




RESEARCH ARTICLE

Submicroscopic and Asymptomatic *Plasmodium* Parasitaemia Associated with Significant Risk of Anaemia in Papua, Indonesia

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. The data used in this paper were collected from a household survey, with IP owned by the coauthors of this paper affiliated to the Papuan Health and Community Development Foundation and Menzies School of Health Research. Further details can be obtained from the corresponding author at rprice@menzies.edu.au. The authors confirm that the data included within the paper and its Supporting Information files constitute the minimal dataset necessary to replicate the study's findings.

Abstract

Submicroscopic *Plasmodium* infections are an important parasite reservoir, but their clinical relevance is poorly defined. A cross-sectional household survey was conducted in southern Papua, Indonesia, using cluster random sampling. Data were recorded using a standardized questionnaire. Blood samples were collected for haemoglobin measurement. Plasmodium parasitaemia was determined by blood film microscopy and PCR. Between April and July 2013, 800 households and 2,830 individuals were surveyed. Peripheral parasitaemia was detected in 37.7% (968/2,567) of individuals, 36.8% (357) of whom were identified by blood film examination. Overall the prevalence of *P. falciparum* parasitaemia was 15.4% (396/2567) and that of *P. vivax* 18.3% (471/2567). In parasitaemic individuals, submicroscopic infection was significantly more likely in adults (adjusted odds ratio (AOR): 3.82 [95%CI: 2.49–5.86], $p < 0.001$) compared to children, females (AOR = 1.41 [1.07–1.86], $p = 0.013$), individuals not sleeping under a bednet (AOR = 1.4 [1.0–1.8], $p = 0.035$), and being afebrile (AOR = 3.2 [1.49–6.93], $p = 0.003$). The risk of anaemia (according to WHO guidelines) was 32.8% and significantly increased in those with asymptomatic parasitaemia (AOR 2.9 [95% 2.1–4.0], $p = 0.007$), and submicroscopic *P. falciparum* infections (AOR 2.5 [95% 1.7–3.6], $p = 0.002$). Asymptomatic and submicroscopic infections in this area co-endemic for *P. falciparum* and *P. vivax* constitute two thirds of detectable parasitaemia and are associated with a high risk of anaemia. Novel public health strategies are needed to detect and eliminate these parasite reservoirs, for the benefit both of the patient and the community.

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Introduction

Significant gains have been made over the last decade in the control and elimination of malaria. Early diagnosis, highly effective treatment and intense vector control have led to reductions in the number of malaria cases, as well as severe disease and associated mortality [1]. In 2014, 18 countries in the Asia-Pacific committed to eliminate malaria from the region by 2030; however, the challenges to achieve this goal are immense [2]. The final stages of malaria elimination are often prolonged, with low-grade transmission sustained by a parasite reservoir present in asymptomatic individuals who may not readily engage or attend healthcare facilities to be treated. Novel strategies are needed to identify and eliminate these asymptomatic parasite reservoirs [3].

The established standard for malaria diagnosis in the field remains blood film examination. Lower limits of detection range from 10–20 parasites/ μL in research settings to more than 100/ μL in clinical practice [4]. Recent advances in molecular techniques have allowed the development of highly sensitive methods for detecting peripheral parasitaemia at submicroscopic levels (0.1–10 parasites/ μL) [5–8]. The application of molecular approaches in surveillance surveys has resulted in an inevitable increase in the prevalence of detectable parasitaemia, often 2–10 fold higher than the prevalence obtained by conventional methods [9]. Although most of the available data on subpatency have been derived from the studies of *P. falciparum*, similar observations have been made for *P. vivax* [10, 11].

Transmission can occur from individuals with asymptomatic microscopic parasitaemia, many of whom are semi-immune and maintain low levels of parasitaemia and gametocytaemia for sustained periods of time [12]. Submicroscopic parasitaemia can also be transmitted to the mosquito vector (albeit less efficiently) and given the higher prevalence, contributes significantly to the overall infectious reservoir [13, 14].

The clinical relevance of submicroscopic infections is less clear. In cross-sectional surveys, low-level parasitaemia may represent early detection of an expanding parasite biomass that will ultimately result in clinical disease. However, repeated exposure and acquisition of partial immunity can result in persistent low-grade infection, and although these individuals may not have fever, chronic carriage can result in anaemia, bacterial co-infection and cognitive impairment [15]. In pregnancy, asymptomatic parasitaemia has also been shown to increase the risk of maternal anaemia and the delivery of low birth weight babies [16, 17]. Thus, carriage of asymptomatic parasitaemia potentially has profound implications for the health of individuals and for the control and elimination of malaria [18–20].

We conducted a large cross-sectional survey in southern Papua, Indonesia, an area co-endemic for *P. falciparum* and *P. vivax*, to identify the risk factors for microscopic and submicroscopic carriage for both species, and thereby populations at greatest risk of harbouring undetected parasitaemia by conventional microscopic surveys. We also aimed to quantify the degree of anaemia associated with asymptomatic and submicroscopic parasitaemia, to gauge the clinical relevance of these low level infections.

Materials and Methods

Study Site

Timika, the main city of the Mimika District, is located in southern Papua province, Indonesia. It has a population of 202,350 composed of different ethnicities broadly including Highland Papuans and Lowland Papuans, and Non-Papuans. Nearly 90% of the population lives in lowland areas where unstable malaria transmission occurs. In 2013, the annual incidence of parasitaemia was 450/1000, with *P. falciparum* and *P. vivax* causing 60% and 40% of cases,

respectively [21], without significant seasonal fluctuation in incidence. Patients with uncomplicated malaria due to any *Plasmodium* species are treated with dihydroartemisinin-piperaquine (DHP) as the first-line treatment regimen.

Cross-Sectional Survey Methods

Sample selection. Households were chosen by cluster random sampling. First, the four largest subdistricts were chosen purposively. Consecutively, the numbers of cluster per district was calculated according to their respective relative population. Finally, 25 houses were chosen randomly within each cluster, following WHO recommendations. Clusters constituted whole existing villages, except in Mimika Baru subdistrict where large populated villages had to be divided into census blocks.

Data and sample collection. People living in the house, eating from the same kitchen, and residing in the study area for at least 6 months were defined as household members and were included in the study. Socio-demographic information and medical history including fever episodes in the last 24 hours and in the last month were recorded from all the members through a standardized questionnaire. Physical examination including weight, height, mid-upper arm circumference, axillary temperature and blood pressure were assessed immediately after completion of the questionnaire. Venous blood from one adult and 200 μ L capillary blood collected in a Microtainer™ from the remaining members of each household were collected and used for blood film examination, haemoglobin measurement, G6PD testing, and molecular analysis. Participants with a positive blood smear for *Plasmodium spp.* were treated with DHP. Those with anaemia were given iron supplementation according to local protocols.

Laboratory Methods

Parasite species was assessed from Giemsa-stained thick blood films. Peripheral parasitaemia was determined from the number of parasites per 200 white blood cells, assuming a white cell count of 7,300 cells/ μ L. All positive films and 10% of the negative slides were cross-checked by a second microscopist at the Eijkman Institute for Molecular Biology in Jakarta and discrepancies reviewed by two expert microscopists for final assessment. Haemoglobin levels were determined using a calibrated portable HemoCue® machine (Ångelholm, Sweden). G6PD status was assessed using the G-6-PDH Deficiency Screen by Spot Test Kit® (Trinity Biotech).

Genomic DNA (gDNA) was extracted from ≥ 50 μ L packed red blood cell (RBC) pellets using the QIAamp 96 DNA Blood Kit (Qiagen). *Plasmodium* species confirmation was undertaken in duplicate with 2 μ L gDNA template using a nested PCR protocol as described elsewhere [22]. *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* small-subunit rRNA DNA clones (MRA-177, MRA-178, MRA-179, and MRA-180; ATCC, Manassas, VA) were used as positive-control materials. The limit of detection (LOD) of the assay was 0.2–2 parasites/ μ L, as evaluated by using well characterized samples of *P. falciparum* and *P. vivax* (Piera *et al.* manuscript in preparation). If a duplicate result was discrepant, the assay was repeated. A sample was positive when 2/4 replicates were positive. In case of a 1/4 positivity rate, the assay was repeated a third time and the sample categorized as positive if 2/6 total replicates were positive, but negative if 1/6 replicates were positive.

Definitions used in the study

An individual was diagnosed as aparasitaemic if both the microscopy and PCR result were negative. Microscopic (patent) parasitaemia was defined in those participants with positive microscopy irrespective of PCR confirmation, assuming that these patients would be detected at a clinical encounter. Submicroscopic (subpatent) parasitaemia was defined in participants

who were microscopy negative, but positive PCR result. Where there was discrepancy between the species determined by microscopy and PCR, the diagnosis was classified according to the PCR result. Symptomatic malaria was defined as a positive malaria result by microscopy or PCR associated with a fever or history of fever in the last 24 hours.

Anaemia was diagnosed according to WHO guidelines, defined as a haemoglobin (Hb) concentration less than 11 g/dL in children under 5 years old or pregnant women, less than 11.5 g/dL in children between 5–11 years, less than 12 g/dL in children between 12–14 years old or adult women (>15years) and less than 13 g/dL in adult men. Moderate and severe anaemia was diagnosed when the Hb concentration was less than 10g/dL and 7g/dL in children under 5 years old or pregnant women and less than 11g/dL and 8g/dL respectively for the remaining groups. The remaining cases of anaemia were classified as mild [23].

Statistical Analyses

Questionnaire and laboratory data were entered into EpiData 3.02 software (EpiData Association, Odense, Denmark). Data were analysed using SPSS v.20.0 for windows software (IBM SPSS Statistics) and STATA 14.1 software (Stata Corp, College Station, TX). Normally distributed data were compared by Student's *t*-test and data not conforming to a normal distribution compared by the Mann-Whitney U test. Categorical data were compared by chi-squared test with Yates' correction, or by Fisher's exact test. A multivariable logistic regression model (with robust standard errors to account for clustering by the 16 villages and within household) was used to determine adjusted odds ratios for risk factors associated with anaemia and subpatency. To identify risk factors for the multivariable logistic regression model, variables associated significantly with anaemia or subpatency in a univariable analysis were entered into the equation and the model constructed by a forward stepwise analysis. Analyses for the outcome subpatency were restricted to those individuals with detectable parasitaemia. Multiple fractional polynomial models were used to illustrate the nonlinear association between age and the log odds of detectable parasitaemia, subpatency and anaemia.

Ethics

All biological samples and patient data were collected with written, informed consent from the patient or a parent or guardian. Ethical approval for this study and the process of informed consent, was obtained from the Eijkman Institute Research Ethics Commission, Eijkman Institute for Molecular Biology, Jakarta, Indonesia, (Ref: KE/FK/763/EC) and the Human Research Ethics Committee of the Northern Territory (NT) Department of Health & Families and Menzies School of Health Research, Darwin, Australia (Ref: HREC-2010-1434).

Results

Between April and July 2013, 800 households were surveyed comprising 4,010 individuals of whom 2,830 (70.6%) were present at the time of interview. Absentee individuals ($n = 1180$) were more likely to be adult males 52.3%, (617) or school age children 5–15 years 25.7%, (304). In total, 2,796 of 2,830 (99.8%) participants underwent blood film examination (S1 Fig). Twenty one infants under 5 year old, 2 children aged 5 to 15 years, 3 adults and another with unknown age declined finger prick sampling. The characteristics of household individuals present and sampled during the survey and those absent from the household are presented in S1 Table.

Microscopy and PCR Confirmation of Parasitaemia

The age distribution of the 2,796 participants peaked in infancy and early adulthood (Fig 1A), with 59.2% (1654) of the individuals females and 45.6% (1,275) indigenous Papuans (19.2% (536) Highlanders and 26.4% (739) Lowlanders).

Peripheral asexual parasitaemia was diagnosed by microscopy in 12.2% (340) of participants, with *P. falciparum* mono-infection present in 145 (5.2%), *P. vivax* in 160 (5.7%),

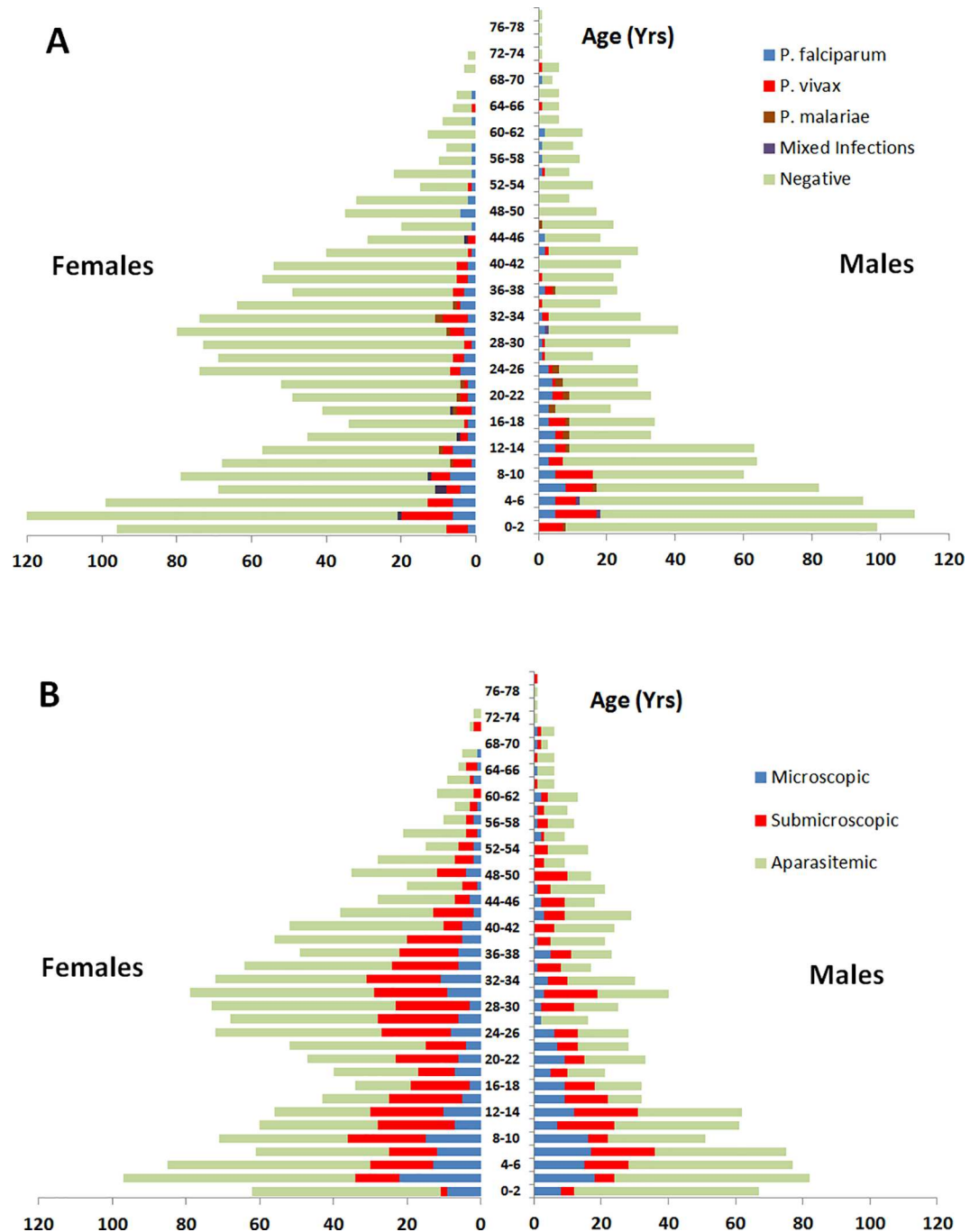


Fig 1. Age stratified prevalence of malaria diagnosed by microscopy and PCR. Microscopy (A) and microscopy and PCR (B).

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Table 1. Malaria diagnosis and demographic features of survey participants^a

n (%)	Sex		Pregnancy Status ^b		Age-Group (Years)				Ethnicity			All
	Male	Female	Non-Pregnant	Pregnant	<1	1–5	5–15	>15	Highland Papuan	Lowland Papuan	Non-Papuan	
Microscopic Parasitaemia (Blood Film Positive)^c												
<i>P. falciparum</i>	78 (7.6)	88 (5.7)	48 (4.9)	3 (6.7)	2 (4.3)	19 (5.4)	57 (9.5)	88 (5.6)	40 (8.1)	74 (10.7)	52 (3.8)	166 (6.5)
<i>P. vivax</i>	76 (7.4)	88 (5.7)	38 (3.9)	2 (4.4)	4 (8.7)	43 (12.2)	53 (8.8)	64 (4.1)	26 (5.3)	51 (7.4)	87 (6.3)	164 (6.4)
<i>P. malariae</i>	13 (1.3)	7 (0.5)	5 (0.5)	0 (0.0)	0 (0.0)	1 (0.3)	3 (0.5)	16 (1.0)	8 (1.6)	6 (0.9)	6 (0.4)	20 (0.8)
Mixed species	3 (0.3)	4 (0.3)	1 (0.1)	0 (0.0)	0 (0.0)	2 (0.6)	3 (0.5)	2 (0.1)	3 (0.6)	4 (0.6)	0 (0.0)	7 (0.3)
Submicroscopic Parasitaemia (Blood Film Negative and PCR Positive)												
<i>P. falciparum</i>	92 (8.9)	138 (9.0)	91 (9.3)	6 (13.3)	0 (0.0)	11 (3.1)	55 (9.2)	164 (10.5)	74 (14.9)	75 (10.9)	81 (5.9)	230 (9.0)
<i>P. vivax</i>	114 (11)	193 (12.6)	129 (13.2)	2 (4.4)	3 (6.5)	25 (7.1)	89 (14.8)	190 (12.1)	55 (11.1)	98 (14.2)	154 (11.1)	307 (12.0)
<i>P. ovale</i>	0 (0.00)	1 (0.07)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	1 (0.2)	0 (0.0)	0 (0.0)	1 (0.04)
<i>P. malariae</i>	4 (0.4)	12 (0.8)	11 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	3 (0.5)	13 (0.8)	9 (1.8)	4 (0.6)	3 (0.2)	16 (0.6)
Mixed species	25 (2.4)	32 (2.1)	22 (2.2)	0 (0.0)	0 (0.0)	3 (0.8)	14 (2.3)	40 (2.6)	12 (2.4)	30 (4.4)	15 (1.1)	57 (2.2)
Blood Film Negative and PCR Negative												
	628 (60.8)	971 (63.3)	633 (64.7)	32 (71.1)	37 (80.4)	249 (70.5)	322 (53.7)	991 (63.2)	267 (53.9)	347 (50.4)	985 (71.2)	1,599 (62.3)
Total	1,033 (40.2)	1,534 (59.8)	978 (38.1)	45 (1.8)	46 (1.8)	353 (13.8)	600 (23.4)	1,568 (61.1)	495 (19.3)	689 (26.8)	1,383 (53.9)	2,567 (100)

n = number.

^a All survey participants with a blood sample

^b 22 adult women without negative PCR result

^c Following reclassification of species with PCR result and inclusion of 17 infections with only sexual stages present.

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P. malariae in 24 (0.9%) and mixed species infections in 11 (0.4%; [Table 1](#)). Gametocytes were present in 2.2% (61/2,796) of individuals; with 88.9% (8/9) of *P. vivax* gametocytes present with asexual stages, compared to only 66.7% (32/48) of *P. falciparum* gametocytes ($p < 0.001$). The geometric mean asexual parasitaemia was 738 parasites/ μ L [95% CI: 543–1,001] for *P. falciparum* and 459 parasites/ μ L [95% CI: 360–586] for *P. vivax*. Fever during the last month was reported in 5.5% (8/145) of those with *P. falciparum*, 5.0% (8/160) of those with *P. vivax*, and 3.8% (94/2,456) of those negative by microscopy ([Table 1](#)).

In total, 12.8% (357/2,796) of individuals had parasitaemia (either asexual or sexual stages) detectable by microscopy; [Table 1](#) and [S1 Fig](#). PCR analysis was available in 88.6% (2,476/2,796) of cases, although 91 of the remaining individuals were positive by microscopy and their microscopic parasitaemia categorized accordingly. Species was confirmed by PCR in 88.7% (236/266) individuals with microscopic parasitaemia. In the remaining 30 cases, 11 *P. falciparum* infections were re-categorized as *P. vivax* by PCR, 10 *P. vivax* as *P. falciparum*, 4 *P. malariae* as *P. falciparum*, 2 mixed infections as *P. falciparum* mono-infections, and 2 mixed infections as *P. vivax* mono-infections. The remaining individual had *P. malariae* detected by microscopy, but was negative by PCR and was categorized as having microscopic *P. malariae* with a false-negative PCR result. The characteristics of the 2,567 participants who could be classified by both microscopy and PCR are presented in [Table 1](#) and the age distribution in [Fig 1B](#).

In total, 37.7% (968/2,567) individuals had detectable parasitaemia by microscopy or PCR, the prevalence being greatest in children aged 5–15 years (46.3%, 278/600) compared to 28.3% (113/399) in children <5 years, and 36.8% (577/1,568) in adults ($p < 0.001$); [Table 1](#), [Figs 1 and 2A](#). The odds of detectable parasitaemia was greater in Papuans compared to Non-Papuans (OR = 2.30 [95%CI: 1.95–2.70]; $p < 0.001$). There was no overall difference in the risk of parasitaemia between individuals according to sex, pregnancy, fever in the last month, or bednet ownership. Detectable parasitaemia was significantly less prevalent in the 66 (2.6%) individuals with severe or intermediate G6PD deficiency compared to those who were G6PD normal (OR = 0.44 [95%CI: 0.24–0.80]; $p = 0.005$). The protective effect of G6PD deficiency was more apparent in *P. vivax* infection (OR = 0.32 [95%CI: 0.13–0.81]; $p = 0.010$) than in *P. falciparum* (OR = 0.46 [95%CI: 0.20–1.08]; $p = 0.067$).

Risk factors for submicroscopic infections

Risk factors for subpatency were assessed in 968 individuals with detectable parasitaemia, of whom 611 (63%) had submicroscopic infection ([Table 1](#)). *P. vivax* parasitaemia was more likely to be submicroscopic (65.2%, 307/471) compared to *P. falciparum* (58%, 230/396; (OR = 1.35 [95%CI: 1.03–1.78], $p = 0.038$). *P. malariae* parasitaemia was submicroscopic in 44% (16/36) of participants.

Overall the risk of submicroscopic infection in those with detectable parasitaemia increased steadily with age (37% (42/113) in children <5, 58% (162/278) in children 5–15 years, and 71% (407/577) in adults; $p < 0.001$, [Table 2](#)). However, the association between subpatency and age was significant for *P. falciparum* (χ^2 test for trend, $p < 0.001$), but not *P. vivax* ($p = 0.524$; [Fig 2B](#)). There was a higher risk of submicroscopic infections in females compared to males (OR = 1.5 [95% CI: 1.1–1.9]; $p = 0.006$) and in those who did not own a bednet compared to those who did (OR = 1.4 [95% CI: 1.0–1.8, $p = 0.025$]; [Table 2](#)).

Fever during the preceding month was reported by in 4% (103/2567) of participants. Only 3.3% (32/968) of individuals with detectable parasitaemia reported fever in the preceding 24 hours, of whom the parasitaemia was microscopic in 65.6% (21/32) and submicroscopic in 34.4% (11/32) ($p = 0.001$). Those with a fever or history of fever in the preceding 24 hours were more likely to be parasitaemic than those without parasitaemia (OR: 2.8 [95%CI: 1.6–5.0]; $p < 0.001$); [Table 2](#).

Risk factors for anaemia

In total haemoglobin concentration was measured in 2757 individuals, of whom 2564 (93.0%) also had documentation of both microscopic and submicroscopic parasitaemia. In 193 cases no sample was collected for PCR. Haemoglobin (Hb) concentrations varied significantly with gender, age, ethnicity, G6PD status, and malaria species ([Table 3](#) and [Fig 3](#)). Anaemia was present in 32.8% (904/2757) of the participants and categorized as mild in 13.1% (365), moderate in 15.8% (443) and severe in 3.4% (96) ([S2 Table](#)). The risk of anaemia was greater in children compared to adults (OR = 1.4 [95% CI: 1.2–1.7]; $p < 0.001$), Papuans compared to Non-Papuans (OR = 4.05 [95% CI: 3.4–4.8]; $p < 0.001$), and females compared to males (OR = 1.18 [95% CI: 1.0–1.4]; $p = 0.039$) ([Table 3](#)).

Individuals with detectable parasitaemia were more likely to be anaemic than aparasitaemic individuals (OR = 2.7 [95% CI: 2.1–3.4]; $p < 0.001$). In those with microscopic infection, the peripheral parasitaemia of any species was negatively correlated with the haemoglobin concentration ($r = -0.184$; $p = 0.001$), with a geometric mean parasitaemia of $739 \mu\text{l}^{-1}$ [95%CI 560–974] in anaemic individuals compared to $407 \mu\text{l}^{-1}$ [95%CI 327–507] in non-anaemic individuals; $p = 0.001$. Combining microscopic and submicroscopic infections, *P. falciparum* was

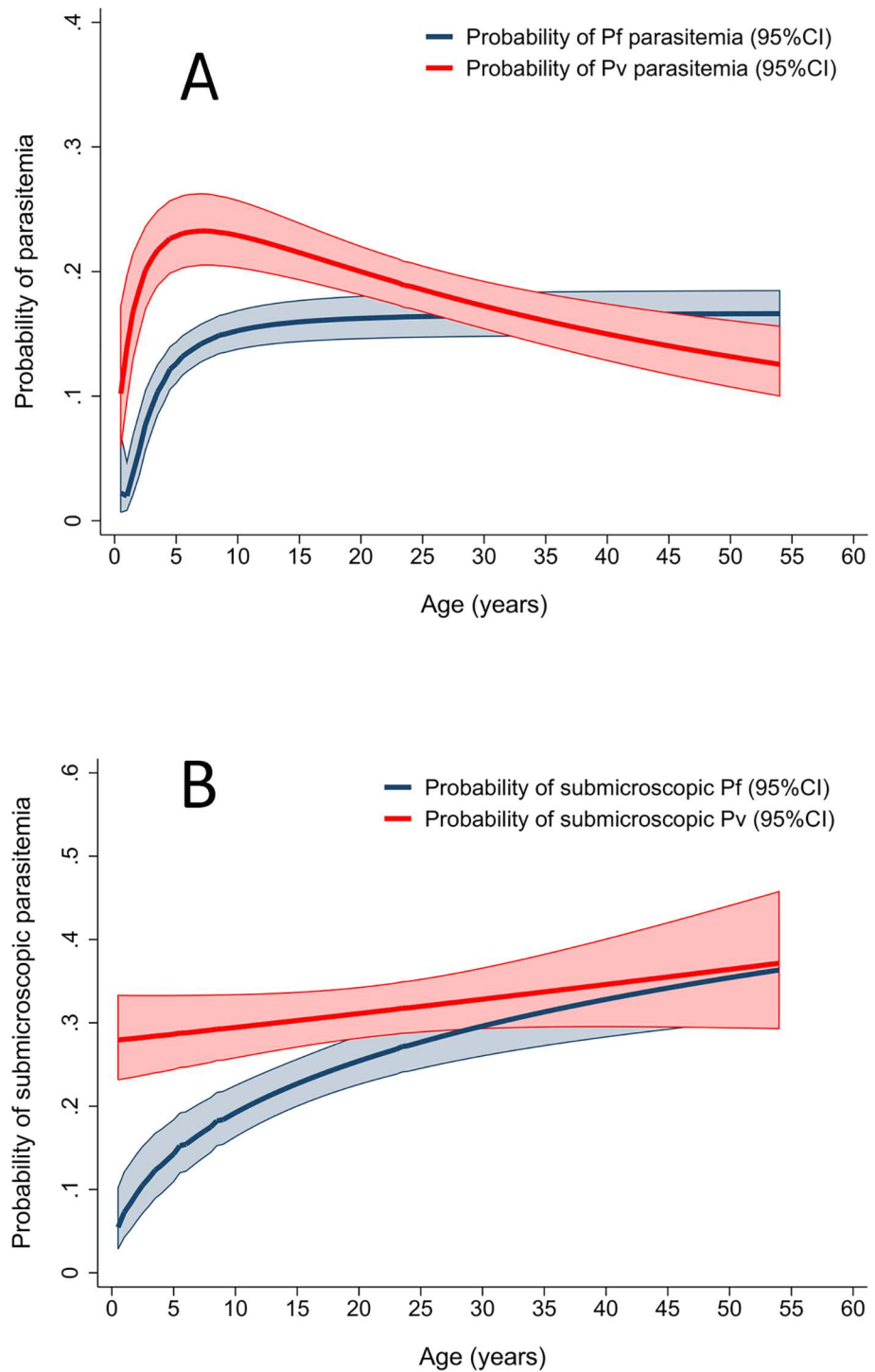


Fig 2. Multiple fractional polynomial curves showing the probability of *P. falciparum* and *P. vivax* parasitaemia. Probability of parasitaemia by age (A) and probability that detectable *P. falciparum* and *P. vivax* parasitaemia is submicroscopic by age (B).

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associated with a significantly lower mean Hb concentration (mean = 11.9 g/dL) compared to *P. vivax* (mean = 12.6 g/dL); $p = 0.003$), but similar to *P. malariae* (mean = 11.9 g/dL) and mixed infections (mean = 11.9 g/dL); Table 3.

Table 2. Risk factors for submicroscopic infection in 968 individuals with detectable parasitaemia.

Characteristics	Submicroscopic% (n/total)	Univariable Analysis		Multivariable Analysis	
		Crude OR [95% CI]	p	AOR [95% CI]	p
Age					
<5 years	37.2 (42/113)	Reference		Reference	
5 to 15 years	58.3 (162/278)	2.36 [1.51–3.70]	<0.001	2.35 [1.6–3.5]	<0.001
>15 years	70.5 (407/577)	4.04 [2.65–6.17]	<0.001	3.82 [2.2–6.7]	<0.001
Gender					
Male	58 (235/405)	Reference		Reference	
Female	66.8 (376/563)	1.5 [1.1–1.9]	0.006	1.41 [1.1–1.8]	0.002
Ethnicity					
Non-Papuan	63.6 (253/398)	Reference			
Highland Papuan	66.2 (151/228)	0.9 [0.6–1.2]	0.544		
Lowland Papuan	60.5 (207/342)	1.1 [0.8–1.5]	0.404		
Bednet use					
Yes	58.3 (197/338)	Reference		Reference	
No	65.7 (414/630)	1.4 [1.0–1.8]	0.025	1.4[0.9–2.1]	0.199
G6PD status^a					
Normal	63.3 (602/951)	Reference			
Deficient	64.3 (9/14)	1.0 [0.3–2.9]	1		
Fever in the last 24hr					
Yes	34.4 (11/32)	Reference		Reference	
No	64.1 (600/936)	3.4 [1.6–7.1]	0.001	3.2 [1.6–6.5]	0.001
Fever in last month					
Yes	52.6% (20/38)	Reference			
No	63.5% (591/930)	1.6 [0.8–3.0]	0.117		
Pregnancy^b					
Non Pregnant	73.3 (253/345)	Reference			
Pregnant	61.5 (8/13)	0.58 [0.2–1.8]	0.35		

Abbreviations: CI = confidence interval; OR = odds ratio; AOR = adjusted odds ratio; n = number.

^a Data missing on 3 cases

^b In 358 adult women.

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After controlling for age, sex and ethnicity, individuals with either microscopic or submicroscopic *P. falciparum* parasitaemia had a greater risk of anaemia compared to aparasitaemic individuals (AOR = 2.9 [95% CI: 1.8–4.6]; $p < 0.001$) and 1.9 [95% CI: 1.4–2.7]; $p < 0.001$, respectively; Table 4 and Fig 4). However, in those with *P. vivax* infections, only those with microscopic parasitaemia had an increased risk of anaemia (AOR = 1.8 [95% CI: 1.1–3.0]; $p = 0.016$). These trends were similar when restricting the analysis to severe or moderate anaemia (S3 Table). The adjusted risks were also similar after excluding 50 individuals with history of fever within the preceding 24 hours (S4 Table).

Discussion

Our study highlights that microscopic blood film examination alone detected only 37% (357/968) of individuals with parasitaemia in this cross sectional survey. Whilst the overall parasite prevalence was greatest in children and Papuan highlanders, the proportion of submicroscopic infections was greatest in older participants, females and those who did not own a bednet. Only 3.3% of participants reported a fever, but asymptomatic microscopic parasitaemia with either

Table 3. Haemoglobin concentration of 2757 individuals according to risk groups.

Risk factors	Total	Mean Hb g/dl	Anaemic % (n)	Univariable Analysis	p
		(SD)		Crude OR [95% CI]	
Age (years)					
<5	497	11.2(2.0)	42.5 (211)	Reference	
5-15y	661	12.1(2.17)	33.3 (220)	0.7 [0.5–0.9]	0.002
>15	1599	13.2(2.62)	29.6 (473)	0.6 [0.5–0.7]	<0.001
Gender					
Male	1123	12.9(2.7)	30.5 (343)	Reference	0.039
Female	1634	12.3(2.42)	34.3 (561)	1.18 [1.0–1.4]	
Ethnicity					
Non-Papuan	1501	13.3(2.2)	19.2 (288)	Reference	
Highland Papuan	529	11.6(3.0)	52.7 (279)	4.70[3.8–5.8]	<0.001
Lowland Papuan	727	11.9(2.5)	46.4 (337)	3.63[3.0–4.4]	<0.001
G6PD status					
Normal	2685	12.6(2.55)	33 (887)	Reference	0.099
Deficient	72	13.5(2.4)	23.6 (17)	0.63 [0.4–1.1]	
Bednet used					
Yes	956	12.5(2.6)	35.0 (335)	Reference	0.067
No	1801	12.6(2.5)	31.6 (569)	1.17 [1.0–1.4]	
Fever in the last 24hr					
Yes	54	12.4(3.6)	42.6 (23)	Reference	0.143
No	2703	12.6(2.5)	32.6 (881)	1.5 [0.9–2.6]	
Fever in last month					
Yes	112	12.3(2.6)	42.0 (47)	Reference	0.040
No	2645	12.6(2.5)	32.4 (857)	1.5 [1.0–2.2]	
Pregnancy					
Non Pregnant	1000	12.7(2.5)	32.1 (321)	Reference	0.103
Pregnant	45	11.1(2.31)	44.4 (20)	1.69 [0.9–3.1]	
<i>Plasmodium</i> species *					
Negative	1599	12.8 (2.4)	27.6 (442)	Reference	
<i>P. falciparum</i>	394	11.9 (3.0)	51 (201)	2.7 [2.2–3.4]	<0.001
<i>P. vivax</i>	470	12.6 (2.6)	32.3 (152)	1.3 [1–1.6]	0.049
<i>P. malariae</i>	36	11.9 (2.9)	47.2 (17)	2.3 [1.2–4.5]	0.014
<i>P. ovale</i>	1	9.1	100 (1)	-	-
Mixed infections	64	11.9 (3.3)	48.4 (31) (6)	2.5 [1.5–4.1]	0.001

Abbreviations: n = number; Hb = haemoglobin; SD = standard deviation; OR = Odds ratio.

* 193 cases did not have microscopic or PCR diagnosis.

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P. falciparum or *P. vivax* was associated with a three-fold increased risk of anaemia compared to aparasitaemic individuals. Similar to previous studies of both clinical malaria and asymptomatic infection, the degree of parasitaemia was correlated with anaemia [15, 24]. Whilst these findings are being increasingly recognised and reported, our study strengthens this relationship by demonstrating that the risk of anaemia, including that of moderate severe anaemia, extends to those with submicroscopic parasitaemia, for *P. falciparum* but not for *P. vivax*.

Plasmodium density can be controlled by acquired host immunity; hence, individuals who are repeatedly exposed to malaria have lower parasite densities compared to less-exposed individuals from the same region[25]. In our survey, older participants were almost 4 times more

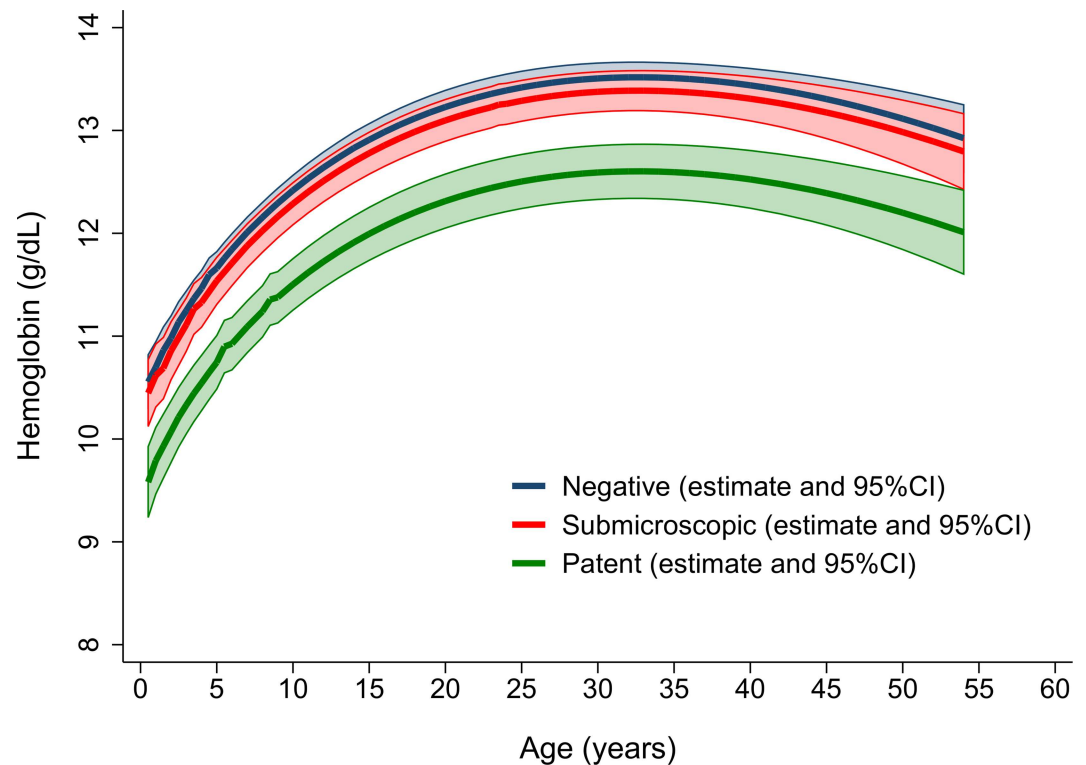


Fig 3. Multiple fractional polynomial curves showing the haemoglobin concentration according to microscopic and submicroscopic parasitaemia and age.

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likely to harbor submicroscopic infections than young children who had had less exposure to infection. The odds of submicroscopy was also significantly, albeit marginally, higher in those with *P. vivax* parasitaemia versus *P. falciparum* infection (OR = 1.35). Similar observations have been reported by other studies in areas of low transmission in the Solomon Islands, Thailand and Myanmar [20, 26] and likely reflect the earlier development of immunity following recurrent *P. vivax* infection compared to *P. falciparum* [27], as well as the lower peripheral parasitaemias associated with *P. vivax* [28]. An increasing body of work emphasizes that the proportion of detectable submicroscopic infections varies hugely, dependent upon the sensitivity of the underlying methodology [5]. Critical factors include the sample volume, collection and storage method, amplification method, and abundance of the target genetic material [20, 29]. Our assay was able to detect peripheral parasitaemias as low as 0.2 parasites/ μ L, similar to the sensitivity reported from previous studies using 200 μ l of capillary blood [20]. Individuals with asymptomatic and submicroscopic infections at these levels have been shown to be capable of infecting mosquitoes [9]. Hence, if elimination is to be achieved in a timely manner, suitable strategies are needed to actively identify and treat all infected individuals or populations, rather than passively waiting for symptomatic patients to present to healthcare facilities. Several public health measures have been proposed. Mass screen and test (MST) relies on rapid microscopic diagnosis or rapid diagnostic tests (RDTs) to identify parasitaemic individuals in need of treatment, including both symptomatic and asymptomatic infections [30]. However, in view of the high proportion of submicroscopic infections, MST will eliminate only a fraction of the parasite reservoir; thus its impact on transmission is likely to be limited [31]. Although diagnosis can be improved using more sensitive field-adapted diagnostics such as LAMP [32],

Table 4. Adjusted odds ratios for having anaemia according to WHO standards.

	n	Age Group (Years)										Overall				
		< 5					5 to 15					> 15				
		AOR	95% CI	P	AOR	95% CI	P	AOR	95% CI	P	AOR	95% CI	P			
Negative	1599															
Any Microscopic Parasitaemia ^a		4.4	2.2–8.8	<0.001	3.4	1.9–6.0	<0.001	1.6	1.2–2.3	0.002	2.3	1.7–3.2	<0.001			
Microscopic <i>P. falciparum</i>	164	8.6	1.8–40	0.006	4.6	2.0–10.2	<0.001	2.0	1.2–3.3	0.005	2.9	1.8–4.6	<0.001			
Microscopic <i>P. vivax</i>	163	3.4	1.5–8	0.005	2.3	1.2–4.5	0.018	1.1	0.6–2.2	0.776	1.8	1.1–3.0	0.016			
Microscopic <i>P. malariae</i>	20	-	-	-	-	-	-	-	-	-	2.7	[1.3–5.6]	0.007			
Microscopic Mixed Species	7	-	-	-	4.0	1.2–13.3	0.025	-	-	-	7.3	1.3–42	0.026			
Any Submicroscopic Parasitaemia ^b		1.00	0.5–2.1	1	2.1	1.3–3.3	0.002	1.1	0.8–1.4	0.591	1.3	0.9–1.7	0.115			
Submicroscopic <i>P. falciparum</i>	230	4.6	1.1–19	0.033	2.9	1.5–5.8	0.002	1.6	1.1–2.2	0.006	1.9	1.4–2.7	<0.001			
Submicroscopic <i>P. vivax</i>	307	0.6	0.3–1.2	0.124	1.6	0.9–2.6	0.083	0.7	0.5–1.1	0.090	0.9	0.6–1.2	0.386			
Submicroscopic <i>P. malariae</i>	16	-	-	-	-	-	-	-	-	-	1.0	0.3–3.7	0.964			
Submicroscopic Mixed Species	57	1.7	0.2–19	0.661	1.8	0.6–5.5	0.307	1.5	0.6–3.6	0.367	1.6	0.7–3.7	0.312			

Abbreviations: n = number; AOR = adjusted odds ratio; CI = confidence interval.

All models control for sex and ethnicity, with robust standard errors to account for clustering within households. In the overall models age group was also included.

^a3 cases had missing Hb measurement

^bOne submicroscopic case of *P. ovale* was not included in the table.

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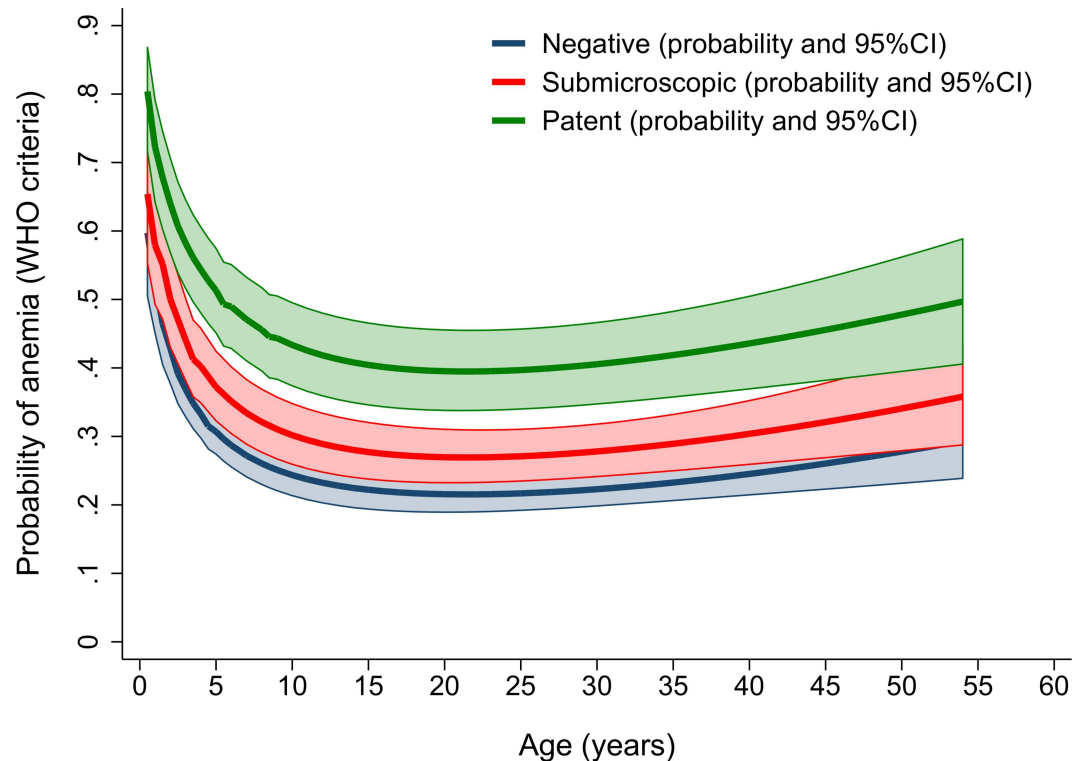


Fig 4. Multiple fractional polynomial curves showing the probability of anaemia by microscopic and submicroscopic parasitaemia and age.

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the sensitivity of these methods will also still miss the majority of individuals with low level parasitaemia[20].

Alternative strategies are needed to identify individuals and populations at greatest risk of infection using highly sensitive diagnostics so that community-based interventions can then be applied. Interventions such as mass drug administration (MDA) or targeted malaria elimination (TME) have been proposed [31], but these are extremely challenging since they usually require three days of antimalarial treatment administered to individuals who are either unaware that they are infected, or are not infected at all. This has cost implications, as well as exposing apparently well individuals to potential drug-related adverse effects. Rather than personal gain, the individual incentive to comply is, therefore, either altruistic (i.e., for the benefit of the community), or to gain some protection against malaria in the future. Our study provides evidence to mitigate this dilemma, by demonstrating that low-levels infections were associated with a significant morbidity, a three-fold increased risk of anaemia. Individuals with submicroscopic *P. falciparum* infection were also at greater risk of anaemia. Although in our study, anaemia was not associated with submicroscopic *P. vivax* parasitaemia, this has been reported previously from Brazil [33]. Treatment of patients with asymptomatic microscopic and submicroscopic parasitaemia, therefore, provides potential benefit for both the individual and the community.

Our observations could not be explained by recent symptomatic illness since fever in the preceding month was reported in only 4% (103/2567) of individuals. Rather the anaemia in parasitaemic individuals is likely to have arisen from a combination of low-grade chronic hemolysis, dyserythropoiesis, and reduced iron absorption [34–37]. Interestingly, detectable parasitaemia was significantly less prevalent in individuals with severe or intermediate G6PD

deficiency (OR = 0.44), and overall the risk of moderate anaemia was significantly higher in G6PD normal individuals (OR = 6.7 [1.6–28]; $p = 0.001$). Hence, whilst G6PD deficiency increases the risk of oxidative stress and drug-induced haemolysis, this appears to be counter-balanced by its protective effect against malaria-attributable anaemia.

The degree of *P. falciparum* associated anaemia is a function of peripheral parasitaemia [24] and this was apparent in our Papuan study with anaemia increasing with higher peripheral parasitaemia. In *P. vivax* infections, the level of parasitaemia is significantly lower than that with *P. falciparum* and anaemia appears to be related more closely to recurrent and chronic infection [34, 35]. Although we were able to quantify the level of microscopic parasitaemia by microscopy, we did not use a semi-quantitative PCR assay to determine the degree of submicroscopic parasitaemia [26, 38, 39]. It is plausible that in submicroscopic *P. vivax* infections the level of parasitaemia, whilst detectable by PCR, was at levels too low to cause significant haemolysis. Our study was also limited by being restricted to a single cross sectional observation with almost 30% of household members absent at the time of the survey. Prospective longitudinal studies will be needed to define better the persistence of submicroscopic infections over time and the associated of with recurrent parasitaemia. Since those individuals absent at the time of the survey were more likely to be adult males with a greater risk of submicroscopic infection, our estimates of parasite prevalence and the proportion of parasitaemia undetected by microscopy are likely to be conservative.

In conclusion, the prevalence of parasitaemia determined by PCR was approximately twice that detected by microscopy. Although most participants in the study were asymptomatic, those with any parasitaemia, including submicroscopic infections, were at significant risk of anaemia. Our analysis identified groups at significantly greater risk of submicroscopic infection, who should be targeted in surveys utilising ultrasensitive diagnostics aiming to quantify hidden reservoirs of infection. The magnitude of submicroscopic infections detected by our studies and others highlights the urgent need to develop new public health strategies and appropriate community-based interventions for the elimination of malaria.

Supporting Information

S1 Data.
(CSV)

S1 Fig. Study profile.
(TIF)

S1 Table. Characteristics of household individuals present and sampled during the survey and those absent from the household.
(DOCX)

S2 Table. The distribution and severity of anaemia.
(DOCX)

S3 Table. Adjusted odds ratios for severe or moderate anaemia in symptomatic and asymptomatic malaria.
(DOCX)

S4 Table. Adjusted odds ratios for severe or moderate anaemia in asymptomatic malaria stratified by age group.
(DOCX)

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