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Age-related susceptibility to severe malaria associated with galectin-2 in highland Papuans

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Abstract

Background—Age and host genetics are important determinants of malaria severity. Lymphotoxin-alpha (LTα) has been linked to the development of cerebral malaria (CM) and other severe malaria (SM) syndromes. Mutations in genes regulating LTα production contribute to other acute vascular diseases and may contribute to malaria pathogenesis.

Methods—We tested the association between rs7291467, a single nucleotide polymorphism (SNP) in the LTα-related gene encoding galectin-2 (*LGALS2*), disease severity and function in a case-control study of ethnic Highland Papuan adults and children with SM (n=380) and asymptomatic malaria-exposed controls (n=356), originating from a non-malaria-endemic region but residing in a lowland malaria-endemic area of Papua, Indonesia.

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Results—The *LGALS2* SNP showed significant association with susceptibility to SM (including CM), in children (OR 2.02 [95% CI:1.14-3.57]) but not adults. In SM, the C-allele at rs7291467 was associated with enhanced galectin-2 transcript levels. In a separate group of Tanzanian children originating from a malaria-endemic region, we found preservation of the major ancestral *LGALS2* allele and no association with susceptibility to CM.

Conclusions—Results suggest differences in the inflammatory contribution to the development of SM between children and adults in the same population, and potential differences between individuals originating from malaria-endemic and non-endemic areas.

Keywords

Malaria; Galectin-2; Inflammation; Gene regulation; Age; Severe Malaria; Cerebral Malaria

Introduction

Severe malaria (SM) caused by *Plasmodium falciparum* can have many manifestations and involve multiple organs, directly resulting in more than a million deaths each year [1,2]. These manifestations include cerebral malaria (CM), severe malaria anaemia (SMA), acute respiratory distress syndrome (ARDS), hyperparasitemia, hypoglycaemia, black water fever, metabolic acidosis, jaundice and renal failure [1]. Recent studies in South-East Asia have shown that complications of SM are age-specific; convulsions and anaemia being more prominent in children, whereas adults were more likely to experience hyperparasitemia, renal failure and jaundice [3]. The virulence and drug-resistance phenotype of *P. falciparum* and the genetic background and age of the patient are all likely to be important determinants of the severity of *P. falciparum* infection and the type of SM syndrome that develops [3-7].

Cerebral malaria has been the focus of many genetic studies, which have identified genes, loci and single nucleotide polymorphisms (SNPs) associated with its development in individuals infected by *P. falciparum*. Evidence from both mouse models of experimental CM (ECM) [8,9], as well as from SM patients [10-13], have identified both TNF and LTα as important pro-inflammatory cytokines linked to pathology, although only one study [13] has reported on LTα levels in human malaria patients. TNF family members have long been considered candidate disease susceptibility genes. However, gene products that regulate the production and/or bioavailability of these cytokines may also influence disease outcome. One such molecule is galectin-2, which co-localises with LTα in the cytoplasm of cells and is thought to aid in the trafficking of LTα out of cells [14]. A point mutation (rs7291467) in the first intron of the gene encoding galectin-2 (*LGALS2*) results in enhanced galectin-2 mRNA transcription and protein expression in the presence of the C allele when compared to transcription and protein expression levels in the presence of the T allele [14]. Thus, altered galectin-2 expression associated with rs7291467 influences LTα protein export from the cell and, ultimately, the level of inflammation in the periphery [14]. Significantly, the rs7291467 SNP has also been associated with risk of acute vascular disease [14].

In CM patients of all ages, post-mortem histological studies show marked microvascular sequestration of parasitised red blood cells associated with endothelial activation [15-19]. However, some differences in histopathology have been noted between adults and children. In children, there appears to be more evidence for an inflammatory response, with leukocytes and platelets present in the cerebral microvasculature of some patients [18-21]. In contrast, adults with CM are commonly reported to have only sparse leukocyte and platelet sequestration in cerebral vessels [16,21,22]. Moreover, plasma levels of TNF are, in general, higher in children with CM [10,12,23] than in adults [24,25]. Thus, there appear to be age-related differences in

inflammatory responses in CM, and therefore potential differences in the contribution of inflammation to this disease between children and adults.

Previous genetic case-control series that have examined the potential contribution of inflammation-related genes to the risk of severe malaria have mostly evaluated either children or adults, usually in different populations, making it difficult to compare the importance of such genetic determinants in different age groups. Here we examine these relationships in adults and children from the same population, and test the following *a priori* hypotheses: (i) a SNP in *LGALS2*, the gene encoding galactin-2, previously shown to regulate the expression of *LTA*, is associated with SM, and (ii) the contribution of inflammatory mechanisms in the development of CM differs between adults and children.

Materials and Methods

Ethics Statement

Written informed consent was obtained from all study subjects or their next of kin, parent or guardian. Studies were approved by the Ethics Committees of the National Institute of Health Research and Development (Ministry of Health, Jakarta, Indonesia), Menzies School of Health Research (Darwin, Australia), Queensland Institute of Medical Research (Brisbane, Australia), Muhimbili University of Health Sciences (Dar es Salaam, Tanzania), National Institute for Medical Research (Dar es Salaam, Tanzania), University of Utah Medical Center (Salt Lake City, USA) and Duke University Medical Center (Durham, USA).

Study participants and sample preparation

Between 2003 and 2007, subjects with severe falciparum malaria (SM) and asymptomatic malaria-exposed controls were recruited in a case-control study in Timika, a lowland region of Papua, Indonesia, with endemic unstable malaria transmission of multidrug-resistant *P. falciparum* and *P. vivax* and annual malaria incidence of 876 per 1000 person-years [26,27]. All subjects were of Highland Papuan origin, a population not historically exposed to malaria transmission prior to the 1970s, but who during the study period had been exposed to malaria following migration to the lowland region of Papua (Table 1).

For both adults and children in Papua, SM was defined as the presence of *P. falciparum* parasitemia with one or more modified WHO criteria of severity [20,28] in the absence of an evident alternative diagnosis. Adult controls were defined as having no clinical features of malaria, with no fever or history of fever in the previous 14 days; with or without asymptomatic parasitemia; no past history of severe malaria, life-threatening illness, or hospitalisation for any illness requiring intravenous therapy; living for at least two years in a known malaria-endemic area of Papua; and being non-pregnant. Controls for children were those > 6 months of age seen in outpatient clinics with mild acute respiratory infection with no other cause present, a normal white cell count, an absence of parasitemia on microscopy or by rapid diagnosis test (HRP2). In an *a priori* analytical plan, children were defined as ≤ 13 years of age and adults as ≥ 14 years of age. DNA samples from a Tanzanian case-control study comprising children with CM enrolled in Dar es Salaam using WHO criteria as previously described [23] and asymptomatic malaria-exposed, healthy control children from Mikocheni Primary school in the Kinondoni Municipality of the Dar es Salaam region were also studied [29].

Blood was collected in lithium heparin. Following a 12 minute centrifugation at 2000 rpm, plasma was stored at −80°C within 30 mins of collection. Genomic DNA was extracted from blood using a DNA QIAamp blood mini kit (Qiagen, Valencia, CA), according to manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were isolated over

a Ficoll Hypaque gradient, washed in 1× PBS and the resulting pellet was frozen directly at −80°C or resuspended in 50 μL PBS and 500 μL RNA*later* (Qiagen), stored at 4°C overnight and then frozen at −80°C until tested.

Genotyping

SNP rs7291467 was typed using iPLEX™ chemistry and analyzed using a Sequenom MassARRAY Compact Mass Spectrometer (Sequenom Inc, San Diego, CA, USA). The 2.5 ml PCR reactions were performed in standard 384-well plates using 10 ng genomic DNA, 0.5 unit of Taq polymerase (HotStarTaq, Qiagen), 500 mmol of each dNTP, and 100 nmol of each PCR primer. Standard PCR thermal cycling conditions and post-PCR extension reactions were carried out as described previously [30]. The iPLEX reaction products were desalted and spotted on a SpectroChip (Sequenom). Data were processed and analysed by MassARRAY Workstation (version 3.4) software (Sequenom).

RNA extractions from PBMC and cDNA synthesis

PBMCs in RNA*later* stored at −70°C were thawed, pelleted in a table-top centrifuge and the RNA*later* was removed. Samples were homogenised by passing lysates through blunt 19-gauge needles. RNA was extracted from PBMC samples using an RNeasy Kit with an on-column DNA digestion (Qiagen), according to the manufacturer's instructions. cDNA was synthesised from 0.7 μg of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and cDNA integrity was verified by amplifying β-actin by PCR.

Real-time RT-PCR

Real-time RT-PCR was used to quantify the amount of galectin-2, LTα and β-actin mRNA transcripts in PBMC samples. Transcripts were detected in 1 μL cDNA/ sample using Taqman Gene Expression Assays (Applied Biosystems) and Taqman Universal PCR Master Mix (Applied Biosystems), according to manufacturer's instructions. All calculated values for galectin-2 and LTα were normalised against the number of β-actin transcripts in the corresponding sample.

Plasma Cytokine Detection

Plasma samples were thawed and LTα levels measured using a BD Cytometric Bead Array Human LTα Flex Set with BD CBA Human Soluble Protein Master Buffer Kit (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's instructions. Each sample was thawed once and two duplicate samples run in each array assay. Supernatants from stimulated EBV-transformed lymphoblastoid cell lines (LCLs) were included as controls. LCLs were cultured at 10^5 cells/ well and maintained at 37°C, 5% (v/v) CO_2. Cells were stimulated with complete RPMI1640 containing 200nM PMA and 125 nM calcium ionomycin (Sigma) for 48 hours. At each time point, samples were spun to pellet cells and supernatants collected and stored at −70°C.

Statistical Analyses

Haploview version 3.32 (Whitehead Institute for Biomedical Research, USA; http://www.broad.mit.edu/mpg/haploview) was used to perform all statistical tests relating to this SNP analysis [31]. Genotype frequency for SNP rs7291467 was tested for departures from Hardy-Weinberg equilibrium in both cases and controls separately. A global P-value of < 0.05 was considered statistically significant. The Mann-Whitney *U*-test was used to determine significant differences in mean galactin-2 mRNA expression. $P \leq 0.05$ was considered significant.

Results

A SNP in intron 1 of the gene encoding galectin-2 is a SM susceptibility marker in highland Papuan children

We examined the rs7291467 point mutation/polymorphism in the first intron of *LGALS2* that results in enhanced galectin-2 mRNA transcription and protein expression in the presence of the C allele when compared to transcription and protein expression levels in the presence of the T allele [14] (Figure 1). Given the important role of LTα in ECM [9], and the role of galectin-2 in LTα protein export from the cell, we hypothesised that rs7291467 would be associated with SM in humans. More than 99% of samples from highland Papuans were successfully genotyped for this polymorphism with a minor allele frequency (C allele) in control subjects of 0.23 (Table 2). Differences in allele frequency were observed between control subjects and SM patients, as well as between control subjects and, more specifically, CM patients (Table 2). Highland Papuan patients with the rs7291467 C allele were at greater risk of SM (OR = 1.36 [95% CI 1.08-1.73], $P = 0.010$) and CM (OR = 1.57 [95% CI 1.12-2.21], $P = 0.009$). Although the increased risk of SM was apparent in children with CM (OR = 2.43 [95% CI 1.089-5.43], $P = 0.028$) and non-CM manifestations (OR = 1.94 [1.08-3.48], $P = 0.025$), no significantly increased risk was observed in adults, particularly when focusing on adults with non-CM manifestations [OR = 1.13 [0.84-1.54], $P = 0.417$].

The presence of the C allele at rs7291467 results in enhanced galectin-2 transcript levels

In vitro experiments have demonstrated that rs7291467 regulates galectin-2 transcription [14]. Therefore, we next investigated whether this association was present in this study. PBMCs were collected from control individuals and SM patients upon admission to hospital. These samples were from individuals included in the genotype study. Normalised galectin-2 mRNA levels were measured in these cells and the results were subsequently grouped based on the presence of the C or T allele at rs7291467 in both adults and children (Figure 2). Galectin-2 mRNA levels were significantly higher in cells with the C allele than those with the T allele in the adult SM PBMC samples (Figure 2; $P < 0.05$). Although not statistically different, a similar trend in galectin-2 mRNA levels was observed in PBMCs from children with SM when grouped by genotype (Figure 2).

Next we sought to determine whether presence of the C allele at rs7291467 had an effect on the level of circulating LTα in patients with malaria. Although LTα could be readily detected in stimulated EBV-transformed LCL supernatants (indicating that the LTα protein detection assay was effective), the LTα levels in the study isolates were all below the detection threshold in all plasma samples, and the association with the rs7291467 C allele could not therefore be tested.

SNP rs7291467 is not associated with cerebral malaria in children historically from a malaria endemic area

The rs7291467 SNP was associated with disease severity in a highland Papuan population historically not exposed to malaria and, therefore, without selective pressure by *P. falciparum*. To assess the association in patients originating from and resident in a malaria-endemic region, 245 healthy children (HC) and 77 children with CM from Dar es Salaam, Tanzania, were genotyped for rs7291467 in *LGALS2*. Unlike the Papuan study population and a previous British population [32], the T allele was found to be the minor allele amongst the Tanzanian study population. A comparable low frequency has previously been reported in Japanese [14], Chinese and Nigerian populations as detected by HapMap (http://www.hapmap.org/), and non-Papuans in our study (data not shown). These observations suggest different selective pressure at this locus in highland Papuans compared with other populations. Furthermore, the frequency of the C allele was similar between the HC subjects

(0.726) and CM cases (0.740), and not associated with childhood CM in this population from Tanzania (Table 3; $P > 0.05$).

Discussion

A SNP in the LTα-related gene encoding galectin-2 showed significant association with susceptibility to SM (including CM) in children of highland Papuan ethnicity, a population historically unexposed to malaria. However, *LGALS2* was not associated with susceptibility to SM in adult highland Papuans. We cannot rule out a weaker association between the rs7291467 C allele and adult SM that might be found by studying larger numbers of adults. Nevertheless, these data suggest that galectin-2 may be a contributing factor in the development of SM, as well as CM more specifically, in highland Papuan children.

There was no association between this SNP and susceptibility to CM in Tanzanian children originating from and living in a malaria endemic region. The major ancestral *LGALS2* SNP allele was preserved in the Tanzanian population, but was the minor allele in highland Papuans, indicative of different selective pressures experienced by the two groups. The presence of the *LGALS2* T allele was shown to be associated with lower levels of PBMC galectin-2 mRNA in adult Papuans with SM, suggesting that rs7291467 is a functionally relevant SNP that influences the transcription of galectin-2 mRNA during SM in highland Papuans. This is consistent with the previous finding that the *LGALS2* T allele is associated with impaired *LGALS2* transcription [14]. Taken together, our results suggest differences in the inflammatory contribution to the development of SM between children and adults in the same population, as well as potential differences between individuals from malaria endemic and non-endemic areas.

Galectins are a family of proteins that have one or more carbohydrate recognition domain(s) and an affinity for β-galactosides [33-36]. They have important roles in cellular adhesion, migration, growth regulatory mechanisms, immune modulation, negative selection and apoptosis [37-41]. SNP rs7291467 in *LGALS2* was examined in this study because of the downstream impact of this SNP on LTα protein expression from cells, and its previous association with acute vascular disease [14]. Studies involving *P. berghei* ANKA infection of ECM-susceptible C57BL/6 mice detected a critical role for LTα, but not TNF, in ECM pathology [9]. The key source of LTα appeared to be from a radio-resistant cell population in the brain of *P. berghei* ANKA-infected mice [9]. Thus, our failure to detect LTα in plasma from SM patients could indicate that localised production of LTα in inflamed tissue may be most relevant for disease pathogenesis. Indeed, no difference in LTα mRNA accumulation was found in PBMC's from healthy and SM patients (data not shown). Since ECM in C57BL/6 mice is LTα-dependent, this model may be useful to examine the role of galectin-2 in ECM disease pathogenesis.

Based on the sequenced gene encoding galectin-2 from *Pan Troglodyte* (chimpanzees; Ensembl gene ENSPTRG00000014338, http://www.ensembl.org), the ancestor allele for rs7291467 appears to be the C allele (major allele in Tanzanian study population/ minor allele in highland Papuan study population). This suggests that the T allele is the true polymorphism. The high frequency of the rs7291467 ancestral C allele (the high galectin-2-expressing allele) in the Tanzanian population may reflect the preservation of this allele for a particular survival advantage. The very high frequency of the rs7291467 T allele (the low galectin-2-expressing allele) in the highland Papuan study population suggests that this allele may have undergone positive selection. Alternatively, the high frequency may be the result of genetic drift.

Most previous genetic case control reports have studied children or adults alone. The unique epidemiology of a recently migrated population, not historically exposed to malaria, has

allowed us to examine the association of the same SNP with severe disease in different age groups in the same population. Age-related differences in the acquisition of malaria-specific immunity have been previously reported [42-44], but these studies generally relate to populations residing in malaria endemic areas. An analysis of malaria-naïve adults migrating to a malaria endemic area suggested that the acquisition of immunity was much faster in adults than in children, even though adults suffered more severe disease episodes during early malaria infections [45] [46]. Hence, there may be components of malaria-induced host immune responses that contribute to protection or disease in an age-specific manner and galectin-2 and/or related molecules may be an example of such molecules, albeit in a specific human population.

In summary, we report that a SNP in *LGALS2* (rs7291467) confers an age-related susceptibility to SM in highland Papuan children. Since the association between the rs7291467 C allele and SM (including CM) appears to be stronger in children, despite a similar frequency of the SNP in adult and child controls from the same study population, there may be different contributions of inflammation to childhood and adult SM. Furthermore, the different frequencies of rs7291467 alleles in highland Papuan and Tanzanian study populations suggests that different pressures may have selected for different alleles in these two groups. Further study of the gene encoding galectin-2 and its associated SNPs in broader and larger population cohorts may help identify these selective pressures, and hence provide a better understanding of the role of galectin-2 in SM, generally, and CM pathogenesis, specifically.

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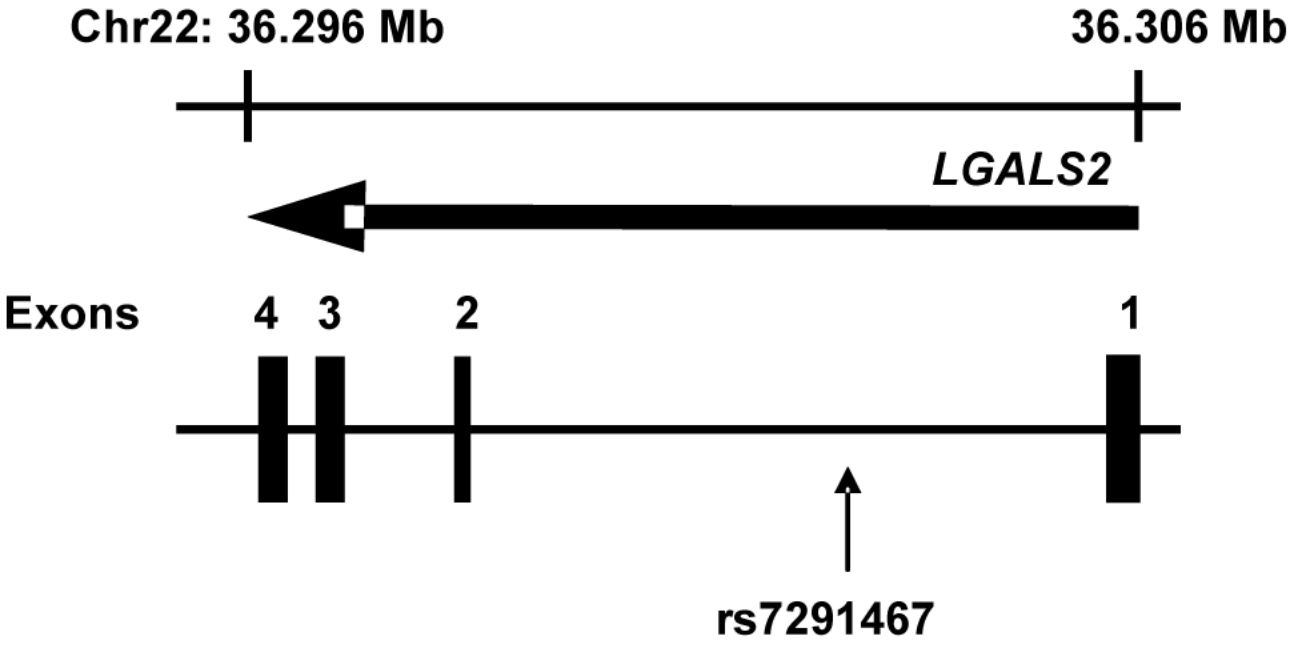

Figure 1. Chromosomal location of the rs7291467 SNP

rs7291467 is located within intron 1 of the *LGALS2* (galectin-2) gene, 3279 bp downstream from the transcription start site on chromosome 22.

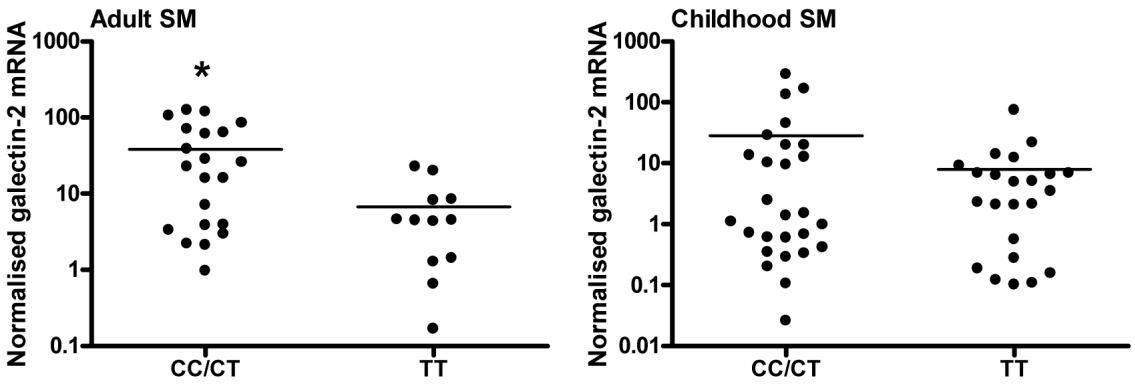

Figure 2. Galectin-2 mRNA levels in PBMCs isolated from adult SM patients are differentially regulated by the allele present at rs7291467

Total RNA was extracted from PBMCs isolated from highland Papuan SM adults or SM children. The expression of galectin-2 mRNA was normalized against 10,000 β-actin transcripts and grouped based on the rs7291467 allele present in each of the individuals. *, $P < 0.05$

Table 1

Characteristics of Papuan patients at hospital admission.

Parameter	Adult Severe Malaria	Adult Control	Childhood Severe Malaria	Childhood Control
Age, years (median, range)	25 (14-73)	26 (16-59)	3.0 (0.5 – 13)	4.0 (0.6 – 12)
Female:male (ratio)	96:166 (0.57)	83:222 (0.37)	60:58 (1.03)	26:25 (1.04)
Temperature, °C (mean, SD)	37.2 (1.4)	35.9 (0.5)	37.5 (1.2)	36.5 (1.0)
P. falciparum parasite density, /μL (geomean, 95% CI)	33800 (24400 – 46800)		45700 (28800 – 72400)	
Individuals with *P. vivax* patent co-infection, no.	23		16	
Hemoglobin, g/dL (median, range)	9 (2 – 17)	12.5 (4.7 – 17.4)	4.9 (2.1 – 12.9)	9.9 (5.3 – 12.5)
Proportion with criterion defining severe malaria subgroup, % (no.)				
Cerebral malaria	32 (85)		18 (21)	
Severe anaemia	6 (16)		52 (61)	
Jaundice	40 (105)		5 (6)	
Renal Failure	40 (106)		2 (2)	
Hypoglycaemia	4 (10)		0.9 (1)	
Hyperparasitemia	23 (60)		40 (47)	
Shock	14 (37)		0.9 (1)	
Blackwater Fever	0.4 (1)		0 (0)	
Acidosis	15 (40)		4 (5)	
Respiratory	22 (59)		25 (29)	
Individuals with ≥ 2 subgroups	49 (129)		33 (39)	
Case fatality rate, % (no.)	16 (42)		4 (5)	

Table 2

rs7291467 (*LGALS2*) and allelic association with severe malaria in a Papuan population.

MAF[a] Phenotype	Controls (no.[b])	Cases	Association[c]	OR	Fisher's	*P*-value[d]
All severe malaria patients	0.23 (356)	0.29 (380)	6.65	1.36 (1.08 to 1.73)	0.01	0.010
Cerebral malaria	0.23 (356)	0.32 (106)	6.85	1.57 (1.12 to 2.21)	0.01	0.009
Adult severe malaria	0.24 (305)	0.27 (262)	2.24	1.23 (0.94 to 1.61)	0.15	0.134
Cerebral malaria	0.24 (305)	0.31 (85)	3.60	1.44 (0.99 to 2.11)	0.07	0.058
Non-cerebral malaria	0.24 (305)	0.26 (177)	0.66	1.13 (0.84 to 1.54)	0.44	0.417
Childhood severe malaria	0.19 (51)	0.32 (118)	5.99	2.02 (1.14 to 3.57)	0.02	0.014
Cerebral malaria	0.19 (51)	0.36 (21)	4.82	2.43 (1.09 to 5.43)	0.03	0.028
Non-cerebral malaria	0.19 (51)	0.31 (97)	5.01	1.94 (1.08 to 3.48)	0.03	0.025

[a] Minor allele (C allele) frequency.

[b] Number of participants.

[c] Association X^2 with severe malaria subgroups.

[d] *P*-value obtained for single marker, $P < 0.05$ is considered significant.

Table 3

rs7291467 and association with CM in Papuan and Tanzanian children.

Phenotype	Number		C allele Frequency		Association[a]	OR	*P*-value[b]
	Controls	Cases	Controls	Cases			
Papuan Childhood SM	51	118	0.19	0.32	5.99	2.02 (1.14-3.57)	0.014
Papuan Childhood CM	51	21	0.19	0.36	4.82	2.43 (1.09-5.43)	0.028
Tanzanian Childhood CM	245	77	0.73	0.74	0.13	0.93 (0.62-1.40)	0.721

[a] Association X^2 with severe malaria subgroups.

[b] *P*-value obtained for single marker, $P < 0.05$ is considered significant.