

Influence of age on the haemoglobin concentration of malaria-infected patients in a reference centre in the Brazilian Amazon

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Anaemia is amongst the major complications of malaria, a major public health problem in the Amazon Region in Latin America. We examined the haemoglobin (Hb) concentrations of malaria-infected patients and compared it to that of malaria-negative febrile patients and afebrile controls. The haematological parameters of febrile patients who had a thick-blood-smear performed at an infectious diseases reference centre of the Brazilian Amazon between December 2009-January 2012 were retrieved together with clinical data. An afebrile community control group was composed from a survey performed in a malaria-endemic area. Hb concentrations and anaemia prevalence were analysed according to clinical-epidemiological status and demographic characteristics. In total, 7,831 observations were included. Patients with Plasmodium falciparum infection had lower mean Hb concentrations (10.5 g/dL) followed by P. vivax-infected individuals (12.4 g/dL), community controls (12.8 g/dL) and malaria-negative febrile patients (13.1 g/dL) ($p < 0.001$). Age, gender and clinical-epidemiological status were strong independent predictors for both outcomes. Amongst malaria-infected individuals, women in the reproductive age had considerably lower Hb concentrations. In this moderate transmission intensity setting, both vivax and falciparum malaria are associated with reduced Hb concentrations and risk of anaemia throughout a wide age range.

Key words: malaria - anaemia - haemoglobin - *Plasmodium vivax* - *Plasmodium falciparum*

Malaria is one of the most important parasitic diseases worldwide, causing significant clinical and socio-economic burden throughout the different regions where it is endemic (Hay et al. 2004). Anaemia figures amongst the most frequent and worrying malaria complications, especially affecting children and pregnant women in high intensity transmission areas (Menéndez et al. 2000, Quintero et al. 2011, Douglas et al. 2012), although in areas of moderate to low transmission adults are also at risk of presenting this complication, especially if no previous immunity exists (Caicedo et al. 2009). Several mechanisms may explain anaemia in malaria infection, including haemolysis of parasitised and non-parasitised red blood cells (RBCs), antibodies-mediated haemolysis,

reduced bone marrow function and rosetting (Menéndez et al. 2000, Anstey et al. 2012, Douglas et al. 2012, Marin-Menéndez et al. 2013).

Most studies of malaria-related anaemia have focused on its occurrence in relation to *Plasmodium falciparum* in the African continent (Menéndez et al. 2000), demonstrating that even asymptomatic infection leads to lower haemoglobin (Hb) values in children (Gansane et al. 2013). However, recent studies have demonstrated the importance of other plasmodia species in areas where multiple species occur, especially *Plasmodium vivax*, which causes anaemia preferentially amongst children (Tjitra et al. 2008, Kochar et al. 2010, Lin et al. 2010, Douglas et al. 2012, Lanca et al. 2012). Few studies have examined the influence of age on malaria-related anaemia in areas of reducing malaria incidence and where *P. vivax* predominates. In Brazil, there has been a continuous reduction of malaria transmission, accompanied by a steep decrease on the proportion of *P. falciparum* infections (Oliveira-Ferreira et al. 2010). With this study we aimed to investigate the influence of age and gender on malaria-related anaemia in a Brazilian Amazon region where *P. vivax* is the predominant parasite.

SUBJECTS, MATERIALS AND METHODS

Patients and study sites - Two sets of patients, from two different sites, were selected for this study. In the first set, sick patients seeking for healthcare at the Heitor Vieira Dourado Tropical Medicine Foundation (FMT-HVD) were included. FMT-HVD is a tertiary referral centre for tropical diseases, comprising an outpatient

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clinic, inpatient department and intensive care unit, located in the city of Manaus, state of Amazonas (AM), Brazil. In spite of being a tertiary care centre, this institution still diagnoses around 20% of all the cases of malaria from Manaus, therefore, acting as well as a primary care for the malaria diagnosis and treatment. All patients attending with fever complaints have a Giemsa-stained thick blood slide performed for *Plasmodium* sp. diagnosis. If the test is positive for malarial infection, antimalarials are prescribed and, if negative, other causes are investigated through clinical and laboratorial assessments. In both situations, patients are thoroughly assessed by a physician, who can request laboratory investigation (complete blood counts and biochemical analyses) at their own discretion. Patients with severe signs are admitted to the wards. Since August 2009, the FMT-HVD has adopted an electronic medical record system (iDoctor[®]), which includes each patient's clinical and laboratory data. For this set, the inclusion criteria for patients was: (i) having a thick blood smear performed for *Plasmodium* sp. diagnosis and (ii) having an available Hb measurement result within seven days of a thick blood smear performed between January 2010-December 2011. After inclusion, individuals from this set were classified as non-malaria febrile patients and malaria patients, who were further categorised as infection by *P. vivax* and *P. falciparum*. Previous studies have shown that other common causes of non-febrile malaria include, amongst others, acute viral hepatitis, leptospirosis and dengue fever (Mourão 2007).

The second set of subjects comprised afebrile control individuals in two communities from a rural settlement area endemic for malaria located in the municipality of Careiro, AM (100 km from Manaus). This population is similar to the one seeking care at FMT-HVD. Data were obtained for subjects who participated in a *Plasmodium* infection survey (through thick blood smear) and Hb survey performed in February 2010. Subjects were included if they had not suffered from a malaria episode in the preceding 90 days before haematological evaluation.

Laboratorial procedures and data extraction - In both sets of patients, malaria diagnosis was performed through Giemsa-stained thick blood smear read by trained microscopists. For the hospital-based set, complete blood cell counts, Hb and haematocrit determination were performed in an ISO-9002 certified laboratory using a cell counter (Sysmex KX-21N[®]), while in the community set Hb was measured using a portable HemoCue[®] photometer (Anglholm, Sweden). In a subsample of patients enrolled in the community, HemoCue results were also compared with the automated cell counter, showing high consistency (Rippmann et al. 1997).

As previously mentioned, for the set of patients from FMT-HVD, demographics, clinical and laboratorial data were extracted in, with appropriate anonymisation procedures, from the electronic medical record system (iDoctor[®]), in a single database. For the set of patients from the cohort, data registered in the case report forms had been entered on an OpenClinica[®] database, from where the clinical and laboratorial data of each survey was obtained.

Data management and statistical analysis - Data for each observation were obtained on the following characteristics from each dataset: age, gender, malaria diagnosis and Hb. Each individual from the hospital set or the cohort set was identified by the hospital identification number or by a unique identification number, respectively. If more than one Hb measurement was available within the seven-day interval for each of the hospital observations, the closest to the thick blood smear was selected.

The main objective of this study was to investigate the association between malaria status and Hb concentration (g/dL) in relation to age. Three groups of patients were defined: (i) hospital malaria-infected patients, comprising patients with confirmed diagnosis of *Plasmodium* sp. infection, further categorised in *P. vivax*-infected and *P. falciparum*-infected, (ii) hospital febrile non-malaria patients, comprising patients with negative thick blood smear and (iii) afebrile uninfected individuals from the community. Univariable and multivariable analyses using Hb concentration (g/dL) as the outcome were performed for the following variables: age, gender and clinical-parasitological status (afebrile, non-malaria febrile and *P. vivax*-infected and *P. falciparum*-infected). In order not to constrain the shape of the association, fractional polynomials were used to assess the relationship between Hb and age (both as continuous variables), as previously described (Royston et al. 1999, Douglas et al. 2013). As some individuals had more than one observation, robust standard errors with clustering by identification number were calculated using the Huber/White/sandwich estimator. To improve stability, individuals below two weeks of age and above 70 years-old were excluded from the analysis, as these were, respectively, the upper and lower 99 percentiles. Anaemia was defined according to the World Health Organization (WHO) criteria (WHO 2011): (i) Hb < 11.0 g/dL if age < 5-years, (ii) Hb < 11.5 g/dL if age ≥ 5 and < 12 years of age, (iii) Hb < 12.0 g/dL if age ≥ 12 and < 14 years of age, Hb < 12.0 g/dL if women and age > 15 years of age and (iv) Hb < 13.0 g/dL if men and age > 15 years of age. The risk of anaemia was investigated using logistic regression with similar procedures to the ones described for the analysis of Hb concentration. All analyses were performed in Stata[®] v.13.1 (Statacorp[®], USA).

The study was approved by the Institutional Review Board of the FMT-HVD and the Brazilian National Committee of Ethics (protocol 25.001.011.792/2009-15).

RESULTS

Between December 2009-February 2012, there were 42,924 thick blood smears performed and with results available in the FMT-HVD database. After evaluation for inclusion, 6,282 observations that complied with the inclusion criteria were obtained, of which 4,631 (73.7%) tested negative for malaria, 1,067 (17%) were diagnosed *P. vivax* infection and 584 (9.3%) diagnosed with *P. falciparum*. From the community cohort set, from 1,200 individuals surveyed, there were 1,099 observations of individuals with negative thick blood smears and concomitant Hb concentration measurement, who had not presented malaria in the preceding three months

(Fig. 1). The mean time between blood slides and Hb measurements for the different groups was assessed by performing Student's *t* test and ANOVA and no significant difference was detected (data not shown).

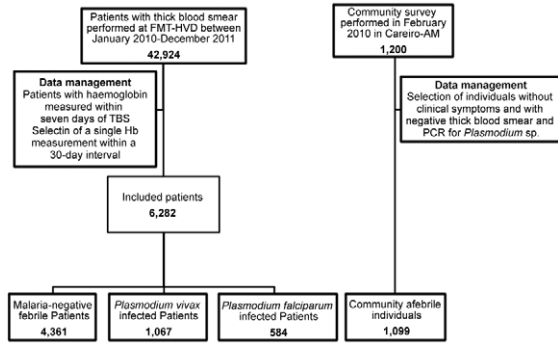


Fig. 1: flowchart of the inclusion of subjects for the study. AM: state of Amazonas; FMT-HVD: Heitor Vieira Dourado Tropical Medicine Foundation; Hb: haemoglobin; PCR: polymerase chain reaction; TBS: tris buffered saline.

Hb concentrations - Age, gender and clinical-epidemiological status were all found to be strongly associated with Hb concentration in both the univariable and multivariable analyses (Tables I, II). Individuals diagnosed with malaria had the lowest Hb concentrations, with *P. falciparum* infection being associated with the lower values {10.5 g/dL [95% confidence interval (CI) 10.3-10.7]}, followed by *P. vivax* infection [12.4 g/dL (95% CI 12.3-12.5)], afebrile community controls [12.8 g/dL (12.7-12.9)] and malaria-negative febrile patients [13.1 g/dL (13.0-13.1)]. Fig. 2 shows how malaria-infected patients presented considerably lower Hb concentrations for the first four decades of life, with the difference in *P. falciparum*-infected patients being more pronounced and longer-lasting when compared to that of *P. vivax* infection, with virtually no difference between malaria-negative febrile patients and afebrile community controls. When comparing the Hb concentration according to gender amongst malaria patients, it is possible to observe that women in the reproductive age had lower

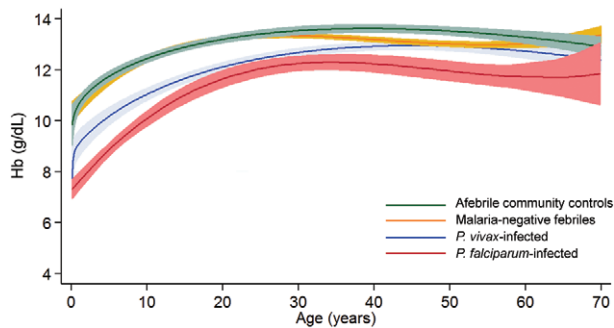


Fig. 2: estimated haemoglobin (Hb) levels and 95% confidence intervals according to age and malaria and clinical statuses (obtained through multivariable fractional polynomial regression).

Hb concentrations when compared to men, with a trend of this difference disappearing after menopause. When compared to negative controls, it is possible to observe that youngsters infected with malaria of both sexes have much lower Hb levels and that the overall Hb levels are sensibly lower throughout all age ranges (Fig. 3).

Anaemia risk - The prevalence of anaemia, as defined by WHO criteria, was associated with gender, age group and malaria infection both in the univariable and multivariable analyses (Tables I, III). *P. vivax* infection and young age presented the strongest association with the risk of anaemia and there was no difference when comparing afebrile community controls with malaria-negative febrile patients. For this outcome the probability of WHO-defined anaemia was considerably higher among individuals under 30 years of age infected with *P. vivax* (Fig. 4A).

For multivariable analysis using Hb concentration below 7 g/dL as the outcome, the only independently associated factors were young age groups (0-5 and 6-10 years-old), malaria-negative febrile status and *P. vivax* and *P. falciparum* infection. The strongest risk factor for presenting Hb below 7.0 g/dL was *P. falciparum* infection [odds ratio (OR) 17.2 (95% CI 6.2-47.5)], followed by age under five years [OR 5.6 (95% CI 3.5-8.9)] and malaria-negative fever [OR 4.7 (95% CI 1.7-13.0)] (Tables I, III). The probability of presenting severe anaemia was considerably higher in children below the age of 15 infected with *P. falciparum* (Fig. 4B).

DISCUSSION

Anaemia is one of the most frequent complications of malaria, occurring as a direct and indirect consequence of the infection and destruction of erythrocytes by *Plasmodium* parasites. In our study, conducted in an area where *P. vivax* is the highly predominant species causing malaria, we have been able to demonstrate that malaria caused by either species is associated with a higher risk of anaemia, when compared to both malaria-negative febrile patients and community controls. Moreover, it was also possible to observe that Hb concentration for malaria-infected individuals had a wider age range when compared to areas of higher transmission intensity, where anaemia typically concentrates at young ages (Tjitra et al. 2008, Lin et al. 2010, Douglas et al. 2013). Amongst malaria-infected individuals, women in reproductive age had considerably lower Hb concentrations than men. Although the overall risk of anaemia was relatively higher for *P. vivax* infection, *P. falciparum* infection was associated with a higher risk of developing severe anaemia, defined as Hb concentrations below 7.0 g/dL.

There are multiple potential mechanisms for malaria-related anaemia, which have not been satisfactorily elucidated. Apart from direct destruction of infected erythrocytes, higher turnover of non-infected RBCs, dyserythropoiesis and other mechanisms have been implicated to cause anaemia in malaria (Menéndez et al. 2000, Douglas et al. 2012). In endemic areas it has been demonstrated that children are amongst the most vulnerable, presenting with a higher risk of malaria-related complications, especially anaemia (Tjitra et al. 2008, Crawley et al. 2010, Lin et al. 2010, Douglas et al. 2013). Data from

TABLE I
Mean haemoglobin (Hb) and prevalence of anaemia according to participants' demographic and clinical characteristics

	n	Mean Hb (SD)	p ^a	Anaemia prevalence ^b (%)	Crude OR (95% CI)	p	Severe ^c anaemia prevalence (%)	Crude OR (95% CI)	p
Clinical-epidemiological status									
Afebrile community	1,099	12.8 (1.8)	Ref.	31.8	Ref.	-	0.4	Ref.	-
Malaria-negative febrile	4,631	13.1 (2.0)	< 0.001	28.8	0.9 (0.8-1.0)	0.055	1	2.9 (1.1-8.1)	0.039
<i>Plasmodium vivax</i>	1,067	12.4 (2.1)	< 0.001	47.2	1.9 (1.6-2.3)	< 0.001	0.8	2.3 (0.7-7.6)	0.161
<i>Plasmodium falciparum</i>	584	10.5 (2.8)	< 0.001	41.7	1.5 (1.3-1.8)	< 0.001	7.9	23.3 (8.5-63.7)	< 0.001
Demographics									
Female	3,209	12.1 (1.8)	< 0.001	37.7	1.4 (1.3- 1.5)	< 0.001	1.8	1.0 (0.7-1.4)	0.997
Male	4,172	13.2 (2.3)	-	30.16	Ref.	-	1.8	Ref.	-
Age group (years)									
0-5	560	10.0 (2.3)	< 0.001	52.9	3.0 (2.6-3.4)	< 0.001	9.1	9.9 (6.4-15.2)	< 0.001
6-10	388	11.7 (1.8)	< 0.001	44	2.1 (1.7-2.5)	< 0.001	2.5	2.5 (1.3-5.0)	0.009
11-20	1,161	12.7 (2.0)	< 0.001	32	1.2 (1.1-1.4)	0.004	1.2	1.2 (0.7-2.3)	0.502
21-40	2,899	13.2 (2.0)	Ref.	27.6	Ref.	-	1	Ref.	-
41-60	1,868	13.1 (1.9)	0.070	31.8	1.2 (1.1-1.4)	0.001	0.8	0.8 (0.5-1.5)	0.531
≥ 61	505	12.7 (2.0)	< 0.001	39.8	1.7 (1.4-2.1)	< 0.001	0.8	0.8 (0.3-2.1)	0.593

a: p-value from univariable linear regression analysis with robust standard errors accounting for clustering at individual level [p-value from univariable logistic regression (likelihood ratio test)]; b: World Health Organization criteria; c: severe anaemia defined by Hb concentration below 7 g/dL; CI: confidence interval; OR: odds ratio; Ref.: this group was taken as reference for the analysis; SD: standard deviation of the mean.

different areas where *P. vivax* and *P. falciparum* are co-endemic have suggested that acquisition of clinical immunity may occur more rapidly to *P. vivax*, as younger children preferentially suffer clinical consequences of infection by this species, with the proportion reversing with older ages (Tjitra et al. 2008, Lin et al. 2010).

In our study we found that *P. vivax* contributed to significant reductions of the Hb concentrations and risk of anaemia through a wider age range, up to the 40 years of age, compared to data from other regions. This could be related to the lower transmission intensity in our area leading to a delayed acquisition of immunity and, therefore, more sustained complications. Notably, women of reproductive age with *P. vivax* infection had considerably lower Hb concentrations, as malaria during pregnancy can be especially severe for both the women and the fetuses (Martínez-Espinosa et al. 2004, Poespoprodjo et al. 2011, Rijken et al. 2012). It has been suggested that chloroquine-resistant *P. vivax* can also be associated

with increased rates of complications (Tjitra et al. 2008) and an acute fall in Hb concentration (Marques et al. 2014), although we were not able to investigate this in the current study. When comparing the most important species causing malaria in our setting, *P. vivax* and *P. falciparum*, there are important differences. Although the former was associated with higher risk of overall anaemia, the latter was associated with lower Hb concentration and increased risk of severe anaemia, highlighting the importance of species-specific diagnosis and the importance of the impressive reduction of the proportion of infections caused by *P. falciparum* achieved by the control programme (Oliveira-Ferreira et al. 2010).

In tropical areas, several other factors can also cause anaemia, including malnutrition, human immunodeficiency virus (HIV) infection, helminthic infections and bacteraemia, amongst others (Cardoso et al. 1994, 2012, Calis et al. 2008). How these factor interact with malarial infection and especially *P. vivax*, has not been com-

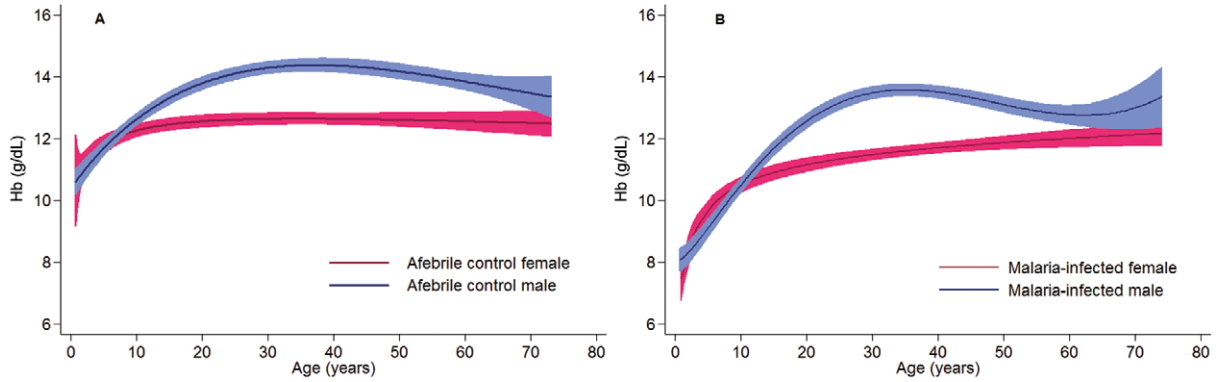


Fig. 3: estimated haemoglobin (Hb) levels and 95% confidence intervals according to age and gender amongst afebrile controls (A) and malaria-infected individuals (B) (obtained through multivariable fractional polynomial regression).

prehensively investigated, with contradictory evidence (Douglas et al. 2012). In a study from the same area of this study, it has been suggested that helminthic infection leads to a less severe decrease in Hb concentration during acute *P. vivax* episodes in children (Melo et al. 2010), while in most *P. falciparum* studies, co-infection with hookworms was found to contribute synergistically to increased risk of anaemia (Stoltzfus et al. 2000, Brooker et al. 2007), illustrating the need to further investigate this association. Although the prevalence of glucose-6-phosphate deficiency and haemoglobinopathies is presumed to be low in the Brazilian western Amazon Region (Haematology and Haemotherapy Foundation, unpublished observations), the well-established knowledge of their influence on the clinical epidemiology of malaria (Mason et al. 2007, Taylor et al. 2012), highlights the need for these factors to be assessed in future studies.

The large number of patients included in our study has allowed us to examine the influence of age on the reduction of Hb in microscopically-confirmed malaria infections in the Brazilian Amazon compared to non-malarial febrile patients and afebrile individuals from a community cohort.

However, important limitations to our study need to be mentioned. Blood cell counts were performed at the discretion of physicians who may have prioritised patients with more severe or intense clinical symptoms and

could possibly lead to selection bias. It is important to bear in mind that febrile patients who tested negative for malaria could be presenting to the hospital affected by a wide range of other conditions associated to anaemia, such as tuberculosis, HIV infection and bacterial infections or even with conditions usually presenting with an increase in Hb concentration, such as dengue fever, with a high incidence in the region of all four known virus serotypes (Bastos et al. 2012). Moreover, the possible causes of fever vary substantially according to age, which could influence some of the findings. The slightly increased Hb concentration observed in the malaria-negative febrile patients, compared to the community cohort individuals, could be due to a high proportion of the former presenting to the hospital with dengue fe-

TABLE II

Multivariate analysis of haemoglobin change according to the clinical-epidemiological status and demographic characteristics

	Coefficient	95% CI	p
Afebrile community	Ref.	-	-
Malaria-negative febrile	0.04	-0.07-0.16	0.427
<i>Plasmodium vivax</i>	-0.66	-0.81-0.51	< 0.001
<i>Plasmodium falciparum</i>	-2.35	-2.58-2.12	< 0.001
Female gender	-1.63	-1.5-1.74	< 0.001
Age (years)	0.023	0.021-0.026	< 0.001

CI: confidence interval; Ref.: this group was taken as reference for the analysis.

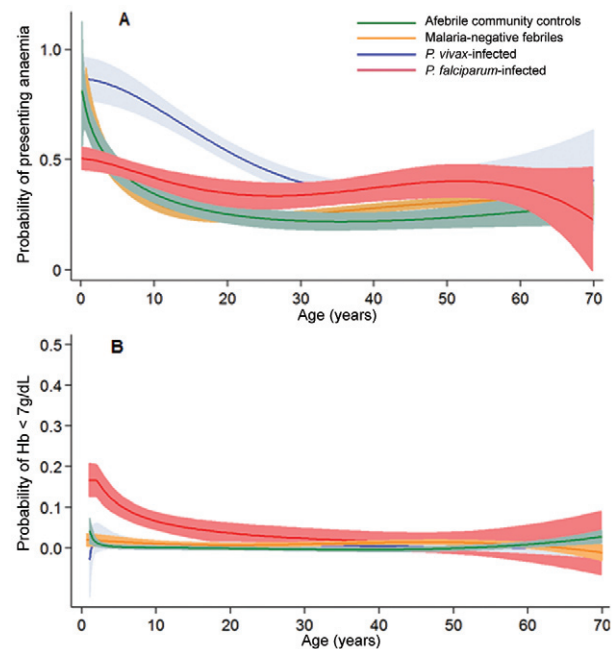


Fig. 4: estimated probability of presenting World Health Organization-defined anaemia (A) and severe anaemia (Hb < 7g/dL) (B) according to age and malaria and clinical statuses (obtained through multivariable fractional polynomial regression).

TABLE III
Odds of World Health Organization (WHO)-defined anaemia and severe anaemia [haemoglobin (Hb) < 7 g/dL] according to the clinical-epidemiological status and demographic characteristics

	WHO-defined anaemia		Severe anaemia (Hb < 7g/dL)	
	Adjusted OR (95% CI)	p ^a	Adjusted OR (95%CI)	p ^a
Afebrile community	Ref.	-	Ref.	-
Malaria-negative febrile	1.1 (0.93-1.2)	0.352	4.7 (1.7-13.0)	0.003
<i>Plasmodium vivax</i> -infected	2.4 (2.0-2.8)	< 0.001	3.5 (1.1-11.3)	0.040
<i>Plasmodium falciparum</i> -infected	1.5 (1.3-1.9)	0.001	17.2 (6.2-47.5)	< 0.001
Female gender	1.4 (1.3-1.6)	< 0.001	1.0 (0.7-1.4)	0.835
Age group (years)				
0-5	2.9 (2.4-3.5)	< 0.001	5.6 (3.5-8.9)	< 0.001
6-10	2.1 (1.7-2.6)	< 0.001	2.0 (0.9-4.0)	0.056
11-20	1.3 (1.1-1.5)	< 0.001	1.3 (0.7-2.4)	0.421
21-40	Ref.	-	-	-
41-60	1.2 (1.1-1.4)	0.004	0.8 (0.4-1.5)	0.536
≥ 61	1.8 (1.5-2.2)	< 0.001	0.7 (0.3-2.1)	0.587

a: p-value obtained from the multivariable logistic regression; CI: confidence interval; Ref.: this group was taken as reference for the analysis.

ver and this unfortunately could not be ascertained in the present study. Additionally, no data were available on some important confounders, especially helminthic infections, nutritional status and haemoglobinopathies, which would ideally be measured in future prospective studies to minimise the effect of residual confounding.

We decided to apply distinct anaemia definitions: the first one from WHO (2011) and the second using an Hb concentration cut-off of 7.0 g/dL, chosen arbitrarily as a proxy of severe anaemia. This approach allowed us to observe that *P. vivax* infection is associated with a higher risk of mild and moderate anaemia, especially at younger age, while there was very strong evidence of *P. falciparum* presenting with a much higher risk of severe anaemia and corroborates with the hypothesis that this species causes a higher proportion of complications.

These findings are of major importance for public health, as even mild and moderate anaemia are associated with important physical and socioeconomic burden in all age groups (Pollitt et al. 1985, Peters et al. 2008, Petranovic et al. 2008, Kassebaum et al. 2013). The pathophysiological particularities of each species causing the differences of clinical presentation between are far from being completely understood and deserve further investigation (Douglas et al. 2012, 2013, Taylor et al. 2013).

The broader age range that presented low Hb concentrations in our settings also highlights the need of more focused attention on the older age groups as the epidemiologic shift in many of the tropical areas in leading to an increase on life expectancy that is being accompanied by higher prevalence of chronic diseases, such as hypertension and diabetes, posing this subpopulation at high risk of malaria-related complications as has been shown before (Lacerda et al. 2012a, b).

In our study we have been able to observe strong evidence of age influencing the extent of the Hb concentration reduction amongst malaria infected patients in an area with decreasing levels of transmission, where there is a large predominance of *P. vivax*. Differently to areas of high transmission intensity, the Hb reduction during acute malaria episodes was observed up to the age of 40 years of age. This finding has important implications in a context of malaria elimination efforts, since older individuals can lose some of the clinical protection observed in areas of higher malaria incidence. Additionally, amongst *P. vivax*-infected individuals, women in the reproductive age had Hb concentrations lower than men. This is especially important as pregnant women are at higher risk of malaria-related complications and also because they are not prescribed primaquine due to risk of

provoking haemolysis in the foetus. Further studies are needed to investigate the mechanisms of anaemia attributed to malaria in tropical settings, especially from areas where a reduction in malaria transmission is changing the clinical epidemiology of the disease.

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