

## ASSOCIATION OF THE ICAM-1<sup>KILIFI</sup> MUTATION WITH PROTECTION AGAINST SEVERE MALARIA IN LAMBARÉNÉ, GABON

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**Abstract.** The intercellular adhesion molecule-1 (ICAM-1) is thought to be a receptor that mediates binding of *Plasmodium falciparum*-infected erythrocytes. Especially in vital organs, the binding of parasitized cells to the endothelium via ICAM-1 may lead to severe disease and death. Recently, a mutation in the coding region of ICAM-1, termed ICAM-1<sup>Kilifi</sup>, was described, causing a change from Lys to Met in the loop that interacts with rhinoviruses, lymphocytes, and parasitized red blood cells. Surprisingly, this mutation was shown to increase susceptibility of Kenyan children to severe malaria in one study. When we compared the distribution of ICAM-1<sup>Kilifi</sup> in two groups of Gabonese children enrolled in a case-control, matched-pair study who presented with either mild or severe malaria, we found that 55% of the patients with mild malaria were carriers whereas only 39% of those with severe malaria were carriers. The difference in the distribution of ICAM-1<sup>Kilifi</sup> homozygous pairs between the groups, as well as the distribution of ICAM-1<sup>Kilifi</sup> carriers, was statistically highly significant ( $P = 0.027$  and  $P = 0.012$ , by the McNemar test). In a group of healthy school children from the same region, a distribution of 52% ICAM-1<sup>Kilifi</sup> carriers to 48% wild-type individuals was found. In a survey for the ICAM-1<sup>Kilifi</sup> in other malaria-endemic regions, this allele was also found in Nigeria and Papua New Guinea, but not in Thailand.

Malaria is one of the most widespread diseases in the world, predominantly in tropical countries. Most of the approximately 400 million clinical cases per year are caused by the highly pathogenic protozoan parasite *Plasmodium falciparum*. Malaria can affect humans differently: up to 10% will have a severe malaria attack, and probably 3 million, especially African children, will die of clinical complications.<sup>1</sup> One of these complications concerns cerebral functions that might lead to coma and death. The pathologic correlate is the binding of *P. falciparum*-infected erythrocytes to brain endothelial cells. The molecular basis of how these infected red blood cells sequester in blood capillaries seems to involve both parasite and host proteins. On the parasite, the variant *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) acts as a ligand that can bind to various receptors. Host cell receptors include CD36, intercellular adhesion molecule-1 (ICAM-1; CD54), the vascular cellular adhesion molecule, thrombospondin, and the polysaccharide chondroitin sulfate-A.<sup>2</sup>

Intercellular adhesion molecule-1 in particular seems to play an important role in malaria pathology. In histopathologic studies, it was shown that ICAM-1 and bound parasitized red blood cells co-localize in brain capillaries of cerebral malaria patients.<sup>3</sup> Furthermore, parasites isolated from cerebral malaria patients showed the highest capacity of binding to ICAM-1.<sup>4</sup> More detailed studies showed that ICAM-1 indeed could bind parasitized erythrocytes at a loop structure that also binds rhinoviruses and the lymphocyte function-associated antigen-1.<sup>5,6</sup>

A mutation was recently described that affects the amino acid sequence of ICAM-1. An A to T transition causing a Lys to Met exchange at position 29 was found in different racial groups in North America and in a Kenyan population in the area of Kilifi.<sup>7,8</sup> This mutation has a very high allelic frequency of 0.322 in Kenya and is predominantly found in African-Americans in the United States. In a cross-sectional study, it was shown that this mutation is associated with

susceptibility to cerebral malaria.<sup>8</sup> However, when this mutant (ICAM-1<sup>Kilifi</sup>) was tested for binding to parasites *in vitro*, no significant difference could be observed between the mutant and wild-type proteins.

We investigated whether ICAM-1<sup>Kilifi</sup> also exists in other malarious areas and how it correlates with disease severity in the central African country of Gabon. We compared severe malaria cases having hyperparasitemia and severe anemia with patients with mild malaria and performed a population survey of this mutation in Papua New Guinea, Thailand, and Nigeria.

### MATERIALS AND METHODS

**Patients.** Patients were recruited for a matched pair, case-control study that took place at the Albert Schweitzer Hospital in Lambaréné, Gabon. One-hundred children in each group were enrolled into the study from 1995 until 1996 if they fulfilled the inclusion criteria: *P. falciparum* malaria with a parasitemia  $> 1,000$  parasites/ $\mu$ l, older than six months of age, and not homozygous for hemoglobin S. Severe malaria was defined as severe anemia (hemoglobin  $< 50$  g/L) and/or hyperparasitemia ( $> 250,000$  parasites/ $\mu$ l corresponding to  $> 10\%$  infected erythrocytes) and other facultative signs of severe malaria such as cerebral malaria, convulsions, hypoglycemia, and respiratory distress.<sup>9</sup> The matching partner was chosen from patients of the same sex, age, and locality, but with mild malaria, and was admitted as soon as possible after the severe case was included in the study. For a mild case of malaria, the following criteria had to be fulfilled: parasitemia between 1,000 and 50,000/l on admission, no schizonts in the periphery, malarial pigment containing circulating leukocytes  $< 50/\mu$ l, not homozygous for hemoglobin S, hemoglobin  $> 80$  g/L, platelets  $> 50/\text{nl}$ , leukocytes  $< 12/\text{nl}$ , lactate  $< 3$  mM, and blood glucose  $> 50$  mg/dl. Exclusion criteria were also signs of severe malaria or other acute infections and prior hospitalization for

any reason to exclude possible severe malaria in the history and intake of antimalarial drugs within the preceding week. Children with chronic diseases or malnutrition were excluded. Parents gave informed consent. The study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital.

Children with severe malaria were admitted to the hospital and treated intravenously with quinine plus clindamycin for four days.<sup>10</sup> Supportive treatment was given as required. Children with mild malaria were treated as outpatients receiving a single oral dose of sulfadoxine and pyrimethamine.<sup>11</sup> All children were observed until parasitologic and clinical cure or death. After the acute phase of the malaria attack, the children joined an ongoing, longitudinal, prospective study that compared reinfection rates.

Routine pediatric micromethods were used to measure hematologic and biochemical parameters. Giemsa-stained thick and thin blood smears were prepared to quantify parasitemia, schizontemia, and malarial pigment containing neutrophils and monocytes.<sup>12,13</sup>

**Isolation of DNA.** The DNA was purified from 200  $\mu$ l of blood from the 200 children representing 100 mild malaria and 100 severe malaria cases using the Qiagen blood kit (Qiagen, Hilden, Germany). A diagnostic polymerase chain reaction (PCR) to investigate the point mutation in the ICAM-1 gene was performed using standard procedures on a Perkin-Elmer (Foster City, CA) DNA Thermocycler.<sup>14</sup> This PCR resulted in a DNA fragment of 304 basepairs. The primers were obtained from Interactiva (Ulm, Germany). The forward primer was 5'-TCT TCC CTC GTT TCT TCT AGG ACC TGG-3'. The reverse primer was 5'-TCC GGG CTC AGT TAC TCA CAG TAC ACG-3'. The PCR conditions were as follows: after an initial denaturation at 92°C for 5 min, 40 cycles were carried out at an annealing temperature of 55°C for 1 min, an extension temperature of 72°C for 1 min, and a denaturing temperature of 92°C for 1 min; the PCR was completed with a final extension at 72°C for 10 min. The reaction volume (50  $\mu$ l) contained 200  $\mu$ M of each dNTP, 10 mM Tris-HCl, pH 8, 20 mM MgCl<sub>2</sub>, 50 mM KCl, and 0.5 units of *Taq* polymerase (Qiagen). The PCR products were subjected to electrophoresis on a 1.2% agarose gel in 1 $\times$  Tris-buffered electrophoresis buffer (90 mM Tris-acetate, pH 8.0, 90 mM boric acid, 2.5 mM EDTA<sup>15</sup>).

Differentiation between the two alleles was done by digestion with the restriction endonuclease *Nla* III (New England Biolabs, Beverly, MA). The digestion was done at 37°C in the reaction buffer supplied by the manufacturer (50 mM KCl, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM dithiothreitol). The *Nla* III-digested PCR products were subjected to electrophoresis on a 1.5% agarose gel according to standard procedures.<sup>15</sup>

**Statistical analysis.** Statview (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis of data. The McNemar test was used for the paired analysis.

## RESULTS

**Severe versus mild malaria in Gabon.** A diagnostic PCR was used to investigate the point mutation in the ICAM-1 gene from 100 children with mild malaria and 100 with severe malaria. The PCR produced a DNA fragment of 304

TABLE 1  
Intercellular adhesion molecule-1 (ICAM-1) gene polymorphism and malaria in Lambaréné, Gabon\*

	K29/K29	K29/M29	M29/M29	n
Severe malaria	61	37	2	100
Mild malaria	45	45	10	100

\* Values are the number of severe and mild malaria cases with different variants of ICAM-1. K29/K29 are homozygotes for ICAM-1 wild-type, M29/M29 are homozygotes for ICAM-1<sup>Kilifi</sup>; K29/M29 are heterozygous for the alleles. McNemar test: homozygous ICAM-1<sup>Kilifi</sup> patients (M29/M29) compared with the remaining patients:  $P = 0.027$ ; all carriers (K29/M29) compared with the ICAM-1 wild-type patients (K29/K29):  $P = 0.012$ .

base pairs. This PCR product was obtained from all 200 samples and digested with the restriction endonuclease *Nla* III. All samples showed an unambiguous restriction pattern as expected for the two alleles for ICAM-1. All DNA fragments could be classified as homozygous ICAM-1 wild type, homozygous ICAM-1<sup>Kilifi</sup>, or heterozygous: *Nla* III cuts the DNA of those with the Kilifi mutation into three fragments of 183, 96, and 25 basepairs; this enzyme cuts the DNA from those with the wild-type alleles into two fragments of 208 and 96 basepairs. The PCR products from heterozygous patients contained fragments of 208, 183, 96, and 25 basepairs.

In the group with severe malaria, 39 patients were carriers of the ICAM-1<sup>Kilifi</sup> mutation: 2 homozygous and 37 heterozygous; 61 patients had the wild-type alleles for ICAM-1 (Table 1). In the group with mild malaria, 55 patients were carriers of the mutation: 10 homozygous and 45 heterozygous. The difference in the distribution of this mutation was statistically significant. When only homozygous patients were compared with the remaining patients, the  $P$  value was 0.027; when all carriers were compared with the patients carrying the wild-type alleles, the  $P$  value was 0.012. The odds ratio of being affected by severe malaria in a carrier of the ICAM-1<sup>Kilifi</sup> mutation was 0.52 (95% confidence interval [CI] = 0.30–0.92). The odds ratio of being affected by severe malaria in an individual homozygous for ICAM-1<sup>Kilifi</sup> was 0.18 (95% CI = 0.04–0.86). Therefore, we concluded a protective role of the ICAM-1<sup>Kilifi</sup> mutation in our study group. Nine patients in the group with severe malaria had cerebral malaria. Three of them were heterozygous and six had the wild-type alleles for ICAM-1. No  $P$  value can be given because of the limited number of patients with this complication.

**Frequency of ICAM-1<sup>Kilifi</sup> mutations in other populations.** We were interested in whether the ICAM-1 mutation is also present in other populations that are affected by malaria. We collected samples from Nigeria, Gabon, Thailand, Papua New Guinea, and Europe. The samples from Nigeria were taken from a population presenting to a hospital with asymptomatic parasite infections or mild malaria. Gabon samples were from healthy school children; Thailand samples were from patients with *P. vivax* malaria; Papua New Guinea samples were from individuals with mild or asymptomatic malaria infections; European samples were from Caucasian blood donors.

The Kilifi mutation was absent in the Thai and European populations; in all other samples, ICAM-1<sup>Kilifi</sup> was found at various frequencies (Table 2). In samples from Papua New Guinea, 1% ( $n = 1$ ) were homozygous and 7% ( $n = 5$ ) were

TABLE 2  
Distribution of intercellular adhesion molecule-1 (ICAM-1) gene polymorphism in malaria-endemic area\*

	Genotype			Frequency	
	K29/K29	K29/M29	M29/M29	K29	M29
Thailand (n = 59)	59 (100%)	0 (0%)	0 (0%)	1	0
Papua New Guinea (n = 71)	65 (91.55%)	5 (7.00%)	1 (1.45%)	0.951	0.049
Gabon (n = 102)	53 (52.00%)	43 (42.15%)	6 (5.85%)	0.730	0.270
Nigeria (n = 190)	86 (45.26%)	88 (46.32%)	16 (8.42%)	0.684	0.316
Kenya† (n = 287)	135 (47.04%)	113 (39.37%)	39 (13.59%)	0.668	0.332

\* K29/K29 are homozygous patients with the wild-type; K29/M29 are heterozygous individuals; M29/M29 indicate homozygous persons with the ICAM-1<sup>Kilifi</sup> mutation. The genotype distribution is given as the number (%).

† Data from Fernandez-Reyes and others.<sup>8</sup>

heterozygous for the ICAM-1<sup>Kilifi</sup> mutation. In the African populations, the frequency of this mutation was 46% (Table 2): 46% (n = 88) of the Nigerian population were heterozygous and 8% (n = 16) were homozygous, and 42% (n = 43) of the Gabonese population were heterozygous and 6% (n = 6) were homozygous. This is comparable with recent data from a study in Kenya, which showed a population of which 39% (n = 113) were heterozygous and 14% (n = 39) were homozygous.<sup>8</sup>

#### DISCUSSION

A mutation was recently found that caused an A ⇒ T transversion in the ICAM-1 gene, causing a Lys to Met change in the amino acid sequence.<sup>7,8</sup> This mutation was found in different populations in Africa and North America. In the North American population, it was found predominantly in African-Americans (n = 41%), but also in individuals with Caucasian (1%, n = 1), Asian (10%, n = 10), or Hispanic (4%, n = 4) backgrounds. The racial background was given by self-identification.

In contrast, no Caucasian was found to carry the ICAM-1 gene mutation.<sup>8</sup> In the two Asian populations studied, only in the Papua New Guinean population was the ICAM-1 gene mutation found. No Thai had the mutation. In North Americans with an Asian background, the ICAM-1<sup>Kilifi</sup> mutation was found.<sup>7</sup> The reason for this discrepancy might be that the Asian-American population is of a greater complexity and will contain more than two different Asian populations than Thai or Papua New Guineans. The allelic frequency in Asian-Americans is very similar to the Papua New Guinean population. Although it is unknown whether there is a substantial migration from Papua New Guinea to the United States, we speculate that other groups may contribute to the gene frequency found in the United States.

A recent study showed that the ICAM-1<sup>Kilifi</sup> mutation is a marker for susceptibility to cerebral malaria in Kenya.<sup>8</sup> Its high frequency in populations in the malarious regions of Africa is explained by a protective effect to pathogens other than *P. falciparum*, such as bacterial infections. In a study conducted in The Gambia, no correlation between ICAM-1 gene polymorphism and disease severity was found.<sup>16</sup> The results obtained with our study group suggest that carriers are protected against severe disease. The criteria for severe disease in the Gabonese study were hyperparasitemia and severe anemia. Both are features of an overwhelming parasite multiplication, which seems to be prerequisite for any other clinical complication that will eventually lead to

death.<sup>9</sup> The Kenyan and Gambian studies compared patients with cerebral malaria and severe anemia with controls. Since the incidence of cerebral malaria in Gabon is rather low, we were not able to collect data from a larger number of these cases. Therefore, we were not able to draw any conclusions about the 9 patients with cerebral malaria in the present study, of whom three were heterozygous carriers of the Kilifi allele and six had the wild-type alleles. However, the validity of our inclusion criteria is corroborated by the distribution of the sickle cell trait in those with severe malaria compared with those with mild malaria.<sup>17,18</sup> We found that the sickle cell trait was present at a higher frequency in the group with mild malaria ( $P = 0.034$ , by the McNemar test). Furthermore, longitudinal data indicate that our patients with severe malaria are more likely to have higher parasitemias and significantly lower hematocrits following malaria attacks than those with mild malaria, and initial cases of severe malaria were infected more often.<sup>19</sup>

There are several explanations for the discrepancies in the results between the Gambian, Kenyan, and Gabonese studies. The different patient groups might play a role since in Kenya and The Gambia cases with cerebral malaria and severe anemia were compared with those with uncomplicated malaria in a cross-sectional study. Our study compared cases with mild malaria and those with severe malaria in a matched case-control study. The effects of the ICAM-1 mutation may be influenced by other genetically determined factors that enhance or weaken binding of ICAM-1<sup>Kilifi</sup> to infected cells, causing susceptibility or protection. This speculative factor might be related to the ICAM-1 mutation. Another reason might be regionally different *P. falciparum* strains that bind with different affinities to endothelial cells in those with the Kilifi mutation. In the recent years, a number of reports have described *P. falciparum* strains that were associated with differences in disease outcome.<sup>17,20,21</sup> Different ligands for ICAM-1 in a parasite population may cause different binding profiles. Little data is currently available that deals with the geographic distribution of the parasite ligand PfEMP1. It would therefore be very interesting to study binding profiles of field isolates with regard to ICAM-1 variants.

Acknowledgments: We thank Silvelia Grummes, Marcel Nkeyi, and Anselme Ndzenge for excellent technical help. We are also very grateful to all colleagues involved in the 1/95C study: Ruprecht Schmidt-Ott, Leopold Lehmann, Bernhard Greve, and Peter Matoušek.

Financial support: This work was supported by grants from the fortune Program of the Medical Faculty of the University of Tübingen.

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