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Association of the GNAS Locus with Severe Malaria

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Abstract

Functional studies have demonstrated an interaction between the stimulatory G protein alpha subunit (G-alpha-s) and the malaria parasite at a cellular level. Obstruction of signal transduction via the erythrocyte G-alpha-s subunit reduced invasion by *P.falciparum* parasites. We sought to determine whether this signal pathway had an impact at the disease level by testing polymorphisms in the gene encoding G-alpha-s (*GNAS*) for association with severe malaria in a large multi-centre study encompassing family and case-control studies from The Gambia, Kenya and Malawi, and a case-control study from Ghana. We gained power to detect association using meta-analysis across the 7 studies, with an overall sample size approximating 4000 cases and 4000 controls. Out of 12 SNPs investigated in the 19kb *GNAS* region, 4 presented signals of association ($P < 0.05$) with severe malaria. The strongest single locus association demonstrated an odds ratio of 1.13 (1.05-1.21), $P = 0.001$. Three of the loci presenting significant associations were clustered at the 5-prime end of the *GNAS* gene. Accordingly, haplotypes constructed from these loci demonstrated significant associations with severe malaria (OR=0.88 (0.81-0.96), $P = 0.005$; and

OR=1.12 (1.03-1.20), $P=0.005$). The evidence presented here indicates that the influence of G-alpha-s on erythrocyte invasion efficacy may, indeed, alter individual susceptibility to disease.

Keywords

G protein; GNAS; signal transduction; malaria; invasion; association

Introduction

Malaria continues to impose a substantial health burden across the globe. It is estimated that annually between 300 and 500 million clinical cases of malaria occur, of which 1.5 to 2.7 million cases are fatal 1. Erythrocyte invasion is an essential gateway to malaria disease and a key target for disease intervention. However, our current understanding of the molecular mechanisms by which *Plasmodium falciparum*, the most virulent species of human malaria parasite, invades human erythrocytes is limited. Erythrocyte invasion is a rapid, multi-step process involving close interactions between the blood stage parasite (the merozoite) and the host erythrocyte (reviewed in Cowman et al. 2). Functional studies have demonstrated that a host erythrocyte G protein signal pathway may be a critical component in parasite invasion and, thus, presents a potential target for disease intervention. Harrison and colleagues demonstrated that successful *in vitro* erythrocyte invasion by *P.falciparum* requires activation of the host stimulatory G protein (Gs) signal transduction pathway mediated by the beta-2-adrenergic receptor (β -2-AR) 3.

G protein-coupled receptor signalling pathways provide a means for intracellular components to respond to extracellular stimuli. The Gs protein is heterotrimeric, consisting of an alpha, beta and gamma subunit. The G alpha subunit (G-alpha-s) sits at the interface between Gs and the G protein-coupled receptor, where it determines receptor specificity. G-alpha-s couples numerous receptors, including β -2-AR, to adenylyl cyclase and is required for receptor-stimulated intracellular cAMP production 4. During *P.falciparum* entry into the erythrocyte, G-alpha-s and β -2-AR are recruited to the newly forming parasitophorous vacuole membrane (PVM), a membrane that shields the internalised parasite from the erythrocyte milieu 3, 5, 6, 7. Within the PVM, G-alpha-s and β -2-AR reside in lipid rich membrane regions known as lipid rafts, which are receiving increasing interest in the scientific community as important domains for signal transduction. Thus, it was suspected that signaling via the host Gs pathway might be functional in erythrocyte invasion. In support of this proposal, Harrison and colleagues demonstrated that peptides that disrupt the interaction of G-alpha-s and β -2-AR block erythrocyte invasion by *P.falciparum* parasites *in vitro* 3.

While functional studies provided valuable information on the interaction between the erythrocyte G-alpha-s protein and the malaria parasite at a cellular level, it remained unclear whether this interaction would impact on disease progression. The possibilities remained that feedback mechanisms would counteract the influence of the Gs signal pathway on malaria infection, or that some *P.falciparum* parasites could use alternative signal pathway(s) to enter the host erythrocyte as has been demonstrated at other stages of erythrocyte invasion 8.

This study describes our investigation into the impact of the G-alpha-s protein on severe malaria disease using genetic epidemiology. We report on the first evidence of association between polymorphism in the G-alpha-s gene (*GNAS*) and severe malaria. To the best of our knowledge, this is the first association study investigating the impact of *GNAS* polymorphism on severe malaria. By virtue of collaborative efforts we were able to design a

highly-powered multi-centre study encompassing large case-control (unrelated severe malaria cases and population controls) and family trio (severe malaria cases with parental controls) studies across several African countries; The Gambia, Ghana, Kenya and Malawi. Although individual studies were reasonably powered to detect modest associations with high frequency alleles, by utilizing meta-analysis across sufficiently homogeneous studies, we were able to further enhance our sample size and, thus, power. Indeed, insufficient sample size is suspected to be one of the main factors responsible for the low rate of replication between association studies 9. For these reasons, meta-analysis is becoming a popular method for resolving discrepancies in genetic association studies 10.

Results

Assay performance

Allele frequencies for the *GNAS* assays are presented in Table 3. In several studies, rs3787493 and rs234630 presented with MAF <5%, but all other assays had frequencies >10%. With the exception of rs3787493 in the Gambian case-control study and rs6026592 and rs234630 in the Kenyan TDT study, all assays conformed to HWE at the 0.1% significance threshold and had genotype failure rates <20%. The rs3787493 assay was excluded from the Gambian case-control study owing to significant deviation from HWE ($P<0.001$), and rs6026592 and rs234630 were excluded from the Kenyan TDT study on the basis of failure rates 20%.

Single-locus associations

Meta-analysis combining the single-locus associations with severe malaria across the studies revealed the three most 5-prime *GNAS* SNPs, rs2057291, rs6128461 and rs6026592, and a single 3-prime SNP, rs3730171, as significant determinants of severe malaria. Table 4 presents the pooled odds ratios of developing severe malaria associated with allelic and genotypic variants of the rs2057291, rs6128461, rs6026592 and rs3730171 loci. The pooled estimate for the rs2057291 A allele indicated protection from severe malaria (OR=0.9 (0.82-0.98), $P=0.011$). The rs6026592 G, rs6128461 C and rs3730171 T alleles all demonstrated significant risk of severe malaria in pooled estimates (OR=1.13 (1.05-1.21), $P=0.001$; OR=1.09 (1.01-1.17), $P=0.022$; OR=1.13 (1.04-1.22), $P=0.005$, respectively). Meta-analysis across the genotype-based models indicated an apparent additive effect of the alleles at rs2057291, rs6128461 and rs6026592 on severe malaria. As demonstrated in Table 4, at each locus, greater deviation from an odds ratio of 1 and accordingly greater significance was observed for the association between the homozygous minor allele state and severe malaria than for the heterozygote state. In contrast to the other loci, despite a strong allelic signal, no significant genotypic associations were demonstrated at the rs3730171 locus. Furthermore, as illustrated in the LD map in Figure 1, the *GNAS* region appears to be split into two blocks of high LD separated by an apparent recombination hotspot, encompassing two SNPs. While rs2057291, rs6026592 and rs6128461 are all located 5-prime to the recombination hotspot, rs3730171 is located 3-prime to the hotspot. This underlying genetic structure may be responsible for the different pattern of association at the rs3730171 locus in comparison to the 5-prime loci. Using Cochran's chi-test of heterogeneity, sufficient homogeneity for the application of meta-analysis ($P<0.05$) was demonstrated between the studies at the loci discussed.

Haplotype distributions

The LD pattern illustrated in Figure 1 was used to inform haplotype construction in the *GNAS* region. Thus, 5-prime haplotypes were constructed from the rs2057291, rs6026592 and rs6128461 loci and 3-prime haplotypes were constructed from the rs6026592, rs3730170, rs3787493, rs234630, rs919196, rs3730171 and rs8386 loci. The Kenya TDT

study was excluded from meta-analysis on the 3-prime haplotypes owing to high genotype failure rates (>20%) at rs6026593 and rs234630. Table 5 presents the frequencies of the major (5% frequency) 5-prime and 3-prime *GNAS* haplotypes across the studies. Three 5-prime haplotypes represented over 98% of the population, while six 3-prime haplotypes represented 85-95% of the population in each study.

Multi-locus associations

Meta-analysis combining the haplotype odds ratios across studies revealed multi-locus associations which concurred with the single locus associations. Figures 2 and 3 present forest plots illustrating the individual study and pooled odds ratios of developing severe malaria associated with haplotypes 122 (GCC) and 211 (ATT), respectively. The size of each square in the forest plot is proportional to the study's weight in the meta-analysis. Horizontal lines reflect the 95% confidence intervals surrounding the odds ratios. The pooled odds ratio is plotted as a diamond, the lateral points of which indicate 95% confidence intervals. As illustrated in Figure 2, the combination of the risk-conferring rs2057291 G, rs6026592 G and rs6128461 C alleles, indeed, confer increased risk of severe malaria (OR=1.12 (1.03-1.20), P=0.005). Figure 3 illustrates the protection from severe malaria conferred by the alternative alleles - rs2057291 A, rs6026592 A and rs6128461 T (OR=0.88 (0.81-0.96), P=0.005). Furthermore (not illustrated here), haplotype 111, which is comprised of a mix of risk (rs2057291 G) and protective (rs6026592 A, rs6128461 T) alleles, exerts no significant impact on severe malaria status (OR=0.98 (0.9-1.06), P=0.949). Cochran's Q-test demonstrated that the studies were sufficiently homogenous to be pooled in meta-analysis at all three 5-prime haplotypes (P>0.05).

In the 3-prime *GNAS* region, using meta-analysis, association was demonstrated at haplotype 1111121 (ATGTTTC) (forest plot not shown here). This association presumably arose from the risk-conferring rs3730171 minor allele (T), as the haplotype conferred increased risk of severe malaria (OR=1.18 (1.02-1.36), P=0.022). However, caution is advised in the interpretation of this association as the studies demonstrated significant heterogeneity at this haplotype with Cochran's Q-test (P = 0.02).

Discussion

Functional studies have demonstrated an interaction between the erythrocyte G-alpha-s protein and the malaria parasite 3, 5, 6, 7. At the cellular level, it appears that erythrocyte Gs signal transduction mediated by the G-alpha-s subunit and the β -2-AR alter erythrocyte invasion by *P.falciparum* 3. However, it remained unclear whether this interaction would impact at the disease level. Using meta-analysis across seven large association studies from The Gambia, Ghana, Kenya and Malawi, we provide evidence for the impact of the G-alpha-s gene, *GNAS*, on malaria disease outcome.

Amongst twelve SNPs investigated in the *GNAS* region, four demonstrated evidence of significant (P<0.05) association with severe malaria using meta-analysis on single-locus and multi-locus genetic models. With allele-based models, the rs2057291 A allele was associated with protection from severe malaria (OR=0.9 (0.82-0.98), P=0.011), while the rs6026592 G, rs6128461 C and rs3730171 T alleles all demonstrated risk of severe malaria (OR=1.13 (1.05-1.21), P=0.001; OR=1.09 (1.01-1.17), P=0.022; OR=1.13 (1.04-1.22), P=0.005, respectively). Genotype-based analysis indicated that the alleles at rs2057291, rs6128461 and rs6026592 were effective in an additive manner. However, despite a strong allelic effect, no genotype-based association was demonstrated at the rs3730171 locus. On investigation of the LD pattern in the *GNAS* region investigated here, it was revealed that the region was split into two blocks of high LD separated by an apparent recombination hot-spot encompassing two SNPs, rs7121 and rs3730168 (see Figure 1). While the rs2057291,

rs6026592 and rs6128461 SNPs all cluster 5-prime of the recombination hot-spot, rs3730171 is located 3-prime to the hotspot. Given this underlying genetic structure, it remained uncertain whether the 5-prime SNPs were picking up the same signal as rs3730171.

Haplotype-based meta-analysis was undertaken on haplotypes 5-prime and 3-prime to the recombination hotspot. The 5-prime haplotypes comprised alleles at rs2057291, rs6026592 and rs6128461, while the 3-prime haplotypes comprised alleles at rs6026593, rs3730170, rs3787493, rs234630, rs919196, rs3730171 and rs8386. Meta-analysis demonstrated 5-prime and 3-prime haplotype associations with severe malaria which concurred with the single-locus associations. Amongst three high frequency (>5%) 5-prime haplotypes, the haplotype comprising the risk-conferring rs2057291 G, rs6026592 G and rs6128461 C alleles (GGC: 122) was significantly associated with increased susceptibility to severe malaria (OR=1.12 (1.03-1.20), P=0.005). Furthermore, the haplotype carrying the alternative, protection-conferring alleles, rs2057291 A, rs6026592 A and rs6128461 T (AAT: 211), demonstrated significant protection from severe malaria (OR=0.88 (0.81-0.96), P=0.005). Amongst six high frequency 3-prime haplotypes, a single haplotype (ATGTTTC: 1111121) was significantly associated with risk of severe malaria (OR=1.18 (1.02-1.36), P=0.022). This haplotype carried the major alleles of all SNP loci with the exception of rs3730171. Thus, it was suspected that the haplotype association reflected the influence of the rs3730171 allele on severe malaria. However, the 3-prime haplotype association should be interpreted with caution, as Cochran's Q-test demonstrated significant inter-study heterogeneity with this haplotype association (P=0.020).

The effect sizes observed at the loci investigated here are all relatively modest. However, as we cannot confirm that any of the loci investigated here are actually functional in malaria disease, we cannot infer a modest effect size of the true functional variant. The rs2057291, rs6026592, rs6128461, and rs3730170 SNPs may be picking up the signal of a functional variant(s) in moderate-to-high LD with them, possibly even at a relatively distal locus. The LD pattern illustrated in Figure 1 only presents a small snapshot of the *GNAS* region. On this scale, the LD between the 5-prime and 3-prime regions appears to be broken up by a recombination hotspot. However, on a larger scale, variants outside the region investigated here may connect the two regions via long-range LD. Further studies are required to elucidate the true functional variant(s) responsible for the signals of association presented here. A detailed understanding of the haplotypic background should facilitate this process.

The P-values presented here were not corrected for multiple testing. The Bonferroni correction, a commonly used correction which assumes independence between markers, was considered too stringent in this study as several SNPs exhibited high degrees of dependence with one another, as measured by LD (D'). In order to reduce our probability of detecting false-positive genetic associations, we took a multi-centre approach to investigate genetic associations. By combining studies using meta-analysis, we were able to increase our overall sample size and, thus, power to detect true positive associations and reject false-positives. Indeed, power calculations indicate that in order to achieve 80% power to detect the genotypic association observed at the rs6026592 and rs6128461 SNPs at the 5% significance threshold, approximately 4000 cases and 4000 controls are required. Furthermore, these variants have substantially high MAFs (~40%). For lower frequency variants, accordingly larger sample sizes are required. Sample sizes in the order of 4000 cases and 4000 controls are currently not possible in individual studies of severe malaria but can be achieved by taking a collaborative multi-centre approach, as demonstrated here.

The *GNAS* locus encodes four alternative transcripts from alternative promoters and first exons that splice onto a common set of downstream exons 12. Thus, although the focus of

this study was the G- α -s transcript, as we were working with genomic DNA, it was not possible to discriminate the influences of other *GNAS* transcripts on severe malaria. In accordance with the functional evidence supporting a role for G- α -s in erythrocyte invasion 1, if we assume that this subunit is at least partially responsible for the severe malaria associations demonstrated here, intervention at this stage of the erythrocyte Gs signal transduction pathway may, indeed, impede malaria disease progression. Given the wealth of pharmacological data currently available on G protein signal pathways 13, this is a promising area of research for anti-malarial drug initiatives.

Materials and Methods

Samples

Patients were recruited from the Royal Victoria Hospital in Banjul, Navrongo War Memorial Hospital, Kilifi District Hospital, and the Queen Elizabeth Central Hospital in Blantyre. Children under the age of 12 years presenting with severe malaria were recruited as cases. Severe malaria was defined as cerebral malaria (CM), severe malarial anaemia (SA), other severe complications such as respiratory acidosis, and fatalities due to malaria infection. CM was defined by a Blantyre coma score ≤ 3 , indicating unrousable coma not attributable to convulsions, hypoglycaemia or meningitis in a patient with *P.falciparum* parasitaemia 14. SA was defined as haemoglobin concentration $<5\text{g/dl}$ in the presence of *P.falciparum* asexual parasitaemia. Severe malaria sub-phenotypes were not investigated here owing to small sample size (see Table 1). In The Gambia, Kenya and Malawi, population controls were obtained from umbilical cord blood samples. In Ghana, for each severe malaria case, two healthy children matched by age, gender and location were obtained as community controls. For the family trios, the patients' mother and father were recruited as controls. The final analysis was restricted to true biological trios confirmed using the *Nuclear* software package 15. Families with $<80\%$ probability of being true parent-offspring trios were excluded from the study. Sample collection and DNA extraction was undertaken by local teams at each study centre. Whole genome amplification of the genomic DNA was undertaken using either Primer Extension Pre-amplification 16 or Multiple Displacement Amplification 17. All samples were obtained with informed consent from a parent or guardian, and approval for sample collection and genotyping was obtained from the relevant research ethics committees in each study population.

SNP selection

The *GNAS* locus, located at 20q13, encodes four transcripts from four alternative promoters 12. SNP selection was focused on the 19kb G- α -s transcript (NM_000516). SNPs were selected using information from the literature and dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/), and reflected a compromise between SNP function, marker spacing and minor allele frequency (MAF). The initial SNP selection consisted of validated markers in the *GNAS* region with MAF $\geq 5\%$. This was narrowed down to an economic subset of SNPs for which genotyping assays could be designed on the mass spectrometry platform. SNP details are presented in Table 2.

Genotyping methods

Genotyping was undertaken using Sequenom's mass spectrometry technology (www.sequenom.com). Genotyping accuracy was assessed by testing the conformation of the observed genotype distributions in the controls to the expected distributions under Hardy-Weinberg equilibrium (HWE). Assays which deviated from HWE at the 0.1% significance threshold were excluded from further analysis.

Association analysis

In all studies, genotype data were available for the sickle cell locus (HbS), the genetic determinant currently most conclusively associated with malaria disease outcome [18, 19]. Thus, where the statistical framework enabled it, adjustments were made for the impact of the HbS locus on severe malaria. Single loci were tested under allele and general genotype models. Multi-locus analysis was undertaken using haplotype models. Only haplotypes with frequency $\geq 5\%$ were investigated. In the case-control studies, allele, genotype and haplotype associations with severe malaria were tested using logistic regression (LR) analysis with adjustment for the HbS locus. In the Gambian and Ghanaian case-control studies, conditional logistic regression (CLR) was undertaken with stratification by ethnicity. All analysis on the case-control studies was undertaken in STATA (STATA, version 9.0; StataCorp, College Station, TX). In the family trios, allele and haplotype-based association tests were undertaken using the transmission distortion test (TDT) [20] in R version 2.2.0 [21]. The TDT framework did not enable correction for the HbS locus. Genotype associations were tested using pseudo case-control analysis with correction for HbS using the GenAssoc package (<http://www-gene.cimr.cam.ac.uk/clayton/software/>) provided in the public domain by the Cambridge Institute for Medical Research, University of Cambridge, UK). Meta-analysis was undertaken across all the case-control and family studies on each of the allele, genotype and haplotype-based tests described above using the methods outlined in Kazeem and Farrell [22]. Inter-study heterogeneity in association was assessed using Cochran's chi-square test (Q-test) under the null hypothesis of homogeneity. Pooled associations with P-values exceeding the 5% significance level were treated with caution. Forests plots were generated in STATA.

Linkage disequilibrium and haplotype construction

MARKER's software (www.gmap.net) was used to calculate the LD between all SNP pairs using the disequilibrium coefficient D' [23] and to plot an LD map of the gene region. LD maps were used to inform haplotype construction. In the case-control studies, haplotypes were reconstructed using SNPHAP (<http://www-gene.cimr.cam.ac.uk/clayton/software/>). Haplotypes with SNPHAP probabilities less than 80% were excluded from analysis. In the family trios, haplotypes were constructed using an Expectation Maximisation algorithm in R.

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Figure 1.

Marker Map illustrating the LD between *GNAS* SNPs in the G-alpha-s region in the Gambia case-control study

LD is measured using D' . Coloured spots connecting SNPs illustrate the absolute D' level between those SNPs. The rs2057291, rs6128461 and rs6026592 loci display moderate-to-high LD amongst one another, as indicated by the dark grey spots. The blank patch encompassing the rs7121 and rs3730168 loci indicates a recombination 'hot-spot'. This hot-spot is observed in all the studies investigated here (Marker maps not shown).

Figure 2.

Meta-analysis combining the odds ratios from associations between haplotype 122 (rs2057291 G, rs6026592 G, rs6128461 C) and severe malaria across studies
Pooled OR=1.12 (95% CI: 1.03-1.2), $P=0.005$. Cochran's Q-test for heterogeneity on 6 degrees of freedom: $P=0.782$.

Figure 3.

Meta-analysis combining the odds ratios from associations between haplotype 211 (rs2057291 A, rs6026592 A, rs6128461 T) and severe malaria across studies
Pooled OR=0.88 (95% CI: 0.81-0.96), P=0.005. Cochran's Q-test for heterogeneity on 6 degrees of freedom: P=0.735.

Table 1

Severe malaria phenotype frequencies

Phenotype	Gambia Case-control	Gambia Trios	Ghana Case-control	Kenya Case-control	Kenya Trios	Malawi Case-control	Malawi Trios	Total
CM	486 (32%)	260 (53%)	30 (4%)	112 (19%)	69 (40%)	562 (50%)	142 (72%)	1661
CM:SA	140 (9%)	137 (28%)	14 (14%)	52 (9%)	26 (15%)	90 (8%)	49 (25%)	508
SA	256 (17%)	75 (15%)	406 (53%)	155 (27%)	38 (22%)	7 (37%)	2 (1%)	939
Other Severe	10 (41%)	14 (3%)	311 (41%)	258 (45%)	41 (24%)	53 (5%)	4 (2%)	691
All Severe	892 (100%)	486 (100%)	761 (100%)	577 (100%)	174 (100%)	712 (100%)	197 (100%)	3799
Controls	628	972	729	914	348	416	348	4355
Total	1520	1458	1490	1491	522	1128	521	8130

The percentage (amongst all severe malaria cases) for each severe malaria sub-phenotype is provided in brackets. *CM* cerebral malaria, *SA* severe malarial anaemia.

Table 2*GNAS* SNP Properties

Assay ¹	Coordinate ²	Alleles ³	Role	Amino Acid Change
rs2057291	56905438	G/A	Intron	-
rs6128461	56910485	T/C	Intron	-
rs6026592	56910606	A/G	Intron	-
rs7121	56912202	T/C	Exon, Coding Sequence	131 I/I
rs3730168	56912334	G/A	Intron, Exon/Intron Boundary	-
rs6026593	56912528	G/A	Intron	-
rs3730170	56913815	T/C	Intron, Exon/Intron Boundary	-
rs3787493	56914543	G/A	Intron	-
rs234630	56917050	T/C	Intron	-
rs919196	56917480	T/C	Intron	-
rs3730171	56917636	C/T	Exon, Coding Sequence	185 I/I
rs8386	56919207	C/T	Exon, Coding Sequence	371 N/N

The majority of the SNPs investigated here were intronic, although three SNPs exhibited synonymous amino acid changes. Relatively uniform marker spacing was achieved across the NM_000516 transcript.

¹ Assay nomenclature based on rs number.

² Coordinate on chromosome 20 as of Ensembl release 36.35i.

³ Major allele/ minor allele.

Table 3

Minor allele frequencies at *GNAS* SNPs

Assay	Alleles ¹	Gambia case-control	Gambia trios	Ghana case-control	Kenya Case-control	Kenya trios	Malawi case-control	Malawi trios
rs2057291	G/A	0.25	0.21	0.25	0.20	0.20	0.27	0.24
rs6128461	T/C	0.39	0.44	0.38	0.47	0.46	0.42	0.40
rs6026592	A/G	0.41	0.46	0.39	0.48	0.46	0.43	0.41
rs7121	T/C	0.20	0.17	0.16	0.22	0.24	0.18	0.20
rs3730168	G/A	0.49	0.49	0.50	0.49	0.48	0.47	0.50
rs6026593	A/G	0.48	0.50	0.49	0.39	-	0.44	0.48
rs3730170	T/C	0.22	0.23	0.22	0.19	0.17	0.18	0.20
rs3787493	G/A	<i>0.04</i> ²	<i>0.03</i>	<i>0.02</i>	0.07	0.06	<i>0.04</i>	0.05
rs234630	T/C	<i>0.03</i>	<i>0.03</i>	<i>0.01</i>	0.05	-	<i>0.04</i>	-
rs919196	T/C	0.34	0.36	0.36	0.29	0.27	0.30	0.30
rs3730171	C/T	0.19	0.19	0.32	0.25	0.26	0.22	0.22
rs8386	C/T	0.12	0.10	0.22	0.18	0.19	0.13	0.13

The majority of assays demonstrated high MAF (> 5%). Furthermore, inter-study allele frequencies demonstrated high similarity.

¹ Major allele/ minor allele.

² Assays with MAF <5% presented in italics.

Table 4

Pooled allele and genotype-based odds ratios of association with severe malaria at rs2057291, rs6026592, rs6128461 and rs3730171

Assay	Test Variant	Pooled OR	Pooled z value (P-value)
rs2057291	A allele	0.9 (0.82-0.98)	-2.55 (0.011)
	AG genotype	0.95 (0.84-1.06)	- 0.96 (0.340)
	AA genotype	0.75 (0.58-0.96)	- 2.29 (0.022)
rs6026592	G allele	1.13 (1.05-1.21)	3.34 (0.001)
	AG genotype	1.11 (0.98-1.25)	1.65 (0.098)
	AA genotype	1.2 (1.03-1.39)	2.33 (0.02)
rs6128461	C allele	1.09 (1.01-1.17)	2.30 (0.022)
	CT genotype	1.13 (1.00-1.27)	1.98 (0.048)
	CC genotype	1.23 (1.05-1.43)	2.86 (0.01)
rs3730171	C allele	1.13 (1.04-1.22)	2.79 (0.005)
	CT genotype	1.08 (0.96-1.21)	1.24 (0.217)
	CC genotype	1.14 (0.91-1.43)	1.13 (0.258)

Table 5
Frequency distribution of the major (> 5%) 5-prime and 3-prime *GNAS* haplotypes

Haplotype Region	<i>I</i> _{Haplotype}	Sequence	Gambia CC	Gambia TDT	Ghana CC	Kenya CC	Kenya TDT	Malawi CC	Malawi TDT
5-Prime Region	111	GAT	21018 (35 %)	434 (37.1%)	967 (35.3%)	914 (31.9%)	149 (36%)	617 (30.9%)	161 (36.6%)
	122	GGC	11198 (41.2%)	443 (36.8%)	1127 (41.1%)	1352 (47.1%)	164 (38.5%)	885 (44.3%)	145 (32.4%)
	211	AAT	655 (22.5 %)	269 (24.3%)	630 (23.0%)	580 (20.2%)	95 (23.4%)	486 (24.3%)	122 (29.3%)
3-Prime Region	1111111	ATGTTCC	666 (24.1%)	325 (22.6%)	421 (15.9%)	677 (24.3%)	-	497 (24.3%)	117 (23.9%)
	1111121	ATGTTTC	213 (7.7%)	125 (8.1%)	248 (9.4%)	190 (6.8%)	-	227 (11.1%)	39 (7.6%)
	1111122	ATGTTT	282 (10.2%)	171 (11.8%)	588 (22.2%)	521 (18.7%)	-	270 (13.2%)	65 (13%)
	2111111	GTGTTCC	477 (17.3%)	254 (17.9%)	355 (13.4%)	311 (11.2%)	-	311 (15.2%)	83 (16.7%)
	2111211	GTGTTCT	297 (10.8%)	140 (10.1%)	335 (12.7%)	245 (8.8%)	-	161 (7.9%)	41 (8.2%)
	2211211	GCGTCCC	557 (20.2%)	247 (17.1%)	556 (21.0%)	506 (18.2%)	-	376 (18.4%)	73 (15.2%)

In both the 5-prime and 3-prime regions, these major haplotypes accounted for the majority of the haplotypes (>85%) in each population.

*I*_{Haplotype} Major allele/ minor allele.

¹ 1 = major allele, 2 = minor allele.

² Assays with MAF <5% presented in italics.

² Frequencies summed across cases and controls.