

# Common apolipoprotein E polymorphisms and risk of clinical malaria in the Gambia

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**A**polipoprotein E (apoE) is a protein involved in the transport and metabolism of plasma cholesterol and triglycerides. The apolipoprotein E gene (APOE), located at chromosome 19q13.2, has three major alleles called  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ , defined by two single nucleotide polymorphisms (SNP) located in exon 4 at positions 3937 (T/C) and 4075 (C/T). The corresponding apoE isoforms differ at amino acid positions 112 (Cys for apoE2 and apoE3, Arg for apoE4) and 158 (Arg for apoE3 and apoE4, Cys for apoE2), and have been shown to have different functional and biochemical properties.<sup>1–4</sup> APOE polymorphisms have been studied in relation to several genetic diseases and disorders. The APOE  $\epsilon 4$  allele has been associated with an increased risk of Alzheimer's disease, coronary heart disease, and death after myocardial infarction.<sup>5–7</sup> Conversely, the APOE  $\epsilon 2$  allele was found to have a protective effect on the occurrence of Alzheimer's disease. However, in other studies, APOE  $\epsilon 2$  was associated with an increased risk of cardiovascular diseases and with some blood lipid abnormalities.<sup>8,9</sup> It has been reported recently that APOE  $\epsilon 2$  homozygote children from Ghana became infected with *Plasmodium falciparum* at an earlier age than those carrying other APOE genotypes.<sup>10</sup>

In this study, performed in the Gambia, the APOE  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  allele distributions were analysed in children with mild (338 cases) or severe (530 cases) malaria, and in individually matched control children (560 subjects). Additionally, the APOE Th1/E47 polymorphism located in the APOE promoter region<sup>11</sup> was studied in a subset of children consisting of 183 severe malaria cases and 179 controls.

## MATERIALS AND METHODS

### Study subjects

Between August 1989 and September 1990, 1428 children aged from 1 to 10 years were enrolled at the Royal Victoria Hospital of Banjul and at the Medical Research Council Hospital of Fajara, in the Gambia. Malaria was diagnosed if a patient with an appropriate clinical picture had parasitaemia  $>2500/\mu\text{l}$ , and relevant laboratory investigations did not suggest other diagnoses. Cerebral malaria was defined by a Blantyre coma score of more than 3 (persisting for more than 30 minutes after effective treatment of hypoglycaemia or convulsions) or repeated prolonged seizure (for more than 30 minutes) in a child with *P. falciparum* parasitaemia and no other apparent cause of fits or coma. Severe malaria anaemia was defined as a haemoglobin level of less than 50 g/l on admission in a child with parasitaemia. Children with mild malaria had an uncomplicated febrile illness with *P. falciparum* parasitaemia and no other apparent cause of fever.

The group of severe malaria patients was matched to two control groups of children for age and area of residence. The mild controls were recruited at both hospitals and health centres in the study area. These children had mild, mostly infective, illnesses that did not require hospital admission and they did not have malarial parasites in their blood on microscopy. Severely infected controls were inpatients at the

## Key points

- Apolipoprotein E (ApoE) is involved in the transport of plasma cholesterol and triglycerides. ApoE polymorphisms have been associated with blood lipid abnormalities and with an increased risk of Alzheimer's disease or cardiovascular disease, in many studies. Recently, the APOE  $\epsilon 2$  allele has been associated with an increased risk of early infection by *Plasmodium falciparum* in Ghanaian children.
- In this study, APOE  $\epsilon 2$ ,  $\epsilon 3$ ,  $\epsilon 4$ , and APOE Th1/E47 polymorphic markers were genotyped for 1428 children in a Gambian case control study, to evaluate the effect of APOE polymorphisms on the risks of mild and severe malaria.
- In this Gambian sample, APOE allele frequencies were 12.8%, 64.4%, and 22.8% for  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ , respectively. No differences in frequencies were observed between the controls and either the mild malaria cases or the severe malaria cases. Moreover, no differences were observed between the mild malaria cases and the severe malaria cases. However, in a particular subgroup of children developing both cerebral malaria and severe malarial anaemia, the APOE  $\epsilon 3/\epsilon 4$  genotype was found to be more frequent (42.9%) than in the controls (24.8%) ( $2 \times 2 \chi^2 = 7.57$ ;  $p = 0.006$ ) or in the mild malaria cases (27.2%) ( $2 \times 2 \chi^2 = 5.06$ ;  $p = 0.024$ ).
- Our results suggest that APOE polymorphisms do not influence protection against cerebral malaria and severe malarial anaemia, but could possibly play a role in protection against a particularly severe form of severe malaria.

two hospitals, with a large range of other acute, mainly infective, illnesses but without evidence of current or recent malaria infection.

The ethnic composition of the population in this area is mixed: Mandinka (42%), Jola (14%), Wolof (14%), Fula (12%), and several less common ethnic groups. The children from the different groups were not matched for ethnic group; instead, analyses stratified with this variable were carried out. Details of this case control study have been described previously.<sup>12</sup>

The study had the approval of the Gambian Government and of the MRC Joint Ethical Committee, and consent was obtained from the parent or guardian of each child entered.

### Apolipoprotein E genotyping

Base positions mentioned in this paper refer to the APOE reference sequence GenBank AF261279. Three APOE SNP

markers, located at positions 832 (APOE-Th1/E47), 3937 (apoE+112), and 4075 (apoE+158), were studied by ligation detection reaction (LDR).<sup>13</sup> For the APOE-Th1/E47 (G/T) SNP, PCR was performed in 15 µl of reaction mixture containing: 50 ng genomic DNA; 10 mM Tris-HCl; 50 mM KCl (pH 8.3, GeneAmp 10X Buffer II, Perkin Elmer, Beaconsfield, UK); 1.0 mM MgCl<sub>2</sub>; 80 µM of each dNTP; 1.0U AmpliTaq Gold DNA polymerase; and 0.33 µM each of forward (5'-TACTTTCTTTCTGGGATCCAGG-3') and reverse (5'-ACTCAAGGATCCCAGACTTG-3') primers.<sup>11</sup> For the APOE 3937 (T/C) and 4075 (C/T) SNPs, a single PCR was performed in 20 µl of reaction mixture containing: 50 ng of genomic DNA; 10 mM Tris-HCl; 50 mM KCl (pH 8.3); 1.5 mM MgCl<sub>2</sub>; 50 µM of each dNTP; 1.0U AmpliTaq Gold DNA polymerase; and 0.25 µM each of forward (5'-AGATGC GGGCACGGCTGTCAAGGA-3') and reverse (5'-CCCTCGC GAGCCCCGGCTGTACAC-3') primers.<sup>14</sup> PCR amplification reactions were performed using an MJ Research tetrad thermal cycler as follows: 94°C for 14 minutes; 39 cycles of 94°C for 60 seconds; 60°C (APOE-Th1/E47) or 67°C (APOE 3937 and 4075) for 60 seconds; 72°C for 60 seconds (APOE-Th1/E47) or 90 seconds (APOE 3937 and 4075); and finally 72°C for 7 minutes.

The SNP genotyping was carried out using the LDR method.<sup>13</sup> For each SNP, the allele specific oligonucleotide probe pairs were distinguished by different fluorescent labels (FAM/HEX) and by their lengths. The common probes were phosphorylated at their 5' extremities (P). LDR reactions were performed as previously described,<sup>15</sup> and LDR products were analysed using an ABI 3700 (Applied Biosystems, Warrington, UK). The following probes were used for APOE-Th1/E47: APOE-Th1/E47-G 5'-(FAM)-AAAAAAAACCCAGAA TGGAGGAGGGTGTCTGG-3', APOE-Th1/E47-T 5'-(HEX)-AA AAAAAACCCAGAATGGAGGAGGGTGTCTGT-3', and APOE-Th1/E47-common 5'-(P)-ATTACTGGGCGAGGTGTCCTCCCTT CAAAAATAAAAAAAAAA-3'. For APOE 3937 we used: APOE 3937-C 5'-(FAM)-AAAAAAAAGCGCGACATGGAGGACGT GC-3', APOE 3937-T 5'-(HEX)-AAAAAAAAGGCGCGGAC ATGGAGGACGTGT-3', and APOE 3937-common 5'-(P)-GC GGCCGCTGGTGCAGTACCAAAAAA-3'. For APOE 4075 we used: APOE 4075-C 5'-(FAM)-AAAAAAAACGATGCCG ATGACCTGCAGAAGC-3', APOE 4075-T 5'-(HEX)-AAAAA AAAAAAGCGATGCCGATGACCTGCAGAAGT-3', and APOE 4075-common 5'-(P)-GCCTGGCAGTGTACCAGGCCGAAAA AAAATAAA-3'. Non-APOE sequence nucleotides were added to the LDR probes to increase the size of the LDR products. The APOE ε2 allele was defined by a T at position 3937 and a T at position 4075. The APOE ε3 allele was defined by a T at position 3937 and a C at position 4075. The APOE ε4 allele was defined by a C at position 3937 and a C at position 4075. The APOE ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4 genotypes were defined according to individuals' APOE 3937 and APOE 4075 genotypes (for more information see <http://www.well.ox.ac.uk/hill/Malaria/APOEgenotyping.htm>). Allele frequencies were then calculated using genotype data.

### Statistical analyses

Allele and genotype frequencies were compared between children with malaria and the control children, using the Pearson's  $\chi^2$  test. Logistic regression analysis was then performed to take into account the effect of confounding factors such as age, gender, ethnicity, and household location.<sup>16</sup> Statistical analyses were carried out with SPSS version 11 (SPSS Inc, USA) and EpiInfo version 1.1.2 (CDC, USA).

### RESULTS

Three SNPs located either in the promoter region (APOE Th1/E47) or in exon 4 (APOE 3937 and 4075) of the APOE gene were genotyped. APOE 3937 and 4075 markers were typed in

530 severe malaria cases, 338 mild malaria cases, and 560 individually matched control children. These genotypes were used to define individuals' APOE ε2, ε3, and ε4 alleles as described. No differences were observed between controls with mild non-malaria diseases (247 patients) and controls with severe non-malaria diseases (313 patients) for allele frequency and genotype distribution (data not shown). Consequently, these control subjects were grouped as a single control group for subsequent analyses.

The APOE ε2 (12.8%), ε3 (64.4%), and ε4 (22.8%) allele frequencies in this Gambian sample were found to be closer to those reported in Ghanaian samples (14.5%, 61.4%, and 24.1%, respectively, for ε2, ε3, and ε4) than others reported in African or non-African populations.<sup>10 17</sup> As shown in table 1(A), APOE allele frequencies were found to be similar in the control group and in children with severe and with mild malaria. Furthermore, no differences were observed between the different severe malaria subgroups and either the controls or the mild malaria cases (table 1(A)). APOE genotypes also were compared between these different study groups. Although the APOE ε3/ε4 genotype was apparently more common in severe malaria (29.4%) and cerebral malaria (29.0%) cases than in the mild malaria cases (27.2%) or in the controls (24.8%), no statistically significant differences were observed between these study groups for any APOE genotype. The APOE genotype distribution was, however, found to be significantly different between the control group and children developing both cerebral malaria and severe malarial anaemia ( $\chi^2 = 11.42$ ,  $p = 0.044$ ). In this particular subgroup of children, the APOE ε3/ε4 genotype was found to be more frequent (42.9%) than in the controls (24.8%) ( $2 \times \chi^2 = 7.57$ ,  $p = 0.006$ ) and the mild malaria cases (27.2%) ( $2 \times \chi^2 = 5.06$ ,  $p = 0.024$ ) (table 1(B)). These results were confirmed by binary logistic regression where additional factors, such as ethnicity, household location, gender, and age, were simultaneously included in the analyses (data not shown).

The APOE Th1/E47 SNP was genotyped in a subset of 183 severe malaria cases and 179 control children (table 2(A)). This polymorphism is located in a consensus sequence of a potential transcriptional (Th1/E47) factor binding site, and the APOE Th1/E47-T allele was previously reported to be associated with an increased risk of Alzheimer's disease.<sup>11 18</sup> As shown in table 2(A), the allele frequencies were similar in severe malaria cases and in control children (75.4% and 76.0%, respectively, for the G allele;  $\chi^2 = 0.03$ ,  $p = 0.87$ ). Similarly, as shown in table 2(B), genotype frequencies were not found to be significantly different between the severe malaria cases and the control children ( $\chi^2 = 1.95$ ,  $p = 0.38$ ). Because of the absence of significant difference between this subset of 183 severe malaria cases and 179 controls, the rest of the Gambian case control samples were not genotyped for this marker.

### DISCUSSION

The results reported here demonstrate that, in this Gambian case control study, genetic polymorphisms within APOE do not significantly influence host resistance or susceptibility to severe malarial anaemia and cerebral malaria, the two most common forms of severe malaria. Surprisingly, when severe malaria cases were subdivided in clinical categories, the APOE ε3/ε4 genotype was found to be more common in children with both cerebral malaria and severe malarial anaemia (42.9%) than in controls (24.8%) and mild malaria cases (27.2%). However, these results must be considered with caution because they are based on a relatively small number of cases (49) and could be due simply to chance, or to a biased distribution of the APOE genotype in this particular sample. Indeed, when a correction factor is applied

**Table 1** Distribution of APOE  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  alleles in the Gambia

Subjects*	Severe malaria	Cerebral malaria†	Severe malarial anaemia†	Severe anaemia + cerebral malaria†	Mild malaria	Controls	Total
<b>(A) Alleles</b>							
Total	1060	642	272	98	676	1120	2856
$\epsilon 2$	134 (12.6)	80 (12.5)	41 (15.1)	7 (7.1)	78 (11.5)	155 (13.8)	367 (12.8)
$\epsilon 3$	682 (64.3)	416 (64.8)	170 (62.5)	67 (68.4)	452 (66.9)	705 (62.9)	1839 (64.4)
$\epsilon 4$	244 (23.0)	146 (22.7)	61 (22.4)	24 (24.5)	146 (21.6)	260 (23.2)	650 (22.8)
$\chi^2‡$	0.76	0.84	0.30	3.52	3.21	–	–
P	0.68	0.66	0.86	0.17	0.20	–	–
$\chi^2§$	1.19	0.64	2.55	1.86	–	–	–
p	0.55	0.72	0.28	0.39	–	–	–
<b>(B) Genotypes</b>							
Total	530	321	136	49	338	560	1428
$\epsilon 2/\epsilon 2$	6 (1.1)	3 (0.9)	1 (0.7)	1 (2.0)	2 (0.6)	9 (1.6)	17 (1.2)
$\epsilon 2/\epsilon 3$	86 (16.2)	55 (17.1)	24 (17.6)	4 (8.2)	56 (16.6)	96 (17.1)	238 (16.7)
$\epsilon 2/\epsilon 4$	36 (6.8)	19 (5.9)	15 (11.0)	1 (2.0)	18 (5.3)	41 (7.3)	95 (6.6)
$\epsilon 3/\epsilon 3$	220 (41.5)	134 (41.7)	57 (41.9)	21 (42.9)	152 (45.0)	235 (42.0)	607 (42.5)
$\epsilon 3/\epsilon 4$	156 (29.4)	93 (29.0)	32 (23.5)	21 (42.9)	92 (27.2)	139 (24.8)	387 (27.1)
$\epsilon 4/\epsilon 4$	26 (4.9)	17 (5.3)	7 (5.1)	1 (2.0)	18 (5.3)	40 (7.1)	84 (5.9)
$\chi^2¶$	5.1	3.7	3.2	11.42	5.1	–	–
p	0.40	0.60	0.67	0.044	0.41	–	–
$\chi^2**$	2.39	0.96	5.35	8.62	–	–	–
p	0.79	0.97	0.38	0.13	–	–	–

\*Percentages within each disease or control group are given in parentheses.

†Children (24) with severe hypoglycaemia only were excluded from this analysis.

‡ $3 \times 2 \chi^2$  test (df = 2) for comparison with the control group.

§ $3 \times 2 \chi^2$  test (df = 2) for comparison with the mild malaria group.

¶ $6 \times 2 \chi^2$  test (df = 5) for comparison with the control group.

\*\* $6 \times 2 \chi^2$  test (df = 5) for comparison with the mild malaria group.

for the number of clinical groups (4) compared with controls in this study, this finding loses statistical significance. These results apparently contrast with a recent report from Ghana.<sup>10</sup> However, different and hence complementary phenotypes were studied in the Gambia (mild and severe clinical malaria) and in Ghana, where children with the APOE  $\epsilon 2/\epsilon 2$  genotype were found to be infected with *P. falciparum* sooner than other children.<sup>10</sup> This difference observed between children with different APOE genotypes could be explained by the fact that the different APOE isoforms have been shown to have different physiological properties and, in particular, have been shown to have different affinities for some of their receptors such as LRP and apoER2.<sup>3,4</sup> Moreover, APOE receptors have been shown to bind to the

circumsporozoite protein (CSP) from plasmodium sporozoites. In mice models, sporozoites were shown to be less able to invade hepatic cells from LDLR knockout mice.<sup>19</sup> Therefore, it is possible that a particular apoE isoform, such as apoE2, is less efficient than the other isoforms in competing with the sporozoites for binding to their receptor on hepatic cells, and hence influence host resistance to malaria infection. In Ghana, APOE polymorphisms were not found to influence individual blood infection levels.<sup>10</sup> In our study, the APOE  $\epsilon 3/\epsilon 4$  genotype was found to be more frequent in a small group of children (49) developing cerebral malaria and severe malarial anaemia simultaneously. This genotype was however not found to be significantly more frequent in children developing cerebral malaria only or severe malarial anaemia only, suggesting that APOE genotypes could have a role in the genetic control of this particular form of severe malaria; alternatively, this may simply be a chance result.

These results suggest that APOE polymorphisms do not influence protection against cerebral malaria and severe malarial anaemia, but could have a role in protection against the early stages of the infection and possibly against a particularly severe form of severe malaria (cerebral malaria plus severe malarial anaemia). Further studies are required to assess the role of APOE polymorphisms in the risk of severe malaria in other populations.

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**Table 2** Distribution of APOE Th1/E47 polymorphism in the Gambia

Subjects*	Severe malaria	Controls	Total
<b>(A) Alleles</b>			
Total	366	358	724
G	276 (75.4)	272 (76.0)	548 (75.7)
T	90 (24.6)	86 (24.0)	176 (24.3)
$\chi^2†$	0.03	–	–
P	0.87	–	–
<b>(B) Genotypes</b>			
Total	183	179	362 (56.3)
G/G	100 (54.6)	104 (58.1)	204
G/T	76 (41.5)	64 (35.8)	140 (38.7)
T/T	7 (3.8)	11 (6.1)	18 (5.0)
$\chi^2‡$	1.95	–	–
p	0.38	–	–

\*Percentages within each disease or control group are given in parentheses.

† $2 \times 2 \chi^2$  test (df = 1) for comparison with the control group.

‡ $3 \times 2 \chi^2$  test (df = 2) for comparison with the control group.

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## DATA ACCESS

GenBank: AF261279; OMIM: 107741

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## ECHO.....

### DQB1 alleles affect susceptibility to pulmonary TB in Europeans



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The first study of its kind in white Europeans has suggested that the allelic frequency of histocompatibility genes is linked to susceptibility to pulmonary TB.

The frequency of DQB1 alleles in a white Polish population showed a significant positive association of DQB1\*05 and pulmonary TB, with patients almost three times more likely to have DQB1\*05 as controls (odds ratio (OR) 2.84; 95% confidence interval (CI) 1.57 to 5.15). This has been reported in many other ethnic populations.

On the other hand, patients were significantly less likely to have DQB1\*02 (OR 0.39; 95% CI 0.21 to 0.71), which has not been noted elsewhere. Other alleles—DQB1\*03, DQB1\*04, and DQB1\*06—had similar frequencies in patients and controls.

This preliminary study included 38 unrelated patients with newly identified active pulmonary TB in one hospital and 58 unrelated controls of similar age. Individuals in both groups were excluded, as necessary, to ensure uniform socioeconomic and ethnic background. All had had BCG immunisation. HLA typing was by low resolution PCR with sequence specific primers (PCR-SSP) of blood samples.

The study provides yet more evidence that variation in the HLA system affects response to infection in pulmonary TB. Only 1 in 10 of infected persons will develop the active disease, and genetic differences in immunological defences, and therefore the HLA system, have been implicated. The frequencies of DQB alleles have been studied in Asian and American ethnic groups, but not in Europe, despite an increasing incidence of TB there.

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