

Differential *var* Gene Expression in Children with Malaria and Antidromic Effects on Host Gene Expression

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Among 62 children with mild malaria, cerebral malaria, or severe malarial anemia, we analyzed the transcription of different *var* gene types. There was no difference in parasitemia level or body temperature between groups. However, a significantly different expression pattern was observed in children with cerebral malaria, compared with that in patients in the other 2 groups: children with cerebral malaria had lower expression of the upsA subtype but higher expression of the upsB and upsC subtypes. Furthermore, expression of human genes responsive to tumor necrosis factor and hypoxia correlated with distinct ups types.

Malaria caused by the protozoan parasite *Plasmodium falciparum* affects individuals in different ways. Infections can be asymptomatic or can manifest themselves via severe signs and symptoms—including severe malarial anemia and cerebral malaria, both of which very often lead to the death of the patient. In Africa, where the burden of the disease is highest, severe malaria is mostly encountered in children of young ages, whereas asymptomatic infection is found in persons of all ages but is predominantly encountered in semi-immune adolescents and adults. The outcome of an infection is determined by both host and parasite factors. A main factor of parasite virulence is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is thought to in-

teract with several host cell receptors in microvessels of various organs, causing cytoadherence and consequent tissue damage [1].

Binding to various receptors is mediated by the structural features of the Duffy binding–like domains (DBLs) or the cysteine-rich interdomain regions of PfEMP1. PfEMP1 is encoded by the *var* gene family; this family consists of ~60 genes, only one of which is expressed at any given time point in the parasite. *var* genes can be grouped according to their chromosomal localization and their 5′ transcribed region into upsA, upsB, or upsC subtypes. Previous studies have provided evidence that these subtypes are associated with various disease outcomes. Two studies, one in Papua New Guinea and one in Tanzania, compared asymptomatic infections with clinical malaria (mild or severe) and showed preferential expression of either group A *var* genes or group A and B *var* genes in clinical cases [2, 3]. In another study in Kenya, investigators found no association when comparing severe and mild disease [4], whereas in Mali an association between group A *var* genes and cerebral malaria was observed in children [5]. Such conflicting results may be due to differences in geographic region or to physiological differences among the groups of patients with severe malaria, who might have a mixture of various, not-well-defined symptoms; alternatively, the group might simply be too small in size. In the present study, we compared *var* gene expression between children with mild malaria and children with cerebral malaria or severe anemia, all of whom were comparable with respect to age, parasitemia level, and body temperature. We also examined correlations between *var* gene types and expression of human genes relevant to the response to tumor necrosis factor (TNF), hypoxia, and arginine metabolism.

Methods. African children from Gabon aged 6 months to 6 years who presented to the Centre Hôpitalier de Libreville or the Albert Schweitzer Hospital in Lambaréné for malaria diagnosis were eligible for the study. Children with either cerebral malaria (Blantyre Coma Scale [BCS] score, <3), severe malarial anemia (hemoglobin level, <5 g/dL), or uncomplicated malaria were recruited after parental informed consent was obtained. Entry criteria were a temperature of >37°C and the presence of *P. falciparum* parasitemia, as determined by examination of thick blood smears. Exclusion criteria were asymptomatic *P. falciparum* infection and indications for concurrent diseases. Five milliliters of peripheral blood was obtained from each child by health personnel and was transferred into PAXGene RNA tubes (Qiagen), in accordance with the manufacturer’s instructions. RNA was extracted using the PAXGene Blood RNA kit (Qiagen), in accordance with the manufacturer’s instructions. Comple-

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Table 1. Clinical Data on the 3 Patient Groups

Parameter	Cerebral (n = 21)	Anemic (n = 26)	Uncomplicated (n = 15)	P
Temperature, °C	38.1 ± 1.0	38.5 ± 1.0	38.4 ± 1.1	.54
BCS score ^a	2 ± 0	5 ± 0	5 ± 0	<.001
Parasitemia level, parasites/μL				
Median ± absolute deviation	49,200 ± 45,700	26,250 ± 20,738	66,500 ± 58,500	.22
Range	1500–855,000	1500–236,000	3600–300,000	
Hemoglobin level, g/dL ^a	6.8 ± 2.2	3.7 ± 0.6	8.4 ± 2.3	<.001
Age, months	42 ± 22	30 ± 23	39 ± 17	.07

NOTE. Data are means ± absolute deviation of the mean, unless otherwise indicated. *P* values were calculated by Kruskal-Wallis estimation.

^a Blantyre Coma Scale (BCS) score and hemoglobin level were used for classification of groups.

mentary DNA (cDNA) was synthesized using SuperScript II reverse transcriptase (Invitrogen).

For differential *var* group transcriptional analysis, quantitative real-time polymerase chain reaction (PCR) specific for all 3 *var* gene groups was performed as described elsewhere [3], using seryl-tRNA synthetase and fructose-bisphosphate aldolase transcripts as endogenous controls. Quantitative real-time PCR with minor groove binding (MGB) probes was performed using a ABI Prism 7200 sequence detection system (Applied Biosystems), as described elsewhere [2] with a few modifications. Briefly, cDNA was synthesized from total RNA, and a primary PCR with 16 cycles over the *var* 5' untranslated region (UTR)–DBL1α target sequence was performed before real-time PCR was performed for *var* groups A, B, and C. Primers and probes targeting upsA, upsB, and upsC *var* genes for the MGB probe assay (upsA probe, upsB probe, and upsC probe) have been described elsewhere [2]. As an internal control gene, seryl-tRNA synthetase was used for relative quantification without prior amplification. Primers and probes were designed using Primer Express software (version 2.0; Applied Biosystems) and included p90Probe_for (5'-ACCTCAGAACCACTTATGTGCTT-3'), p90Probe_rev (5'-TGTGCCCTGCTTC-TTTTCTAA-3'), and p90Probe (5'-[6-FAM]AGGTTACCAC-TCAAATACGCTGGATTCTCATCTTG[TAMRA-6-FAM]-3'). Oligonucleotide primers for amplification of *var* gene regions were as follows: for *var* group A, 5_UTR (150 bp), upsA1_for* (5'-AACTTACCATAAATTATCATCAAA-3'), upsA3_probe C5'-[6FAM]-AAACCTTTGGTATAGAAAAAATATT-MGB-3'), and upsAj_rev (5'-TCACCTACAACAAAT[A/G]TAATAAA-3'); for *var* group B, 5_UTR (360 bp), 17deg_for* (5'-CTCAT[A/T]TATAATTTTA[C/G]AAAATA[A/T]A[A/T]AAAAAC-3'), upsB1_probe (5'-[6FAM]-TCTAACAAAAAATAAAC-AACAATTAC-MGB-3'), and RT-17.2_rev (5'-TTA[A/T]GGGAGTAT[A/T]GT[A/G/T]ATATGGTAGAAT-3'); and for *var* group C, 5_UTR (240 bp), RT-5B1.1_for* (5'-AATATTCAT-ATTCCACATT[A/G]TCATATAT-3'), upsC_probe (5'-[6FAM]-

ACATATAATACGACTAAGAAAC-MGB-3'), and 5B1.4_rev (5'-ATTATGTGGTAATATCATGTAATGG-3'). Forward primers marked with an asterisk (*) were identical in primary and real-time PCR.

The expression profile of defined human genes was determined using 2-step Assay-on-Demand gene expression products (Applied Biosystems). After cDNA was synthesized from total RNA by means of SuperScript II reverse transcriptase (Invitrogen), 20-μL reactions were prepared. Each reaction consisted of 20 ng of cDNA (1 ng/μL), 1× TaqMan Universal Master mix, and 1× Assay-on-Demand gene expression mix. For amplification and proof of the target messenger RNA, we used the GeneAmp 5700 sequence detection system (Applied Biosystems) with the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. As an endogenous control, we used hypoxanthine guanine phosphoribosyl transferase 1. After normalization, the data, logarithmized to base 2, were analyzed by analysis of variance (ANOVA) and were corrected by the Bonferroni-Dunn method.

After all samples had been subjected to real-time PCR, the data set was cleaned for subsequent statistical analysis. Data points were not considered if the melting temperature diverged >1°C from the expected value or if the cycle threshold (Ct) value was >30. Transcript abundance was compared between clinical groups after normalization to internal controls (yielding ΔCt values). On the basis of these values, fold changes were calculated by the ΔΔCt method. Comparisons were done using the nonparametric Kruskal-Wallis test; parametric tests were done using ANOVA, with StatView software (version 7.0; SAS Institute). Differences with *P* < .05 were considered significant.

This study was approved by the ethics committees of the International Foundation for the Albert Schweitzer Hospital in Lambaréné, Gabon, and the Medical Faculty of the University of Tübingen, Germany.

Results. In total, 62 patients with either cerebral malaria (*n* = 21), severe malarial anemia (*n* = 26), or mild malaria

($n = 15$) were analyzed. The groups did not differ in age, parasitemia level, or body temperature. Groups differed significantly in the most important criteria, that is, BCS score and hemoglobin level (Table 1).

When the relative expression of the 3 *var* gene groups was analyzed, a significant difference was observed between children with cerebral malaria and those with uncomplicated malaria or severe malarial anemia (Figure 1A). In all patient groups, upsA was the prominent *var* gene type; it was most pronounced in patients with severe malarial anemia or uncomplicated malaria (Figure 1A). The least expressed variant was the upsB type.

When children with cerebral malaria were compared with children with severe malarial anemia or uncomplicated malaria, highly statistically significant differences were observed in the expression level of all 3 ups groups. No statistically significant difference was found in the expression pattern of the 3 variants between children with severe malarial anemia and those with uncomplicated malaria.

Among 25 patients, all of whom had malarial anemia, we correlated RNA levels for a panel of human genes with ups expression; we focused on 10 genes located in the chromosomal region 5q31–33 (mainly transcription factors and stimulus-re-

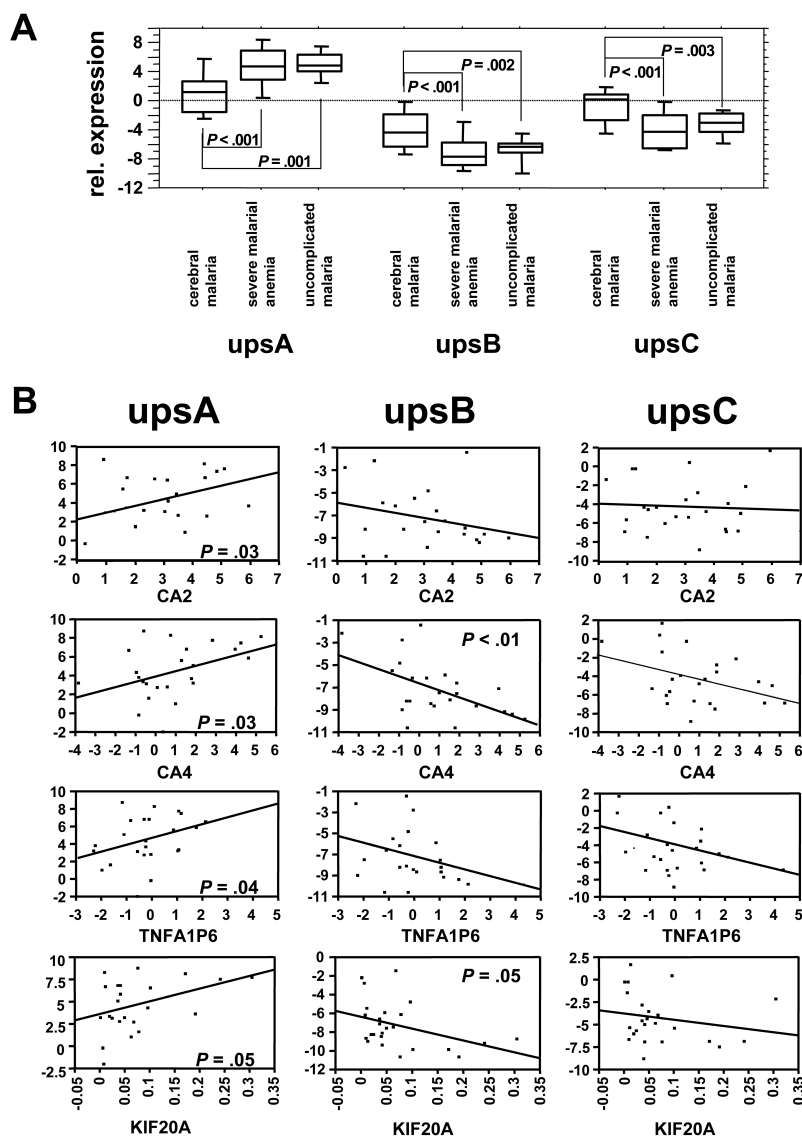


Figure 1. Relative expression of ups variants in patients with malaria of differing severity and influence on host gene expression. *A*, Boxplots showing relative expression in patients with cerebral malaria ($n = 21$), severe malarial anemia ($n = 26$), or uncomplicated malaria ($n = 15$). *P* values are shown only for significant differences. *B*, Correlation of expression of upsA, upsB, or upsC with 4 selected human genes encoding for carbonic anhydrase (CA) 2 and 4, kinesin 20A (KIF20A), and tumor necrosis factor–induced protein 6 (TNFIP6). *P* values are shown only for significant correlations.

sponsive genes). This region has been described as being involved in mediating protection against malaria and contains genes coding for cytokines, receptors belonging to clusters of differentiation (CD), and TNF-responsive molecules. Several single-nucleotide polymorphisms in these genes have been described as being associated with the outcome of malaria. Other genes were chosen on the basis of their involvement in arginine metabolism, because it has been shown that low arginine and low nitric oxide production are common in severe malaria, probably as a result of increased arginase production in severe malaria. Another group of molecules we analyzed were transporters that are involved in metabolite transport and pH regulation. We observed a positive correlation of kinesin 20A (located in 5q31–33), TNF-induced protein 6, and carbonic anhydrase 2 and 4 with upsA and the opposite for the same genes with upsB or upsC (Figure 1B). Other genes were not heavily influenced by ups expression.

Discussion. In African children, cerebral malaria is one of the most dangerous complications of *P. falciparum* infection. It has been hypothesized that the course of a malarial infection is reflected in similar expression patterns for members of certain *var* gene groups associated with anemia and uncomplicated disease, whereas in the case of cerebral complications the *var* gene expression pattern is different.

Our results showing a distinct *var* gene expression pattern in patients with cerebral malaria are in accordance with observations of others [5, 6], who also described an association between defined *var* genes expression patterns and certain clinical presentations. Discrepancies in the expressed subtypes might be explained merely by geographical or genetic differences. In Gabon, a child experiences 1–2 episodes of malaria per year; therefore, the children enrolled in this study will have had a limited number of infections. This might explain the high prevalence of the *var* upsA types, which in another study have been described as being associated with the first infections occurring in early life [7]. In our study, patients with cerebral malaria showed a more even distribution of ups types; this has also been observed in the expressed *var* gene repertoire among patients in another study, which clearly showed that a greater number of *var* genes are transcribed in patients with cerebral malaria, whereas fewer variants are transcribed in patients with severe anemia but at a higher rate [6].

It is possible that ethnically determined host genetic polymorphisms could influence the expression of certain *var* genes, and the most prominent receptor involved in cerebral malaria is intercellular adhesion molecule 1 (ICAM-1) [8]. It has been shown that polymorphisms in ICAM-1 can influence the likelihood that a child might develop cerebral malaria. However, such polymorphisms can be beneficial [8] or detrimental [9] depending on the region considered, and it is interesting to speculate to what degree such host polymorphisms can influ-

ence the expression of *var* genes. Additional studies conducted in different regions with different ethnic groups will be necessary to reach definitive conclusions.

We present data indicating that specific PfEMP1 types may influence the expression of human genes. Interestingly, the different ups variants have antidromic effects on defined genes. Genes regulated in opposite directions in individuals can be observed in microarray studies involving patients with malaria [10], in which individual differences were attributed to cellular activity. Perhaps this countereffect is also caused by the expressed *var* gene type in the particular patient. Two of the genes regulated in this way encode TNF-responsive proteins. TNF itself has been shown to be induced by parasites in a strain-specific manner [11], so perhaps what we see here is a secondary effect of TNF secreted by macrophages and monocytes on contact with parasitized erythrocytes expressing variant PfEMP1 molecules. In addition, carbonic anhydrases can contribute to malarial pathology, since it has been shown that free carbonic anhydrases of erythrocytic origin can mediate hemorrhagic retinal and cerebral vascular permeability [12]. Furthermore, carbonic anhydrases are induced by hypoxia, which is a feature of malaria [13]. Given that upsA types have been correlated with increased expression of carbonic anhydrases and associated with severity of malaria in other studies, part of the pathology may be due to overexpression of these enzymes.

Interaction between parasitized cells and immune cells is common, and suppressive effects on dendritic cells have been described [14]. The opposite effect, leading to an overstimulated immune response and subsequently to an aggravation of malarial symptoms, is also conceivable. That PfEMP1 influences induction by TNF or hypoxia shows a novel facet of the interplay between host and parasite. Because there are conflicting results regarding the role played by PfEMP1 in interactions between immune cells and infected blood cells [15], underlying mechanisms probably depend on cell type and the expressed *var* gene and will be investigated in additional studies.

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