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ORIGINAL ARTICLE

Plasmodium malariae and *Plasmodium ovale* infections and their association with common red blood cell polymorphisms in a highly endemic area of Uganda

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Background: *Plasmodium ovale* and *Plasmodium malariae* infections are scarcely studied in sub-Saharan Africa, where the *Plasmodium falciparum* species predominates. The objective of this study is to investigate the prevalence of *P. ovale* and *P. malariae* infections and their relationship with common red blood cell polymorphisms in a cohort of 509 individuals from Uganda.

Methods: Three cross-sectional surveys were conducted in individuals of 1–10 and >20 y of age from the Apac district at baseline and 6 and 16 weeks after drug treatment. Malaria infections were assessed by polymerase chain reaction and genotyping was performed for the sickle cell allele, α-thalassaemia and glucose-6-phosphate dehydrogenase.

Results: At baseline, the prevalence of infection was 7.5%, 12.6% and 57.4% for *P. ovale*, *P. malariae* and *P. falciparum* species, respectively. Co-infections were present in 14.1% of individuals, all including *P. falciparum* parasites. In children 1–5 y of age, the prevalence of *P. ovale* mono-infections increased significantly from 1.7% to 7.3% over time (p=0.004) while the prevalence of *P. malariae* and *P. falciparum* infections declined significantly during this study. After adjusting for confounding and multiple testing, only α-thalassaemia had a statistically significant increase in the odds of *P. falciparum* infections (odds ratio 1.93 [95% confidence interval 1.26 to 2.94]).

Conclusions: Common red blood cell polymorphisms do not show strong effects on mild *Plasmodium* infections in this Ugandan population. To understand the extent of this result, similar studies should be carried out in other populations using larger cohorts.

Keywords: ACT, co-infections, genetic association, non-falciparum

Introduction

In the last decade, the burden of *Plasmodium falciparum* has decreased in Africa.[1,2] Less is known about the burden of the sympatric and often-neglected malaria species *Plasmodium ovale* and *Plasmodium malariae*. Several studies have reported low prevalence and incidence rates of these species in Africa.[3–8] However, their prevalence may have been underestimated in studies relying on rapid diagnostic tests (RDTs) detecting the histidine-rich protein 2, which is not expressed by *P. malariae* and *P. ovale* parasites.[9] Additionally, infections with *P. malariae* and *P. ovale* are typically asymptomatic and with low parasitaemia.[10,11] The quiescent hypnozoite stage also diminishes the detectability of *P. ovale* infections.[10] In line with a likely

underestimation of these non-falciparum infections, a recent serological study showed a high seroprevalence associated with antigens of these malaria species in Benin.[12] Clearly, more epidemiological investigations are needed to quantify the real contribution of these neglected species to the total burden of malaria in Africa. Molecular methods can improve the detectability of malaria infections and allow the distinction between different malaria species.[13]

Until now only a few studies have attempted to assess the variation of *P. falciparum*, *P. ovale* and *P. malariae* infections over time.[3,4] A landmark study is the 20-y follow-up of the Dielmo community from Senegal.[14] In this community, the prevalence of each malaria species remained high until 2004, when it began to decrease until reaching very low levels in 2010.[4,15] This decrease was attributed to changes in antimalarial drug policies in 2010 when treatment policy shifted to an artemisinin-based combination therapy (ACT). In Mozambique, it was reported that the prevalence of *P. malariae* and *P. ovale* infections decreased substantially during the wet season with a concomitant increase of *P. falciparum* mono-infections.[16] This observation suggests a seasonal change in the composition of the *Plasmodium* pool circulating in the population.[16–18]

Genetic factors might play an important role in the acquisition and clearance of malaria infections and the likelihood of these progressing to clinical disease. In sub-Saharan African, common red blood cell (RBC) polymorphisms, including the sickle cell allele and thalassaemia, have been associated with protection against severe forms of *P. falciparum* malaria.[19–21] Although less convincing, there is also evidence that these genetic variants have an impact on *P. falciparum* asymptomatic malaria and parasite density.[22–24] Little is known about whether these associations can be extended to *P. ovale* and *P. malariae* infections. Since morbidity and mortality are generally low in non-falciparum infections, it is expected that the respective selective pressure is less strong than that derived from *P. falciparum* alone. On the other hand, the likelihood of generating mixed infections in the same individual might trigger pleiotropic effects of existing genetic variants on the control of these infections.

To fill in some of the research gaps concerning *P. falciparum*, *P. ovale* and *P. malariae* infections, this study aims to assess the prevalence of these malaria species and their dynamics after antimalarial treatment in a cohort living in a high-transmission area of Uganda and their association with common RBC polymorphisms.

Methods

Study design

The study was conducted between July and November 2010 in Abedi parish in the Apac district, a highly endemic area of Uganda with perennial transmission.[25] Briefly, the study participants were recruited using predefined strata (1–5 y, n=249; 6–10 y, n=126; >20 y, n=134). The recruitment was based on a random selection of all households that could be mapped within a 5-km radius of the Abedi Health Centre. Households with at least one child from the lowest age stratum and at least one individual from one of the other age strata were considered eligible for participation. The exclusion criteria were a weight-for-height or height-for-age Z-score <−3, severe anaemia (haemoglobin <5.0 g/dL) or the presence of any chronic disease. Excluded individuals were referred to the Apac District Hospital for appropriate clinical management. Additional information about the sampling can be found in the original reference to this study.[25]

Blood samples were taken from the participants by finger-prick at baseline (July 2010) and after 6 and 16 weeks (August and November 2010, respectively), coinciding with the peak time of malaria transmission. Samples were collected into ethylenediaminetetraacetic acid–coated microtainers and blotted onto filter papers (3mm; Whatman, Maidstone, UK). Passive case detection was implemented, with dedicated study staff at the health facility being available 24 h per day throughout the week. At baseline, all study participants received ACT treatment with artemether–lumefantrine due to previously published evidence of submicroscopic infections in the population.[26] Indoor residual spraying (IRS) in the population was conducted a month after the second survey as part of the national malaria control program. The precise dates of spraying were neither monitored nor recorded by the research team from this study.

Molecular analysis and parasite detection

Malaria infection was detected using molecular assays. DNA was first extracted from filter papers using the QIAamp mini kit (QIAGEN, Venlo, The Netherlands). *Plasmodium* species detection was then done by nested polymerase chain reaction (PCR) amplification of the small subunit (18S) ribosomal RNA gene.[13] Parasite density was estimated in *P. falciparum* infections only using microscopy.

Sample genotyping

Human DNA was first extracted from blood samples and then used for genotyping of the sickle haemoglobin gene (*HbS*) using methods described in detail elsewhere.[27] Glucose-6-phosphate dehydrogenase (G6PD) deficiency status was determined by genotyping the single-nucleotide polymorphism resulting in G202A in the *G6PD* gene.[28] The status of α-thalassemia was determined in each participant by assessing the African α-globin deletion $\alpha^{3.7}$.[29]

Statistical analysis

To investigate the association between *P. falciparum*, *P. ovale* and *P. malariae* infections, three-way contingency tables were first constructed for each age group. As a null hypothesis, mutual independence (i.e. the infection status of each malaria species is independent of the remaining ones) was tested in these tables using an appropriate log-linear model. The respective model estimation was performed using the maximum likelihood (ML) method. Likelihood ratios and Pearson's tests were used to assess the goodness-of-fit of the estimated models. In these tests, p-values >0.05 provided evidence for the hypothesis of mutual independence. The Cochran–Armitage test for trend was also used to explore the dynamics of prevalence over time.

For each malaria species, the ratios between probabilities of infection status referring to different time points were estimated using an appropriate log-linear model for categorical variables.

The study of the genetic association was performed using baseline data only, because both treatment at baseline and indoor spraying that occurred afterwards could mask the putative genetic associations. Since there were missing genotypes due to either sample exhaustion or amplification failures, a statistical analysis was performed on these data to understand their potential source of bias. An association between the missing status of each genetic polymorphism and different possible covariates (e.g. infection status of each malaria species or age strata) was performed using a Pearson's χ^2 test for independence in two-way contingency tables where p-values >0.05 were indicative of no association between a given covariate and the missing genotype status under analysis. In addition, a missing completely at random (MCAR) model[30] was fitted to the three-way contingency table referring to the cross-tabulation of the three genetic polymorphisms, including the missing genotype frequencies (Supplementary Table 1). The estimation step was done via the ML method, while the respective goodness-of-fit was assessed by a likelihood ratio test where a p-value >0.05 suggested evidence for the MCAR.

For the data referring to fever and infection status for each malaria species, genetic associations were initially assessed with a univariate analysis using the Freeman–Halton exact test for independence in two-way contingency tables, where p-values <0.05 were indicative of a possible association (unadjusted analysis). This exact test was used due to the unbalanced nature of the frequency tables under analysis, where use of the traditional χ^2 test is not recommended. The unadjusted analysis was then extended to adjust for age and gender and to include different single-locus genetic effects. In particular, a (non-genetic) logistic regression model including gender and age strata was fitted to data of infection status for each *Plasmodium* species. Genetic logistic models including the above covariates and the genetic effect under testing were also fitted to the same data. The genetic effects under testing were defined in terms of the alternative allele and describing the following modes of inheritance: general—each genotype has its own effect; additive—the effect of the allele is additive; dominant—the effect of the homozygotic and heterozygotic genotypes are the same; heterosis—the effect of both homozygotic genotypes is the same.[27,31] In this analysis, evidence for a genetic association was assessed using a likelihood ratio test where the non-genetic model was compared with each genetic model (adjusted analysis).

Similar association analysis was conducted for the parasite density (on a logarithmic scale) in data from individuals with evidence of a *P. falciparum* infection. Since this quantitative trait followed an approximate Gaussian distribution, the unadjusted analysis was performed using analysis of variance tests. The adjusted analysis was performed as described above but using linear regression models.

Since there were three RBC polymorphisms for each phenotype, a Bonferroni correction was used for controlling for multiple testing, which ensured a global 5% significance level for the association analysis. Note that for the association analysis of a given trait and genetic variant, testing five different modes of inheritance is equivalent to selecting the 'best' model for the corresponding data and, as such, it was not accounted for when correcting for multiple testing.

Data were analysed in using Stata version 13 (StataCorp, College Station, TX, USA) and R software version 3.3.2 (http://www.r-project.org). In particular, the package ACD/CatData for the R software (http://CRAN.R-project.org/package=ACD) was used to analyse all categorical variables, including their missing data patterns. The analysis scripts are available from the corresponding author upon request.

Results

Baseline characteristics of the study participants

A total of 509 individuals were recruited in the following three age strata: 1–5 y (n=249), 6–10 y (n=126) and >20 y (n=134) (Table 1); 52.5% of the cohort participants were female and 65.2% owned a bed net. As expected, RBC polymorphisms were common in this survey: 14.6% (67/460) were carriers of the G6PD-defficiency allele, 20.2% (89/441) were carriers of the HbS allele and 45.7% (221/483) were α-thalassemia carriers (Table 1). Twenty-four individuals were both α-thalassemia and sickle cell carriers, 17 had both a homo-/heterozygous α-thalassemia allele and heterozygous G6PD-deficient allele and 7 had both a homo-/heterozygous α-thalassemia trait and homozygous G6PD-deficient allele. The co-existence of a homo-/heterozygous G6PD-deficient allele with a homo-/heterozygous sickle cell allele was rare in the participants. Three individuals of 6, 40 and 46 y of age were carriers of all three haemoglobinopathies. From the initial sample size of 509, there were only 391 individuals with complete genotype data, while the remaining individuals had at least one missing genotype (Supplementary Table 1). Five individuals had no data available on the three genetic polymorphisms under analysis.

To assess any potential source of bias due to missing data, a statistical analysis was performed where the association between different covariates and the missing genotype status was tested (Supplementary Figure 1). There was strong evidence for no association between the missing genotype status and most of the covariates, including infection status for each malaria species. Gender and age group seemed borderline associated with the missing data status of HbS and α-thalassaemia genes. Finally, when analysing Supplementary Table 1, the expected frequencies under an MCAR mechanism were in good agreement with the observed data (likelihood ratio test, p=0.93 with 23 degrees of freedom; Supplementary Table 1). Therefore there was strong evidence that the missing genotypes were generated completely at random. According to this mechanism, the corresponding adjusted percentages of HbS, α-thalassaemia and G6PD carriers were estimated at 20.2%, 37.2% and 18.6%, respectively.

Analysis of the prevalence of infections over time

Overall, the prevalence of any *Plasmodium* infection detected by PCR was 58.9% at baseline (Table 1). The percentages of *P. falciparum*, *P. malariae* and *P. ovale* infections were 57.4% (95% confidence interval [CI] 53.0 to 61.7), 12.7% (95% CI 9.6 to 15.8) and 7.5% (95% CI 5.2 to 9.8), respectively. Non-falciparum mono-infections (*P. malariae* or *P. ovale*) and mixed infections

Table 1. Baseline characteristics of study participants and the respective prevalence of *Plasmodium* infection by age group

Characteristics	1–5 y of age	6–10 y of age	≥20 y of age	Overall
Female	125/249 (50.2)	55/126 (43.7)	87/134 (64.9)	267/509 (52.5)
Low haemoglobin count[a]	97/249 (39.0)	19/126 (15.1)	9/134 (6.7)	125/509 (24.6)
Fever[b]	25/247 (10.1)	7/125 (5.6)	10/134 (7.5)	42/506 (6.1)
P. falciparum parasite density, median (minimum–maximum)[c]	1200 (80–147 200)	480 (40–19 440)	200.0 (40–760)	760 (40–147 200)
Bed net ownership	183/248 (73.8)	57/123 (46.3)	89/134 (66.4)	329/505 (65.2)
G6PD status				
Wild-type	198/231(85.7)	96/113 (85.0)	99/116 (85.3)	393/460 (85.4)
Heterozygous	22/231 (9.5)	8/113 (7.1)	13/116 (11.2)	43/460 (9.4)
Homo-/heterozygous	11/231 (4.8)	9/113 (8.0)	4/116 (3.5)	24/460 (5.2)
Sickle cell allele status				
Wild-type	186/224 (83.0)	83/110 (75.5)	83/107 (77.6)	352/441 (79.8)
Heterozygous	37/224 (16.5)	27/110 (24.6)	24/107 (22.4)	88/441 (20.0)
Homozygous	1/224 (0.5)	0/110 (0.0)	0/107 (0.0)	1/441 (0.2)
α-Thalassaemia status				
Wild-type	134/242 (55.4)	71/123 (57.7)	67/118 (56.8)	272/483 (56.3)
Heterozygous	90/242 (37.2)	46/123 (37.4)	44/118 (37.3)	180/483 (37.3)
Homozygous	18/242 (7.4)	6/123 (4.9)	7/118 (5.9)	31/483(6.4)
Plasmodium infection				
Overall	141/248 (56.9)	107/126 (84.9)	51/134 (38.1)	299/508 (58.9)
P. falciparum	130/240 (54.2)	103/122 (84.4)	48/128 (37.5)	281/490 (57.4)
P. malariae	30/234 (12.8)	25/102 (24.5)	3/121 (2.5)	58/457 (12.7)
P. ovale	20/248 (8.1)	15/126 (11.9)	3/134 (2.2)	38/508 (7.5)
Mono-infection				
P. falciparum	93/229 (40.6)	55/100 (55.0)	40/115 (21.3)	188/444 (42.3)
P. malariae	5/229 (2.2)	1/100 (1.0)	1/115 (0.9)	7/444 (1.6)
P. ovale	3/229 (1.3)	3/100 (3.0)	2/115 (1.7)	8/444 (1.8)
Co-infection				
P. falciparum+*P. malariae*	18/229 (7.9)	19/100 (19.0)	2/115 (1.7)	39/444 (8.8)
P. falciparum+*P. ovale*	9/229 (3.9)	5/100 (5.0)	0/115 (0.0)	14/444 (3.2)
P. malariae+*P. ovale*	0/229 (0.0)	0/100 (0.0)	0/115 (0.0)	0/444 (0.0)
P. falciparum+*P. ovale*+*P. malariae*	5/229 (2.2)	5/100 (5.0)	0/115 (0.0)	10/444 (2.3)

Values are presented as n/N (%) unless stated otherwise.
[a]Haemoglobin <11 g/dL.
[b]Fever ≥37.5°C.
[c]For *P. falciparum*–infected individuals only.

accounted for 3.3% (95% CI 1.6 to 4.9) and 14.2% (95% CI 11.2 to 17.7) of the total infections; co-infections were estimated at 8.8% (95% CI 6.1 to 11.4) for *P. falciparum/P. malariae*, 3.2% (95% CI 1.5 to 4.8) for *P. falciparum/P. ovale* and 2.3% (95% CI 0.9 to 3.6) *P. falciparum/P. malariae/P. ovale*. All of these mixed-species infections included *P. falciparum*.

To understand the association between different malaria species, a hypothesis of mutual independence was tested on data from each age group. Evidence for this hypothesis was found for the 6–10 y and >20 y age groups (p-values >0.1, likelihood ratio test). Infections of different malaria species appear to be associated with each other in the 1–5 y age group (p<0.001, likelihood ratio test).

After curative treatment, the prevalence of *P. falciparum* infections decreased in every age group (Figure 1A–C). Moreover, the overall probability of being *P. falciparum*–infected decreased 29.1% (95% CI 19.6 to 37.4) and 69.0% (95% CI 61.8 to 74.9) at weeks 6 and 16, respectively, in relation to baseline (Figure 1D; see Supplementary Table 2 for detailed data on the respective infection dynamic profiles). A similar decrease over time was observed for *P. malariae* infections in all age groups. In contrast, the prevalence of *P. ovale* infections increased from baseline to week 6 in both the 1–5 y and 6–10 y age groups (Figure 1A and B). However, statistical significance was only reached in the youngest age group (Figure 1D). In this age group, the probability of being *P. ovale* infected increased 94.4%

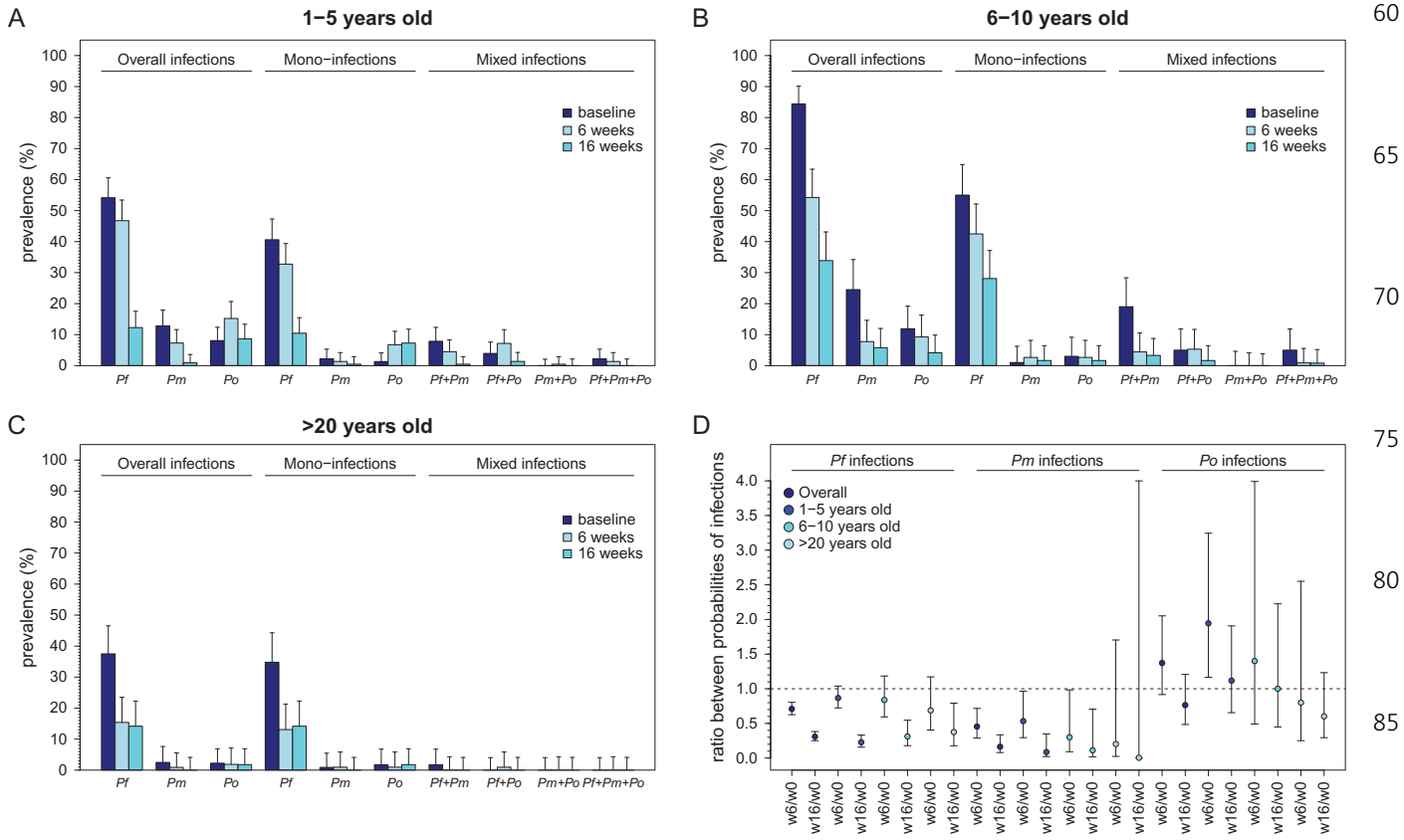

Figure 1. Prevalence of *Plasmodium* infection (overall, single or mixed *P. falciparum*, *P. malariae* and *P. ovale*) over time in different age groups: (A) 1–5 y; (B) 6–10 y and (C) >20 y. (D) Ratios between the estimated probabilities of infections and their 95% CIs, where w6/w0 is the ratio of the probability of infection at week 6 and baseline and w16/w0 is the ratio of the probability of infection at week 16 and baseline.

(95% CI 16.5 to 224.5) in relation to baseline (with a 8.1% prevalence of infections).

Association between haemoglobinopathies and *Plasmodium* infections

Simple univariate analyses detected two putative associations of α-thalassaemia and the *G6PD* gene with *P. falciparum* infections at baseline (Pearson's χ^2 test, p-value >0.01–<0.05; Figure 2A). When these associations were adjusted for gender and age and tested using different genetic effects, only the association referring to α-thalassaemia remained significant according to the Bonferroni correction (Figure 2B and Supplementary Figure 2). The best genetic model—heterosis effect—predicted an increased risk of *P. falciparum* infections in heterozygotic carriers of the α-thalassaemia allele (adjusted odds ratio [OR] 1.93 [95% CI 1.26 to 2.94], p=0.002; Table 2).

The adjusted analysis also revealed a possible association between the sickle cell allele and *P. ovale* infections (Figure 2B and Supplementary Figure 2). The best model for this association (including a heterosis effect) reached the 5% significance level but did not hold when correcting for multiple testing. This model predicted an increased risk of *P. ovale* infections in heterozygotic carriers of the sickle cell allele (adjusted OR 2.37 [95% CI 1.10 to 5.09], p=0.027; Table 2).

Since the three genetic polymorphisms usually have a protective effect against complicated or severe forms of malaria, the above association study was further extended to the analysis of fever and *P. falciparum* parasite density. There were only 42 individuals with fever (Table 1), of which 23 were infected with *P. falciparum* and 4 with *P. malariae*. None of the febrile cases were infected with *P. ovale* parasites. Thus the genetic polymorphisms under analysis were not expected to be strongly associated with fever. The respective association analysis showed a borderline effect of the HbS allele on fever, which did not reach statistical significance after adjusting for multiple testing and confounding (Figure 3). No significant associations were obtained for parasite density after conducting similar adjustments (Figure 3). This finding is explained in part by a strong effect of age on parasite density (Table 1), which reduces the power to detect the effect of these genetic polymorphisms.

Discussion

This study investigated the dynamics of *P. falciparum*, *P. malariae* and *P. ovale* infections and the possible role of common RBC

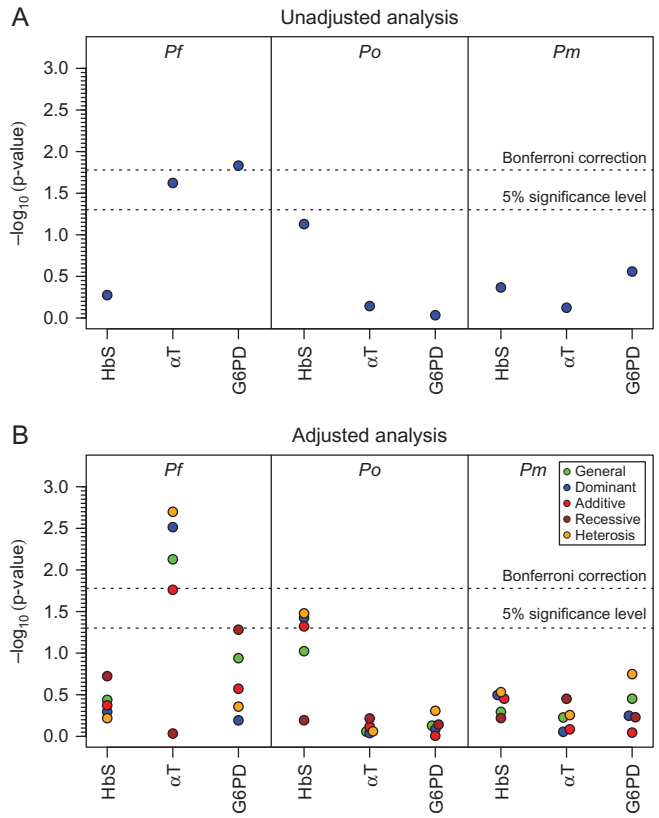

Figure 2. Association analysis between infection status for *Plasmodium* species (phenotype of interest) at baseline and common RBC polymorphisms (HbS: sickle cell gene; αT: α-thalassaemia gene; G6PD: G6PD-deficiency gene). (A) Unadjusted analysis where the association between each polymorphism and each phenotype of interest was assessed using Pearson's χ^2 tests for independence. (B) Adjusted analysis where the association between each polymorphism and each phenotype of interest was assessed using the likelihood ratio tests. These tests refer to the comparison between a 'null' model including gender and age as covariates and different 'genetic models' that include gender, age and the respective genetic effect being tested (general, dominant, additive, recessive and heterosis). The strength of the association was measured by $-\log_{10}$(p-value) (on the *y*-axis), where the lower and upper horizontal dashed lines refer to the 5% significance level and the significance level of each individual test that warrants a 5% global significance for the analysis according to the Bonferroni correction.

Table 2. Adjusted ORs for the association between the α-thalassaemia allele (αT) and *P. falciparum* infection and between the sickle cell allele (HbS) and *P. ovale* infections according to the best models (with heterosis effect) shown in Figure 2B

Association	Predictor	OR (95% CI)	p-Value
αT/*P. falciparum*	Age group (years)		
	1–5	1	–
	6–10	4.60 (2.63 to 8.05)	<0.001
	>20	0.46 (0.28 to 0.74)	<0.001
	Gender (female)	0.72 (0.48 to 1.09)	0.118
	αT (heterozygotic)	1.93 (1.26 to 2.94)	0.002
HbS/*P. ovale*[a]	Age group (years)		
	1–5	1	–
	6–10	1.60 (0.75 to 3.42)	0.229
	>20	0.37 (0.11 to 1.32)	0.125
	Gender (female)	0.45 (0.21 to 0.97)	0.041
	HbS (heterozygotic)	2.37 (1.10 to 5.09)	0.027

[a]Association does not hold after using Bonferroni correction for multiple testing (Figure 2B).

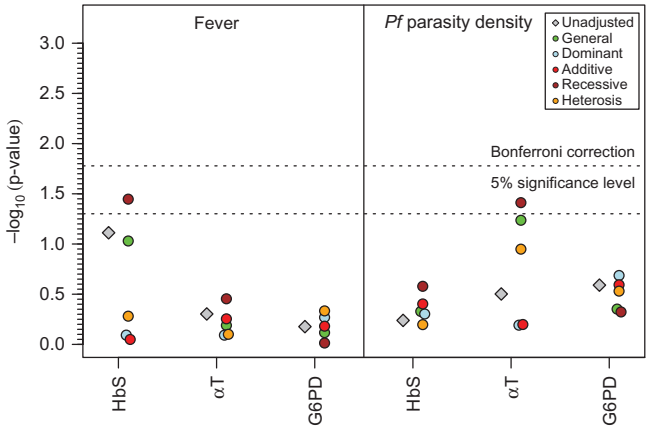

Figure 3. Association analysis of common RBC polymorphisms with fever and *P. falciparum* parasite density. HbS: sickle cell allele; αT: α-thalassaemia gene; G6PD: G6PD-deficiency gene. See Figure 2 for further details.

polymorphisms in susceptibility to these infections in an area of intense malaria transmission in northern Uganda. The results provide evidence for a decline in the prevalence of *P. falciparum* and *P. malariae* infections in all age groups after treatment with ACT, followed by another decrease after community-based IRS. Two comments can be made about these results. The first is that although there was a decline in the prevalence of *P. falciparum* infections, many study participants were infected at week 6. These infections might have resulted from either re-infection or poor response to treatment. Re-infection would appear to be the most reasonable explanation since the population under study is located in an area of intense transmission intensity and sampling coincided with the peak of transmission season. In addition, poor response to treatment has not been reported in the study area.

The second comment concerns the concomitant benefit of ACT in clearing not only *P. falciparum* infections, but also *P. malariae* infections. Similar results were observed in the village of Dielmo, Senegal, where the shift to an ACT-based drug policy led to near elimination of both *P. falciparum* and *P. malariae* in the area.[15] However, a recent study from Uganda reported an increase in the prevalence of *P. malariae* infections in ACT-treated children monitored for malaria and schistosomiasis during an 18-month period in the village of Bukoba, Tanzania.[32] This contrasting finding might be explained by reduced ACT

efficacy due to a specific interaction with schistosomiasis drug treatment given to all participants in this study. Unfortunately, this possible explanation was not explored in the original study.

With respect to *P. ovale* infections, ACT would appear to produce similar qualitative benefits for older age groups. In contrast, the amount of single *P. ovale* infections significantly increased over time in children 1–5 y old. The reason for this increase is unclear but might be related to two alternative hypotheses, as discussed for *P. vivax* infections after ACT in Southeast Asia.[33] The first hypothesis suggests that a high probability of co-infections might lead to suppression of *P. ovale* parasitaemia by concurrent *P. falciparum* parasites, typically in greater densities. The second hypothesis is that *P. falciparum* infections and their subsequent treatment might have activated dormant *P. ovale* hypnozoites. Unfortunately, there were no available genetic data of the infecting parasites, so the validity of this hypothesis could not be assessed.

There was evidence for an association between the prevalence of infection with different malaria species in children 1–5 y of age. Such an association might be a consequence of non-specific immune responses at these early ages. Consistent with this explanation is the similar antibody levels against different *P. falciparum* blood-stage antigens between infected and non-infected children in a previous analysis of the same cohort.[25] In contrast, infections of different malaria species were found to be mutually independent of each other in the remaining age strata. This mutual independence agrees with an acquisition of species-specific immunity due to continuous exposure to malaria parasites in this highly endemic area of Uganda. This acquired immunity is likely to be translated into an increase in specific antibodies against *P. ovale* and *P. malariae* MSP1 antigens with age, as shown in other populations where these malaria species coexist.[34,35]

The overall prevalence of *P. ovale* infections was relatively stable during the study period even in the presence of ACT curative treatment. The reason for this stability is unclear but might be due to combinations of re-infections, recrudescences or relapses. Again, genetic data of the infecting parasites could have helped in distinguishing the contribution of these different events in the infection profile of each individual. However, a recent theoretical study on *P. vivax*/*P. falciparum* co-infection dynamics showed that even when this type of data is available, there is limited statistical power to classify a given infection as a re-infection, recrudescence or relapse with a high probability.[36]

The prevalence of all *Plasmodium* species was highest among children 6–10 y of age, as reported elsewhere.[14,37] This result is likely related to the development of immunity that limits clinical symptoms but does not reduce the chance of being infected. As expected, adults had the lowest prevalence of malaria infections because of well-established malaria immunity due to continuous parasite exposure.

Both α-thalassaemia and the HbS allele are typically in strong association with severe malaria.[38,39] In this study, the analysis was focused on asymptomatic individuals where infection could be detected using PCR. Therefore there was a prior expectation that the association signals would be reduced when compared with those derived from studies targeting symptomatic individuals. This expectation was confirmed with the detection of a single positive association between α-thalassaemia carriage and PCR-detected *P. falciparum* infection. Similar results were observed in northeast Tanzania, where α-thalassaemia carriers have a reduced risk of being infected.[22] In terms of the mechanism, the protection of α-thalassaemia against infection might result from impaired growth of parasites in thalassaemic RBCs.[40] In contrast, the HbS allele was weakly associated with the prevalence of *P. ovale* infections. This is not a surprising result, because the underlying statistical power is likely to be reduced owing to a low prevalence of non-falciparum infections. For *P. falciparum*, the joint analysis of multiple survey points could have allowed detection of the association signals related to infection, as demonstrated in a study from Burkina Faso.[23] Unfortunately, this type of analysis was not performed, as the population under study experienced drug/IRS interventions after baseline, thus masking possible effects of the RBC polymorphisms on infection.

In this study, there was no evidence of an association between common RBC polymorphisms and *P. falciparum* parasite density. This result is not surprising since the infected individuals were asymptomatic, with a few cases of non-malaria fever. Unfortunately there were no data available about the parasite density for non-falciparum infections. These hypothetical data would have allowed us to investigate the pleiotropic effects of RBC polymorphisms on parasitaemia of *P. ovale* and *P. malariae* infections and on the relative contribution of each malaria species to the overall parasitaemia in co-infections. This study could have also benefited from a larger sample size that would have increased the power to detect moderate associations between RBC polymorphisms and infection outcomes.

In conclusion, this study reported the prevalence dynamics of *P. ovale* and *P. malariae* infections in a malaria hyperendemic area in sub-Saharan Africa and a persistence of *P. ovale* infections over the malaria season. There was a possible protective effect of α-thalassaemia against asymptomatic *P. falciparum* infections. Another possible association was found between the HbS allele and *P. ovale* infections, but it did not hold after adjusting for confounding and multiple testing. The use of larger cohorts would help to confirm these associations and detect others with moderate effect. This study also showed that *P. malariae* and *P. ovale* infections are both more common than thought. Although not associated with severe disease, these non-falciparum infections are likely to persist if interventions only target *P. falciparum* parasites, thus slowing down elimination efforts in endemic areas where these infections co-exist.

Supplementary data

Supplementary data are available at Transactions online (http://trstmh.oxfordjournals.org/).

Authors' contributions: NS and CD conceived the study. CP, BNK, BB, TGE, MO and FV carried out the field and lab work. LS, TB, CD and NS carried out the analysis and interpretation of data. LS, CD and NS drafted the manuscript. LS, TB, CD and NS critically revised the manuscript for intellectual content. All authors read and approved the final version of the manuscript. CD and NS are the guarantors of the paper.

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