Non-*O* ABO blood group genotypes differ in their associations with *Plasmodium falciparum* rosetting and severe malaria

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Abstract (250 words)

Blood group O is associated with protection against severe malaria and reduced size and stability of *P. falciparum*-host red blood cell (RBC) rosettes compared to non-O blood groups. Whether the non-O blood groups encoded by the specific *ABO* genotypes *AO, BO, AA, BB* and *AB* differ in their associations with severe malaria and rosetting is unknown. The A and B antigens are host RBC receptors for rosetting, hence we hypothesized that the higher levels of A and/or B antigen on RBCs from *AA, BB* and *AB* genotypes compared to *AO/BO* genotypes could lead to larger rosettes, increased microvascular obstruction and higher risk of malaria pathology. We used a case-control study of Kenyan children and *in vitro* adhesion assays to test the hypothesis that "double dose" non-*O* genotypes (*AA, BB, AB*) are associated with increased risk of severe malaria and larger rosettes than "single dose" heterozygotes (*AO, BO*). In the case-control study, compared to *OO*, the double dose genotypes consistently had higher odds ratios (OR) for severe malaria than single dose genotypes, with *AB* (OR 1.93) and *AO* (OR 1.27) showing most marked difference (P=0.02, Wald test). *In vitro* experiments with blood group A-preferring *P. falciparum* parasites showed that significantly larger rosettes were formed with *AA* and *AB* host RBCs compared to *OO*, whereas *AO* genotype rosettes were indistinguishable from *OO*. Overall, the data show that *ABO* genotype influences *P. falciparum* rosetting and support the hypothesis that double dose non-*O* genotypes confer a greater risk of severe malaria than *AO/BO* heterozygosity.

Key Words: ABO blood group; *ABO* genotype; Severe malaria; Rosetting; Cytoadhesion; *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1)

Introduction

The ABO blood group was the first human blood group system to be discovered, and has since been widely studied (1). Three red blood cell (RBC) expressed carbohydrate antigens characterise the ABO system; the A and B antigens are the products of the enzymatic addition of N-acetyl-D-galactosamine (GalNAc) or D-galactose (Gal), respectively, to a precursor H antigen, while an inactive glycosyltransferase fails to add any sugar residues to the H antigen giving rise to blood group O (2). The resultant A, B, AB and O blood group phenotypes have been associated with numerous diseases (3), most recently including COVID-19 (4), and there is strong evidence to support their role in severe malaria (5). Blood group O has a high frequency in malaria-endemic regions (6), and has been associated with protection from severe malaria in numerous studies (7-15). Both observations are consistent with a malaria-selective pressure for blood group O. Additionally, the A and B antigens interact with molecules on the surface of *Plasmodium falciparum* infected red blood cells (iRBCs), such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), mediating iRBC binding to uninfected RBCs to form clusters called rosettes (16-20). *P. falciparum* isolates vary in their propensity to form rosettes, high levels of rosetting contributing to impaired microvascular blood flow, and severe malaria (21-23). Rosetting iRBCs show a “preference” for uninfected A or B RBCs, and form larger, more stable rosettes than with O RBCs (16-19, 24-26). The protection against severe malaria associated with blood group O may be due to reduced rosetting, and hence reduced microvascular obstruction and pathology (7, 16, 24, 27-29).

Previous studies linking ABO blood group to malaria susceptibility and rosetting have either used serological RBC agglutination assays to determine ABO blood group phenotype (A, B, AB, O), or single nucleotide polymorphisms (SNPs) at the *ABO* gene to derive *ABO* genotype (*OO, AO, AA, BO, BB, AB*), which have then been used to infer ABO phenotypes. While multiple polymorphisms underlie the genetic basis of the ABO system (30, 31), most are rare, and two SNPs rs8176719 and rs8176746 are considered sufficient (>90% accuracy) to infer

ABO phenotype (8-15, 32, 33). However, the concordance between *ABO* genotype and ABO phenotype has not been reported in previous population studies of malaria susceptibility, most of which have been conducted in sub-Saharan Africa.

Similarly, few studies have been conducted which have examined the association between *ABO* genotype (rather than phenotype) and malaria susceptibility, or potential protective mechanisms such as their impacts on rosetting. Prior work shows a gene dosage effect between *ABO* genotype and the number of ABH antigens on RBCs. For example, *AA* and *BB* homozygotes have substantially higher levels of A and B antigen on RBCs than *AO* and BO heterozygotes respectively (34, 35), while *AB* heterozygotes have antigen levels that are more similar to those found in *AA/BB* homozygotes (34-38). The level of A antigen displayed on the RBC surface has been shown to have an impact on rosetting. For example, both rosetting and PfEMP1-binding are substantially reduced in RBCs of the A_2 blood group phenotype, in which fewer A antigen sites are displayed per RBC than cells of the common A_1 phenotype (16, 17, 39). However, to the best of our knowledge, whether RBCs from *AO* and *BO* genotypes differ in their rosette-forming ability compared to RBCs from *AA/BB/AB* genotypes with higher levels of A and B antigens has not yet been tested.

Here, we used a case-control study conducted in East Africa to investigate whether specific *ABO* genotypes are associated with differing levels of susceptibility to severe childhood malaria. In addition, we examined the concordance between *ABO* genotype and phenotype. Finally, through *in vitro* studies, we investigated whether *ABO* genotype influences either *P. falciparum* rosetting or other parasite adhesion-related phenomena including binding to endothelial receptors and the display of PfEMP1 on the iRBC surface (40). We hypothesised that individuals with two non-*O* alleles (*AA/BB/AB*) might have a greater risk of severe malaria than non-*O* heterozygotes (*AO/BO*), due to the former having increased levels of A or B antigens on their RBCs, enabling iRBCs to form larger, more stable rosettes that cause greater microvascular obstruction and pathology.

Methods

Kilifi study area

All epidemiological and clinical studies were conducted in Kilifi County on the coast of Kenya (41). At the time the studies were conducted, malaria transmission followed a seasonal pattern determined by annual long and short rainy seasons (42).

Kilifi case-control study

This study has been described in detail elsewhere (14, 43, 44). 2245 children aged <14 years who presented to Kilifi County Hospital (KCH) with features of severe malaria were recruited as cases between January 2001 and January 2008. Severe malaria was classified as the presence of a blood film positive for *P. falciparum* accompanied by any of the following complications: cerebral malaria (CM, a Blantyre Coma Score of <3), severe malarial anaemia (SMA, a haemoglobin concentration of <5g/dl) or respiratory distress (RD, abnormally deep breathing) (45). In a recent modelling study based on white blood cell and platelet counts in the same cohort of children, it was shown that malaria was probably not the primary cause for the severe disease seen in approximately one third (842 out of 2245) of the "severe malaria" cases (46). For this reason, these cases were dropped from the current analysis, leaving 1403 cases for inclusion in the current study. Children who were born within the same area as cases between August 2006 and September 2010 were recruited at 3-12 months of age to a genetics cohort study served as controls (47). Data on ABO blood group (inferred from genotype data) and severe malaria risk from this study have been published previously (14).

Kilifi longitudinal cohort study

The Kilifi longitudinal cohort study, described in detail elsewhere (48), was established to investigate the immuno-epidemiology of uncomplicated malaria and other common childhood diseases in an area approximately 15 km to the north of KCH (49, 50). Between August 1998

and August 2001, children aged <10 years were recruited into the cohort, including children born from study households during the study period. Cohort members were followed up actively for clinical events on a weekly basis. In addition, members were passively followed for inter-current illnesses at a dedicated clinic at KCH. Finally, the cohort was also monitored for the prevalence of asymptomatic *P. falciparum* carriage through four cross-sectional surveys carried out in March, July and October 2000 and June 2001. Exclusion criteria included death, migration from the study area for more than 2 months and consent withdrawal. Uncomplicated malaria in this cohort was defined as fever (axillary temperature of >37.5°C) with *P. falciparum* infection at any density, in the absence of any signs of severity (51).

ABO phenotyping and genotyping

Serological typing for ABO blood group phenotypes was carried out by slide haemagglutination assays using anti-A and anti-B monoclonal antibodies (Alba Bioscience, Edinburgh, UK). For genotyping, DNA was extracted from fresh or frozen whole blood samples using either an ABI PRISM® 6100 Nucleic acid prep station (Applied Biosystems, Foster City, CA, USA) or QIAamp DNA Blood Mini Kits (Qiagen, West Sussex, United Kingdom) respectively. ABO genotyping was carried out by assessing SNPs rs8176719 and rs8176746, using the SEQUENOM iPLEX® Gold (Sequenom) multiplex system following DNA amplification by whole genome amplification, as described previously (8). rs8176719 in exon 6 of the *ABO* gene on chromosome 9 encodes the 261G deletion giving rise to the *O* allele (30). rs8176746 in exon 7 of the *ABO* gene encodes C796A which distinguishes A and B alleles (30). The two SNPs were used to designate *ABO* genotypes and infer blood groups as described previously in this population (8) and as summarized in Table 1. The common *O* deletion (D) arose on the background of the A allele (C). Therefore, double heterozygotes GD X AC are assumed to have the haplotype GA and DC, giving rise to the *BO* genotype, rather than haplotype GC and DA that gives rise to the *AO* genotype (Table 1). Genotyping for rs334 on *HBB*, which detects the HbS mutation that results in sickle cell trait (HbAS) or

sickle cell anaemia (HbSS), and for the 3.7Kb α-globin deletion at the *HBA* locus, which gives rise to the common African variant of α^+thalassaemia, was conducted by PCR as described elsewhere (52, 53).

Data analysis for epidemiological studies

Agreement between *ABO* blood group genotype and serological phenotype was tested using Cohen's Kappa statistic, for which score of 100% and 0% show complete agreement and no agreement, respectively.

The Pearson's χ^2 or Fisher's exact tests were used to test for differences in the distribution of *ABO* genotypes between severe malaria (including its various clinical sub-types of CM, SMA, RD and mortality) and community controls or across groups in different categorical variables including gender, ethnic group and HbS and α^+thalassaemia genotypes. We tested for differences in age across *ABO* genotypes by use of the Kruskal Wallis test.

Odds ratios (ORs) (with 95% confidence intervals, CIs) for severe malaria and its various clinical sub-types were determined by comparison of genotype frequencies among cases and controls, using a fixed-effects logistic-regression model with adjustments for self-reported ethnicity, gender, α^+thalassaemia and HbAS. The *OO* genotype was taken as the reference group to which the other blood group genotypes (*AO*, *AA*, *AB*, *BO*, *BB*) were compared both individually and as 'non-*O*' genotypes combined. To test the hypothesis that double dose non-*O* genotypes (*AA*, *AB*, *BB*) are associated with a higher risk of severe malaria than single dose non-O genotypes (*AO*, *BO*), odds ratios between single and double dose non-*O* genotypes from the logistic regression analysis above were compared using the Wald test.

In the longitudinal cohort study, the impact of *ABO* genotype on the incidence of uncomplicated malaria was investigated using a random effects Poisson regression model

with the reference *OO* genotype compared to all other *ABO* genotypes assuming a genotypic model of inheritance, or to non-*O* genotypes combined in a recessive model of inheritance using the rs8176719 SNP. Incidence Rate Ratios (IRR) were generated from both a univariate model and a multivariate model which were both adjusted for age, season, ethnic group, and HbS and α^{+}thalassaemia genotypes. All analyses accounted for within-person clustering of events. For the cross-sectional survey, odds ratios investigating the impact of *ABO* genotypes on the prevalence of asymptomatic parasitaemia were generated by use of logistic regression analysis. We conducted both a univariate analysis and multivariate analysis with adjustment for confounding by age, season, ethnic group and HbS genotype. The analysis also accounted for clustering of asymptomatic parasitaemia events within individuals.

Red blood cells

All *in vitro* assays were carried out using malaria negative RBC samples that were collected and processed during May 2009 and May 2010, as described in detail previously (40), as part of the Kilifi longitudinal cohort study, (49, 50). Briefly, whole blood samples were collected into heparinized tubes with plasma aspirated and removed following centrifugation, and white blood cells removed by density centrifugation through Lymphoprep™ (Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway). Purified RBC pellets were washed and either stored at 4°C and used within 4 days for cytoadhesion assays or cryopreserved in glycerolyte and thawed by standard methods (54, 55) before use for rosetting and PfEMP1 expression assays.

Parasites and parasite culture

Rosetting assays were carried out with the *P. falciparum* clone IT/R29 (56-60) expressing the ITvar9 PfEMP1 variant that binds to RBCs to form rosettes (56-61). The ItG *P. falciparum* line used for the *in vitro* static adhesion assays binds to both ICAM-1 and CD36 endothelial receptors and expresses the ITvar16 PfEMP1 variant (56, 57). All parasites were maintained using standard culture methods (55) in blood group O+ human RBCs. Prior to conducting the

in vitro experiments, mature pigmented-trophozoite stage iRBCs were purified to >90% parasitaemia from uninfected and ring-stage iRBCs by magnetic–activated cell sorting (MACS®) (62). The purified iRBCs were then used to invade donor RBCs of different *ABO* genotypes, in duplicate flasks, at a starting parasitaemia of 1.5% for ItG and 3% for IT/R29 and cultured for 24-48 hours to the mature pigmented-trophozoite stage (40). RBCs from a local control *OO* donor were included in all experiments to adjust for day-to-day variation in the assays.

Rosetting Assays

Rosetting was assessed as described previously (40, 55). Briefly, a wet preparation of IT/R29 culture suspension at 2% haematocrit stained with 25μg/ml ethidium-bromide was examined with combined UV/bright field using a Leica DM 2000 fluorescence microscope (x40 objective). A rosette was defined as a mature pigmented trophozoite-stage iRBC binding two or more uninfected RBC, and the number of uninfected RBC bound per rosette was counted for at least 30 rosettes to determine mean rosette size for each donor. The frequency of large rosettes is the percentage of the rosettes counted that had >4 uninfected RBCs per rosette. Sample genotypes were masked to avoid observer bias.

Static adhesion assays

Static adhesion assays were carried out as described in detail previously (40, 63). Briefly, donor RBC samples infected with ItG *P. falciparum* parasites were assessed for binding to purified recombinant proteins CD36 (R & D Systems, UK) and ICAM1-Fc (a gift from Professor Alister Craig, Liverpool School of Tropical Medicine) spotted on bacteriological petri dishes (BD Falcon 351007) at a concentration of 50μg/ml. Each donor sample was tested once in duplicate dishes run on the same day, with triplicate spots of each protein in each dish. For each spot, 6 images of adherent iRBCs were captured across random fields using an inverted microscope (Eclipse TE2000-S, Nikon, x40 magnification), giving 36

images per protein for each donor RBC. Images were processed and analysed using Image SXM software (University of Liverpool, UK) (64) and the results expressed as the mean number of iRBC bound per mm^2 of surface area.

PfEMP1 expression assays

Assessment of PfEMP1 expression by flow cytometry, including gating strategies, in donor RBCs infected with the IT/R29 *P. falciparum* clone has been described in detail previously (40). Briefly, ITvar9 PfEMP1 expression was determined by staining the same preparation of IT/R29 donor iRBC samples tested for rosetting above, with rabbit polyclonal total IgG raised against the ITvar9 variant (65). ITvar9 PfEMP1 expression was defined by both the median fluorescent intensity (MFI) and proportion of iRBC positively staining with anti-ITvar9 IgG.

Data analysis for in vitro experiments

Multivariate regression analysis was used to test the effect of *ABO* genotype on rosetting, cytoadhesion or PfEMP1 expression. Potential confounding variables including HbS and α^+thalassaemia genotypes (40), mean corpuscular volume and complement receptor 1 level (66, 67) and Knops blood group (40, 43) were first examined in univariate analyses. The final model included the variables HbAS and α^+thalassaemia which showed significant associations on univariate analysis ($P<0.05$), and improved the overall model fit tested using the log-likelihood ratio test. Experimental day was included as a covariate to account for day-to-day variation when experiments were conducted over several days. Non-normally distributed CD36 and ICAM-1 binding data were normalized by square root-transformation. To visualise the data, dot plots were generated showing individual data points for each donor, normalized to the mean of control donor genotype *OO* cells run on the same day to account for day-to-day variation. A p value of <0.05 was considered significant in all analyses. Statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria) or Stata v13.1 (StataCorp, Texas, USA), and graphs were generated using Prism v7.0 (Graphpad Inc, San Diego, California).

Blood group preference of the IT/R29 P. falciparum clone.

RBCs were obtained from 13 Scottish donors (4 donors of group O and A, and 5 for group B). After the removal of plasma and white cells as described above, RBCs from the 13 donors, and an aliquot of the uninfected group O RBCs in which the parasites were cultured (hereafter known as 'home O') were labelled with 6-carboxyfluorescein diacetate (C-FDA) (150µg/ml in RPMI 1640 medium), at 2% haematocrit for 15 minutes, washed twice with RPMI 1640 and stored at 4°C. Blood group preference assays were carried out as described (16, 17, 36). Briefly, IT/R29 parasites in RPMI 1640 at 6-10% parasitaemia and a rosette frequency of >60% were stained with 25µg/ml ethidium bromide for 5 minutes with 200µg/ml fucoidan added to disrupt all rosettes (68). Labelled uninfected RBCs were also resuspended at 2% haematocrit in RPMI 1640 with 200µg/ml fucoidan. Equal volumes of parasite culture and labelled cells were mixed in triplicate, and the percentage labelled cells in the resulting mixtures counted (300 cells). After 3 washes, cells were resuspended in RPMI 1640 with 10% AB serum and incubated for 1 hour at 37°C to allow rosettes to reform. For each replicate two counts were made: (1) the percentage labelled cells in the mix (300 cells, using only those cells not in rosettes) and (2) the percentage labelled cells in rosettes (200 cells, only those within rosettes). Graphs show the difference between the percentage labelled cells in rosettes and in the mix (using the mean of the 2 counts for % labelled cells in the mix, before and after washes). The mean and SEM of the triplicate readings are shown for each RBC donor. For statistical analysis of the differences between blood groups, the triplicate values for each donor were averaged and treated as a single data point, such that n=4 for groups O and A, and n=5 for group B. Blood groups were compared using a Kruskal Wallis test with Dunn's multiple comparisons using Prism v7.0 (Graphpad Inc., San Diego, California).

Ethics statement

Informed consent was obtained from the parents or guardians of study participants. All study

protocols were approved by the Kenya Medical Research Institute (KEMRI) National Ethical Review Committee (case control study: SCC1192; cohort study: SCC3149). RBCs and sera from Scottish blood donors were obtained following informed consent, with approval from the Scottish National Blood Transfusion Service Committee for the Governance of Blood and Tissue Samples for Nontherapeutic Use (reference SNBTS 12~35).

Role of the funding source

The funders had no role in study design, collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the paper for publication. DHO, TNW and JAR had full access to the raw data and TNW and JAR had final and full responsibility to submit the manuscript for publication.

Results

ABO genotypes are strongly concordant with ABO phenotypes in Kenyan children

ABO genotype was determined in a case-control study on susceptibility to severe malaria involving >5000 Kenyan children, while ABO blood group phenotypes were assessed on a sub-group of 2761 control children. O was the most common blood group being found in 55.8% of controls, followed by A (23.1%) and B (18.2%), with AB being relatively rare (2.9%) (Table 1). In terms of genotype, *OO* was the most common (54.8% of controls), followed by *AO* (21.0%) and *BO* (17.7%). The *AA* (2.1%), *BB* (1.4%) and *AB* (3.0%) genotypes were rare (Table 1). The overall agreement between genotype and phenotype was 97.1% (Kappa score, 0.95; P <0.0001). The highest agreement was seen for the *OO* genotype with blood group O (agreement, 99.3%), and the lowest was for *BB* genotype with blood group B (92.7%), with the other genotypes showing >93% agreement (Table 2).

The BB and AB genotypes were associated with the highest odds ratios for severe malaria in the case-control study

We next examined the relationship between *ABO* genotype and susceptibility to severe malaria in the case-control study. Associations between ABO blood group and severe malaria for this full dataset, which also included case children who we have subsequently shown to have a low probability of severe malaria, have been reported previously (14). However, our current analysis differs by using a more precise case-definition of severe malaria (46), and by focussing on differences between specific non-*O* genotypes, which were not analysed in the previous study (14). The general characteristics of the cohort are presented in Table 3. There was no significant departure from Hardy Weinberg Equilibrium among controls (χ^2=0.78, p=0.677). A significantly higher proportion of the controls had the *OO* genotype compared to the severe malaria cases (Table 3). *ABO* genotype distributions also differed significantly across gender and ethnicity, for which we adjusted in the association analyses described below. Parasite density in the severe malaria cases did not vary significantly across the different *ABO* genotypes (Table S1).

We tested for associations between *ABO* genotype and severe malaria, including the specific severe malaria syndromes cerebral malaria (CM), severe malarial anaemia (SMA) and respiratory distress (RD), and malaria-specific mortality using a logistic regression model both with, and without, adjustment for the confounders HbS, α^+thalassaemia, gender, and ethnicity. When grouped together, the non-*O* genotypes were associated with an adjusted Odds Ratio (aOR) for all severe malaria of 1.49 (95% CI 1.31-1.70; p<0.001), with similar values for each of the clinical sub-phenotypes individually (Table 4). When considered separately, the *ABO* genotypes that were associated with the highest aOR for severe malaria were *BB* (aOR 2.08; 95% CI 1.29-3.37; p=0.003), and *AB* (aOR 1.93; 95% CI 1.37-2.72; p<0.001), which were also associated with the highest aOR for the specific severe malaria syndromes of CM and RD (*BB)* and SMA (*AB*) (Table 4).

To examine the hypothesis that RBC A/B antigen levels affect malaria susceptibility, we analysed differences in odds ratios between single (*AO, BO*) and double dose (*AA, AB, BB*) non-*O* genotypes using the Wald test. Odds ratios for severe malaria overall for *AA* (aOR=1.46) and *AB* (1.93) were higher than that for *AO* (1.27) and were similarly higher for *BB* (2.08) and *AB* (1.93) compared to *BO* (1.65). However, only the *AB* vs *AO* comparison reached statistical significance (P=0.020, Table 5). Similarly, for the specific severe malaria syndromes, double dose *A* and/or *B* genotypes were associated with higher odds ratios than single dose genotypes in many cases but were not statistically significant (Table S2). Overall, the data show patterns that are consistent with the hypothesis, but do not allow us to reject the null hypothesis.

AA/AB RBCs form larger rosettes than OO RBCs with a blood group A-preferring P. falciparum clone

To the best of our knowledge, whether host RBC *ABO* genotype influences *P. falciparum* rosetting has not been investigated previously. We examined rosette size following parasite invasion into RBCs from 60 donors (*OO*=23, *AO*=18, *AA*=2, *BO*=9, *BB*=1, *AB*=7), using the blood group A-preferring rosetting *P. falciparum* clone IT/R29 (19, 25) (Figure S1). The *AB* and *AA* genotypes were associated with significantly larger rosettes and a higher proportion of large rosettes compared to *OO* (Figure 1, Table 6). In contrast, *AO, BO, and BB* genotype RBCs did not differ from *OO* genotype RBCs in terms of rosette size or the proportion of large rosettes (Figure 1, Table 6). In the case of *BB* and *BO* this was as expected, because IT/R29 is a blood group A-preferring parasite clone (Figure S1).

P. falciparum cytoadhesion and PfEMP1 display do not differ between ABO genotypes

Effects on other parasite adhesion-related properties, such as reduced cytoadhesion to microvascular endothelial cells via receptors such as ICAM-1 and CD36 and reduced display of PfEMP1 on the surface of iRBCs, have been implicated as mechanisms of protection for

various human RBC polymorphisms (40, 69). To determine whether *ABO* genotype might influence malaria susceptibility via these mechanisms, we conducted *in vitro* experiments examining cytoadhesion to ICAM-1 and CD36 and PfEMP1 display in relation to iRBC *ABO* genotype. No genotype-specific differences were seen (Supplementary Figures S2-S3, Supplementary Tables S3-S4).

ABO genotype is not associated with risk of either uncomplicated malaria or asymptomatic infection

Rosetting is primarily a property of parasite isolates causing severe malaria, and no (or very low level) rosetting is seen in *P. falciparum* isolates collected from patients with uncomplicated malaria (27, 70). If *ABO* genotype influences malaria susceptibility via a mechanism related to rosette size and microvascular obstruction, we would predict that *ABO* genotype associations would only be demonstrated in severe disease, and not in mild or asymptomatic infections. We tested this hypothesis using a longitudinal cohort study and cross-sectional surveys carried out in the same geographic area as the case-control study. No significant associations were seen between *ABO* genotype and either uncomplicated malaria or asymptomatic *P. falciparum* infection (Supplementary tables S5-S7).

Discussion

Although associations between ABO blood group, *P. falciparum* rosetting and susceptibility to severe malaria are well-established (9-11, 16, 20, 27, 71-73), to the best of our knowledge, higher resolution analyses to determine their associations with distinct *ABO* genotypes have not been conducted. Our results show a significant effect of *ABO* genotype on rosette size, with *AA/AB* genotype RBCs forming significantly larger rosettes than *OO* genotype RBCs with the A-preferring *P. falciparum* line IT/R29. In contrast, *AO* genotype RBCs did not differ from *OO* genotype RBCs in the rosetting assays. Previous work has shown that rosettes in non-O blood are not only larger but are also more stable and more difficult to disrupt with reagents such as heparin, compared to rosettes in group O RBCs, (16, 18, 19, 25, 28). Rosette

size and stability are of great potential importance in malaria pathology, because experimental studies show that larger rosettes in A compared to O RBCs are more resistant to disruption under shear stress and therefore cause greater obstruction to capillary-sized channels (26). Hence, a direct causal link between host *ABO* genotype, parasite virulence in terms of rosette size, and the primary pathological process of microvascular obstruction in severe malaria is suggested.

Our finding that the *ABO* gene dose affects rosetting supports our prediction that *AA/BB/AB* individuals will be at higher risk of severe malaria than *AO/BO* individuals. In the case-control study we found that for most comparisons, *AA/BB/AB* individuals had higher odds ratios for severe malaria than *AO* and *BO* individuals, but we could not reject the null hypothesis on statistical grounds. Despite recruiting ~1400 severe malaria cases into our study, the frequency of the key *AA*, *BB* and *AB* genotypes in the dataset was low (37, 34 and 61 severe cases respectively) which limited the power of our study. Larger studies will be needed to examine the relationship between *ABO* genotype and severe malaria in more detail and to determine whether *AA*, *BB* and *AB* genotypes are consistently associated with higher ORs for severe disease than *AO/BO* genotypes.

Our *in vitro* rosetting results support previous suggestions that rosetting is a causal factor in the protective association between ABO blood group and severe malaria (7, 16, 24, 28). However, alternative protective mechanisms have been suggested for other polymorphisms (40, 74). We examined two of these mechanisms here, iRBC binding to endothelial receptors (ICAM-1 and CD36) and PfEMP1 expression levels on the surface of iRBCs. We found no evidence for an association between *ABO* genotype and either endothelial receptor binding or PfEMP1 display. Other protective mechanisms for ABO have also been suggested, including effects on RBC invasion (75, 76) and phagocytic clearance of iRBCs (25, 77, 78). However, both invasion- and clearance-related mechanisms would be expected to have an impact by lowering parasite burden, yet no consistent effect of ABO blood group on parasite density has

436 been seen across multiple studies (reviewed by (73)), and we found no significant association
437 in our case-control study. Taken together, existing data support a role for reduced *P.*
438 *falciparum* rosetting in O RBCs and consequent reduced microvascular obstruction, as the
439 key mechanism by which ABO blood group influences the risk of severe malaria.
440
441 An additional aim of our study was to investigate the correlation between *ABO* genotype and
442 blood group phenotype in an African population. We report strong agreement between *ABO*
443 genotype and phenotype, with only 79/2686 samples (2.9%) being discordant. The strongest
444 concordance was for genotype *OO* with blood group O (99.3%), and the weakest for genotype
445 *BB* with blood group B (92.3%). Two American studies using the same SNPs reported a
446 genotype-phenotype concordance of 100% and 92% respectively, although the perfect
447 concordance seen by Risch and colleagues could potentially have been a reflection of their
448 very small sample size (n=30) (32, 33). Some discrepancies in our study, for example, the 42
449 samples genotyped as *AO*, *AA*, *BO* and *BB* but serologically typed as blood group O, could be
450 due to weak A and B subgroups that reduce A and B antigen density on the RBC surface (79-
451 81), which might have resulted in samples being typed as O in standard agglutination assays
452 (82-85). Another possible source of error might have resulted from the assumption made in
453 inferring *ABO* genotype in heterozygotes. 62/79 (78%) of discrepant samples were
454 heterozygous for the *O* deletional allele ("GD" in Table 1). The double heterozygote
455 combination of GD at rs8176719 and AC at rs8176746 can result in either *BO* genotype (GA
456 and DC haplotype) or *AO* genotype from the rare GC and DA haplotype. The *O* deletion is
457 thought to have arisen on the background of an *A* allele (2), so all individuals were assigned
458 as *BO* genotype. However, *O* deletions arising on a *B* allele background have been described
459 (86), and could account for some of the discrepancies we observed. Finally, it is also possible
460 that some samples were erroneously typed by serology. We were unable to access fresh RBC
461 samples from the same individuals to repeat the typing.
462

In conclusion, our combined epidemiological and laboratory studies support the hypothesis that *AO/BO* heterozygotes differ from *AA/AB/BB* individuals in relation to *P. falciparum* rosetting and severe malaria risk. Alternative mechanisms of protection for blood group O and the *OO* genotype, such as binding to endothelial receptors ICAM-1 and CD36 and effects on PfEMP1 display were not supported by the data. Additional studies examining the effects of *ABO* genotype, as well as weak A and B blood groups, may give further insights into the complex host-parasite interactions in severe malaria.

Data Sharing

De-identified participant data (TNW) and *in vitro* datasets (JAR) used during the current study are available from the corresponding authors on reasonable request.

Contributors

DHO, CMN, TNW and JAR designed the research; DHO, CMN, SU, AWM, CF, GN, JO, MS, KOA, NM, NP, BT, GB, KM, SK, DPK, and KR performed the research; DHO, CMN, TNW and JAR analysed the data; and DHO, TNW and JAR wrote the paper.

Declaration of interests

The authors declare no conflict of interest.

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504

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766

767 ***Figure legends***

768 **Figure 1.** *P. falciparum* IT/R29 rosette size and frequency of large rosettes by *ABO* genotype.
769 **(A)** IT/R29 mean rosette size (number of uninfected RBCs per rosette) **(B)** IT/R29 frequency
770 of large rosettes (more than 4 uninfected RBCs per rosette). Purified IT/R29 infected RBCs
771 (iRBCs) were allowed to invade into RBCs from 60 donors (*OO* n=23, *AO* n=18, *AA* n=2, *BO*
772 n=9, *BB* n=1, *AB* n=7) and rosette size and frequency of large rosettes were assessed the next
773 day by fluorescence microscopy. Samples were tested over two consecutive experimental
774 days (day 1=30 and day 2=30) in duplicates. Horizontal bars represent the median rosette size
775 and median frequency of large rosettes for each genotype. The number of donors per
776 genotype are shown in parenthesis. Sample genotype was masked during counting to avoid
777 observer bias. Statistically significant P values from multivariate regression analysis of the
778 data with adjustment for confounders are shown (see Table 6 for all values).
779
780

781 **Figure 1**

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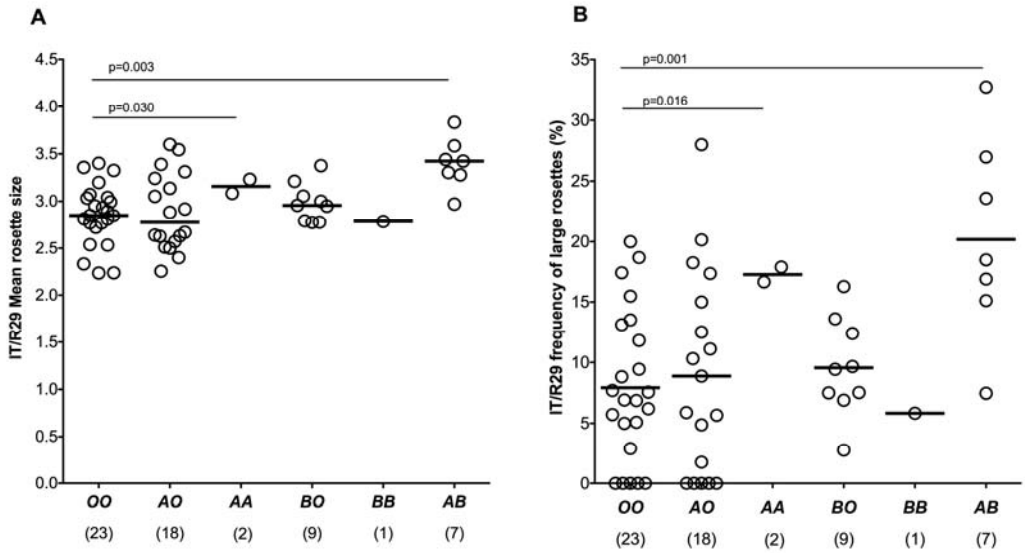

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Tables

Table 1. *ABO* genotype and phenotype frequencies in the case-control study

rs8176719 X rs8176746 haplotype[§]	Inferred *ABO* genotype	Cases N (%)	Controls N (%)	ABO serological phenotype#	Controls N (%)
DD X CC	*OO*	605 (43.3)	2030 (52.5)	O	1540 (55.8)
DD X AA	*OO*	3 (0.2)	1 (0.0)		
DD X AC	*OO*	14 (1.0)	87 (2.3)		
GD X CC	*AO*	306 (21.9)	810 (21.0)	A	637 (23.1)
GG X CC	*AA*	37 (2.7)	83 (2.1)		
GD X AA	*BO*	13 (0.9)	20 (0.5)	B	503 (18.2)
GD*X AC	*BO*	324 (23.2)	663 (17.2)		
GG X AA	*BB*	34 (2.4)	55 (1.4)		
GG X AC	*AB*	61 (4.4)	115 (3.0)	AB	81 (2.9)

[§]*ABO* blood group genotypes were determined using two SNPs at the *ABO* locus; rs8176719 in exon 6 that encodes the 261G deletion giving rise to blood group O with the rs8176719 alleles represented as 261G (G) and 261delG (D); rs8176746 in exon 7 which encodes C796A and distinguishes *A* and *B* alleles. Genotyping at both SNPs was successful in 99.6% 1397/1403) severe malaria cases and 97.8% (3864/3949) community controls. 1 severe malaria case and 8 controls had missing genotype data for the rs8176746 SNP but were homozygous for the 261G deletion in exon 6 and were therefore denoted as OO genotype

* The common O deletion (D) arose on the background of the A allele (C). Therefore, double heterozygotes GD X AC are assumed to have the haplotype GA and DC, giving rise to the *BO* genotype, rather than haplotype GC and DA that gives rise to the *AO* genotype. This assumption is supported by the data shown here, with only 5/6086 children being homozygous for the D deletion on the background of the B allele (AA).

#ABO blood group serological phenotype was determined using standard haemagglutination tests on a sub-group of 2761 (70%) of the community controls. ABO phenotyping was not included as part of the original study design and was only added after the study had begun so some controls were not phenotyped.

Table 2. ABO genotype-phenotype agreement[§]

	ABO genotype					
ABO phenotype	***OO*** **N (%)**	***AO*** **N (%)**	***AA*** **N (%)**	***BO*** **N (%)**	***BB*** **N (%)**	***AB*** **N (%)**
O	1453 (99.3)	25 (4.3)	1 (2.0)	13 (2.8)	3 (7.3)	0 (0.0)
A	4 (0.3)	549 (94.6)	54 (98.0)	17 (3.6)	0 (0.0)	1 (1.4)
B	5 (0.3)	5 (0.9)	0 (0.0)	442 (93.4)	38 (92.7)	2 (2.7)
AB	1 (0.1)	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)	71 (95.9)

[§]Agreement between ABO blood group phenotype and inferred *ABO* genotype was tested using Cohen's Kappa statistic on the 2686 community control samples that were successfully typed by both methods. A score of 100% corresponds to complete agreement while 0% corresponds to no agreement.

Table 3. The general characteristics of children recruited to the case-control study by *ABO* genotype

***ABO* genotype**		***OO (%)***	***AO (%)***	***AA (%)***	***BO (%)***	***BB (%)***	***AB (%)***	***P-value***
Disease category	Controls n=3872	2126 (54.9)	810 (20.9)	83 (2.1)	683 (17.6)	55 (1.4)	115 (3.0)	Reference
	All SM n=1398	623 (44.6)	306 (21.9)	37 (2.6)	337 (24.1)	34 (2.4)	61 (4.4)	<0.001
	CM n=716	326 (45.5)	166 (23.2)	22 (3.1)	160 (22.3)	16 (2.2)	26 (3.6)	0.001
	SMA n=415	181 (43.6)	97 (23.4)	9 (2.2)	99 (23.9)	10 (2.4)	19 (4.6)	<0.001
	RD n=417	181 (43.4)	101 (24.2)	13 (3.1)	99 (23.7)	11 (2.6)	12 (2.9)	<0.001
	Died n=116	52 (44.8)	33 (28.4)	2 (1.7)	23 (19.8)	2 (1.7)	4 (3.4)	0.339
Gender	Males	1407 (52.7)	549 (20.6)	47 (1.8)	514 (19.3)	45 (1.7)	106 (4.0)	
	Females	1342 (51.6)	567 (21.8)	73 (2.8)	506 (19.4)	44 (1.7)	70 (2.7)	0.015
Ethnic group	Giriama	1336 (50.7)	555 (21.1)	62 (2.4)	545 (20.7)	47 (1.8)	90 (3.4)	
	Chonyi	940 (55.4)	364 (21.4)	37 (2.2)	289 (17.0)	23 (1.4)	44 (2.6)	
	Kauma	268 (49.8)	113 (21.0)	10 (1.9)	114 (21.2)	10 (1.9)	23 (4.3)	
	Others	205 (51.2)	84 (21.0)	11 (2.8)	72 (18.0)	9 (2.2)	19 (4.8)	0.077
Sickle genotype	AA	2394 (51.7)	996 (21.5)	104 (2.2)	907 (19.6)	76 (1.6)	150 (3.2)	
	AS	334 (55.0)	111 (18.3)	16 (2.6)	107 (17.6)	13 (2.1)	26 (4.3)	
	SS	19 (57.6)	8 (24.2)	0 (0.0)	6 (18.2)	0 (0.0)	6 (0.0)	0.396
α+thalassaemia genotype	αα/αα	972 (52.3)	383 (20.6)	50 (2.7)	357 (19.2)	33 (1.8)	64 (3.4)	
	-α/αα	1330 (52.1)	546 (21.4)	52 (2.0)	498 (19.5)	35 (1.4)	92 (3.6)	
	-α/-α	418 (53.0)	170 (21.5)	16 (2.0)	149 (18.9)	17 (2.2)	19 (2.4)	0.619
Age in months Median (IQR)		7 (5-11)	8 (5-12)	9 (6-20)	8 (6-16)	8 (5-20)	9 (6-19)	<0.001

The Pearson's Chi square test (or Fisher's exact test when numbers in any category <10) was used to test for differences in the distribution of ABO genotypes across categorical variables of gender, ethnic group and HbS and α^{+}thalassaemia genotype while the Kruskal-Wallis test was used to test for differences in age (as a continuous variable) by ABO genotype. IQR, interquartile range. For severe malaria, including specific clinical sub-types (CM, cerebral malaria; SMA, severe malarial anaemia; RD, respiratory distress & mortality), comparisons were made to community controls used as the reference group.

Table 4. Case-control analysis of the association between *ABO* genotype and severe malaria syndromes

			Crude				Adjusted†			
Case Phenotype	**No. Cases/controls**	***ABO* genotype**	**OR**	**LCI**	**UCI**	**P**	**OR**	**LCI**	**UCI**	***P-value***
All SM	623/2126	*OO*	1				1			
	306/810	*AO*	1.29	1.10	1.51	0.002	1.27	1.07	1.50	0.006
	37/83	*AA*	1.52	1.02	2.26	0.039	1.46	0.95	2.22	0.082
	61/115	*AB*	1.81	1.31	2.50	< 0.001	1.93	1.37	2.72	<0.001
	337/683	*BO*	1.68	1.44	1.97	< 0.001	1.65	1.40	1.95	<0.001
	34/55	*BB*	2.11	1.36	3.27	0.001	2.08	1.29	3.37	0.003
	775/1746	Non-*O*	1.52	1.34	1.71	< 0.001	1.49	1.31	1.70	<0.001
All CM	326/2126	*OO*	1				1			
	166/810	*AO*	1.34	1.09	1.64	0.005	1.29	1.05	1.60	0.018
	22/83	*AA*	1.73	1.07	2.81	0.027	1.60	0.96	2.67	0.070
	26/115	*AB*	1.47	0.95	2.29	0.085	1.47	0.93	2.34	0.101
	160/683	*BO*	1.53	1.24	1.88	< 0.001	1.45	1.17	1.80	0.001
	16/55	*BB*	1.90	1.07	3.35	0.027	1.90	1.03	3.49	0.039
	390/1746	Non-*O*	1.46	1.24	1.71	< 0.001	1.40	1.18	1.65	<0.001
All SMA	181/2126	*OO*	1				1			
	97/810	*AO*	1.41	1.09	1.82	0.010	1.35	1.03	1.78	0.031
	9/83	*AA*	1.27	0.63	2.58	0.773	1.18	0.56	2.52	0.662
	19/115	*AB*	1.94	1.17	3.32	0.011	2.05	1.21	3.45	0.007
	99/683	*BO*	1.70	1.31	2.21	< 0.001	1.62	1.23	2.12	0.001
	10/55	*BB*	2.14	1.07	4.26	0.031	1.71	0.75	3.89	0.204
	234/1746	Non-*O*	1.57	1.28	1.93	< 0.001	1.50	1.21	1.86	<0.001
*All RD**	181/2126	*OO*	1				1			
	101/810	*AO*	1.47	1.13	1.89	0.004	1.41	1.08	1.83	0.012
	13/83	*AA*	1.84	1.01	3.37	0.048	1.68	0.89	3.17	0.113
	12/115	*AB*	1.23	0.66	2.26	0.516	1.25	0.67	2.33	0.489
	99/683	*BO*	1.70	1.31	2.21	< 0.001	1.63	1.25	2.13	<0.001
	11/55	*BB*	2.35	1.21	4.57	0.012	2.01	0.96	4.21	0.064
	236/1746	Non-*O*	1.59	1.30	1.95	< 0.001	1.51	1.23	1.87	<0.001
*Mortality**	52/2126	*OO*	1				1			
	33/810	*AO*	1.67	1.07	2.60	0.024	1.74	1.11	2.73	0.017
	2/83	*AA*	0.99	0.24	4.11	0.984	1.00	0.24	4.21	0.999
	4/115	*AB*	1.42	0.51	4.00	0.505	1.50	0.53	4.26	0.450
	23/683	*BO*	1.38	0.84	2.27	0.209	1.35	0.81	2.25	0.258
	2/55	*BB*	1.49	0.35	6.26	0.589	1.66	0.39	7.08	0.497
	64/1746	Non-*O*	1.50	1.03	2.17	0.033	1.53	1.04	2.24	0.029

SM: Severe malaria; CM: cerebral malaria; SMA: severe malaria anaemia; RD: respiratory distress; OR: Odds Ratio; LCI: Lower Confidence Interval (95%); UCI: Upper Confidence Interval; P: P-value using a logistic regression model. † adjusted for HbS, α+thalassaemia, gender, ethnicity and interaction (HbS and α+thalassaemia). * no interaction term included between HbS and α+thalassaemia genotype.

38 **Table 5.** A comparison of the odds ratio differences for severe malaria between single dose and
39 double dose *non-O* genotypes using the Wald test

Case Phenotype	*ABO* genotype	No. Cases/controls	Odds Ratio comparisons	Wald test P value
All SM	*AO vs AA*	306/810; 37/83	1.27/1.46	0.528
	BO vs BB	337/683; 34/55	1.65/2.08	0.351
	AO vs AB	306/810; 61/115	1.27/1.93	0.020
	BO vs AB	337/683; 61/115	1.65/1.93	0.384

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Table 6. Rosetting of *P. falciparum* clone IT/R29 by *ABO* genotype

IT/R29 rosette size				
N	**Genotype**	**Mean rosette size**	**95% CI**	**P value**
23	*OO*	2.89	2.77 – 3.01	-
18	*AO*	2.92	2.78 – 3.05	0.737
2	*AA*	3.36	2.95 – 3.78	0.030
9	*BO*	2.92	2.73 – 3.11	0.773
1	*BB*	2.52	1.93 – 3.11	0.229
7	*AB*	3.28	3.06 – 3.50	0.003
37	*Non-O*	3.00	2.92 – 3.11	0.157
Frequency of IT/R29 large rosettes				
N	**Genotype**	**% of large rosettes (>4 uninfected RBCs/rosette)**	**95% CI**	**P value**
23	*OO*	8.49	5.75 – 1.22	-
18	*AO*	9.36	6.27 – 12.22	0.674
2	*AA*	20.55	11.20 – 29.91	0.016
9	*BO*	8.45	4.09 – 12.81	0.988
1	*BB*	1.29	-12.01 – 14.59	0.296
7	*AB*	18.23	13.19 – 23.28	0.001
37	*Non-O*	11.27	8.89-13.66	0.143

Differences in IT/R29 rosette size or frequency of large rosettes by *ABO* genotype were tested using multivariate regression analysis with adjustment for confounding by HbAS. 60 RBC donor samples were tested once in duplicate over two successive experimental days (day 1 n = 30 and day 2 n = 30), therefore, experimental day was included as a co-variate to account for day-to-day variation.