1.31 (0.01–380)	.93

**NOTE.** The OR for *var* group A is the estimate from the logistic regression of [case(A,100) control(A,0)]/[control(A,100) case(A,0)], where case(A,100) is the no. of case patients with 100% group A *var* transcripts, control(A,0) is the no. of control subjects with 0% group A *var* transcripts, etc. ORs are either unadjusted or adjusted in multivariate logistic regression for parasitemia.

<sup>a</sup> Statistically significant.

[*P* = .66]). However, in asymptomatic children, we detected a significant positive association between parasitemia levels and proportions of *var* group C transcripts (Spearman's  $\rho$ , 0.51 [*P* = .013])—more parasites were detected in children with increased proportions of *var* group C transcripts (>0.4). By contrast, in children with clinical disease, a negative association was found between parasitemia levels and proportions of *var* group C transcripts.

Rosetting frequencies were >10% in 33% (10/30) of parasites from children with mild or severe disease, and *var* group A transcripts were detected significantly more frequently in those samples. The median level of *var* group A transcripts was 3 times that in children who had lower frequencies of rosetting (*P* = .047, likelihood ratio from logistic regression) (figure 1C). The rosetting phenotype was not correlated with the severity of disease, and we did not observe a significant difference in proportions of *var* group A transcripts between children with severe disease and those with mild malaria; we noted only a small, nonsignificant decrease in proportions of *var* group A transcripts in children with asymptomatic malaria (figure 1A and table 1). When we compared *var* group A transcription in children with different symptoms of severe malaria, a nonsignificant increase in proportions of *var* group A transcripts was found in children with alterations in neurologic function—that is, the median of these proportions was found in the third quartile (50th–75th percentile) of *var* group proportions of all case patients and control subjects.

## DISCUSSION

To test whether parasites in children with clinical disease express different *var* genes, we used qPCR to compare the proportions

of transcripts belonging to *var* group A, B, and C in 65 children from PNG with severe, mild, and asymptomatic malaria. We found significantly higher proportions of *var* group B transcripts in children with clinical malaria (both mild and severe), compared with those in children who were asymptomatic. Conversely, *var* group C transcripts formed a significantly higher proportion of asymptomatic malaria infections. The major differences in *var* group transcripts were between asymptomatic and clinical samples. Quantification of the proportion of *var* group gDNA by qPCR indicated similar distributions of *var* group-specific DNA among samples from children with different clinical outcomes. Therefore, we conclude that the observed differences in *var* transcripts are due to transcriptional regulation during symptomatic malaria and not to primer bias or DNA composition.

There was no significant difference in proportions of *var* transcripts between children with mild and severe malaria. The number of children with severe malaria in the present study might have been too small for the detection of minor differences, and the analytical power was further reduced when various conditions caused by severe malaria were analyzed separately. However, the absence of major differences between mild and severe disease might indicate that the progression of malaria is a multifactorial continuum in which the same *var* gene subset is expressed. The tendency for *var* group B transcripts to be more frequent in children with severe malaria suggests that some *var* group B genes encode for variants that contribute to sequestration in vital organs.

It has previously been suggested that group A *var* genes may be responsible for severe clinical disease [25–27]. We found a tendency toward higher group A *var* transcription only in chil-

dren with alterations in neurologic function, and we observed only a slight increase in proportions of *var* group A transcripts in children with mild malaria, compared with those in asymptomatic children. However, *var* group A transcripts were significantly more frequent in children with rosetting parasites, which confirms observations from culture, in which rosetting was attributed to *var* group A genes (data not shown). In contrast to studies in Africa [18, 23], rosetting has been previously shown in PNG not to be correlated with the severity of disease [28]. CR1 is the main ligand on uninfected erythrocytes for rosetting [29], and CR1 deficiency occurs in 79% of the Madang population [30]. Low CR1 expression (<150 molecules/cell) was measured in 73% of the children from whom parasite rosetting frequencies were measured (22/30; data not shown). The rosettes observed in CR1-deficient hosts might not withstand shear forces in vivo. This could explain the lack of an association between disease and rosetting previously described in PNG, and it could also explain the lack of an association between severe disease and the up-regulation of *var* group A transcripts in these samples.

The prominent presence of parasites transcribing *var* group B genes in children with clinical malaria reflects the ability of these parasites to successfully multiply, leading to morbidity. It has previously been shown that, in clinical malaria, the parasites that predominate express VSAs that are not recognized by an existing antibody repertoire [31]. In 3D7, *var* group B represents the largest *var* group [10], which could be sufficiently diverse to initially evade the immune system. The proportional increase in *var* group C transcripts in asymptomatic children might indicate the presence of parasites expressing less-pathogenic *var* variants with reduced binding abilities.

Nevertheless, we still observed, in 14 of 25 asymptomatic children, more *var* group B than C transcripts. In 7 of these children, a small proportion of *var* group C genes were detected on the genomic level (<10%), which might indicate that our primers targeted fewer *var* group C genes in those parasites. We also noticed that most of these 14 children had lower parasite loads than other asymptomatic children, so we may have just detected a new parasite infection preceding clinical symptoms. However, low parasitemia levels may also reflect a tighter control of parasites expressing *var* group B variants in hosts who already have a moderate anti-PfEMP1 antibody repertoire.

Apart from differences in immunogenicity or binding characteristics, the present findings could also be explained by imprinting processes or specific switching rates inherent to a *var* gene or a specific *var* gene group. For instance, the predominance of *var* group B expression in clinical infection might be explained by the high switch-on rate inherent to variants of this *var* group. Once their repertoire is exhausted by increasing immunity, *var* group C variants might prevail in asymptomatic infections. One could speculate that high switch-off rates are

inherent to *var* group C genes, resulting in a rapid turnover that impedes effective antibody responses. Our previous findings in a longitudinal study of a highly dynamic and transient picture of *var* transcription in asymptomatic children from PNG support this hypothesis [14].

In conclusion, the present findings emphasize the importance of differential PfEMP1 expression in disease manifestation. Here, we show, to our knowledge for the first time, in a malaria case-control study conducted in an area of endemicity, that major differences exist in *var* expression in vivo between parasites from clinical attacks and asymptomatic infections, and we have associated the rosetting phenotype with *var* subgroup A expression. Further analysis of the *var* transcripts that we collected will reveal the composition of *var* genes within the corresponding *var* groups and hopefully shed further light on more-virulent *var* genes.

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