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Strengthening routine malaria surveillance through amplicon sequencing in Western Equatoria State, South Sudan: drug resistance and transmission dynamics

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Title

Strengthening routine malaria surveillance through amplicon sequencing in Western Equatoria State, South Sudan: drug resistance and transmission dynamics.

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Abstract

Background

Malaria is a major public health burden in South Sudan. However, the absence of routine molecular surveillance of malaria parasites limits evidence-based malaria control strategies able to counteract biological threats such as resistance to antimalarial drugs. To address this knowledge gap, a large molecular epidemiology study was conducted using targeted Amplicon Next-Generation Sequencing (AmpSeq).

Methods

Between November 2021 and June 2022 children under five years and pregnant women attending outpatient or antenatal care (ANC) at three Primary Health Care Centres (Mundri, Mvolo, and Lakamadi) in Western Equatoria State were enrolled. Participants underwent rapid diagnostic tests (RDTs) and Dried Blood Spots were collected. Data generated with AmpSeq were used to evaluate *Plasmodium* species, barcode call rate (proxy for parasite density), complexity of infection (COI), genetic differentiation (Fst) and drug resistance markers.

Results

Among 1,873 samples, *Plasmodium* was detected in 64.2% of samples by AmpSeq versus 26.5% by RDT ($p<0.001$). *P. falciparum* accounted for 97.3% of positive infections, while non-*falciparum* species were detected in 11.6%. Positivity and barcode call rate were higher in children than pregnant women ($p<0.001$) whereas COI and Fst were similar between groups. Mundri showed lower positivity, barcode call rate and COI than Lakamadi and Mvolo ($p<0.001$), suggesting lower transmission intensity, while low Fst indicated extensive gene flow across Western Equatoria. Positivity and barcode call rate decreased during drier months, while COI and Fst remained stable, suggesting sustained perennial malaria transmission. Among pregnant women, positivity and barcode call rate decreased with increasing gravidity ($p<0.001$), while COI and Fst were unchanged, consistent with gravidity-dependent immunity. Artemisinin resistance-associated *pfkelch13* mutations were detected in 1.6% of samples, including the WHO-validated A675V marker. Mutant *mdr1* haplotypes associated with resistance to the artemisinin partner drugs occurred in 59.4% of samples, and the quintuple *dhfr-dhps* mutant haplotype associated with sulfadoxine-pyrimethamine resistance reached a 67.5% frequency.

Conclusions

This is the first comprehensive AmpSeq-based molecular investigation of malaria parasites in South Sudan, providing the foundation for routine genomic surveillance. The findings highlight perennial transmission with monthly and regional variations, a substantial proportion of submicroscopic infections, and relevant circulation of non-*falciparum* species. The detection of *pfkelch13* artemisinin resistance mutations warrants the implementation of

a nationwide molecular surveillance system to guide National Malaria Control Program activities. Comparable population structure observed in pregnant women and children support ANC as a platform for molecular surveillance representative of the wider population.

Keywords

Malaria, South Sudan, Molecular surveillance, Antimalarial drug resistance

Abbreviations

AICS	Italian Agency for Development Cooperation
AmpSeq	Amplicon targeted Next-Generation Sequencing
ANC	AnteNatal Care
COI	Complexity of Infection
DBS	Dried Blood Spots
HS	Health System
IPTp	Intermittent Preventive Treatment in pregnancy
NGO	Non-Governmental Organisation
NMCP	National Malaria Control Program
NLPH	National Laboratory of Public Health
OPD	Outpatient Department
PHCC	Primary Health Care Centre
RDT	Rapid diagnostic test
SNP	Single Nucleotide Polymorphism
SSA	Sub-Saharan African
WHO	World Health Organization

Background

Malaria is a life-threatening and poverty-associated infectious disease caused by apicomplexan protozoa of the genus *Plasmodium* and transmitted by infective bites of female *Anopheles* mosquitoes in tropical and subtropical areas of the world. In 2024, malaria was endemic in 80 countries and caused an estimated 282 million clinical cases and 610,000 deaths, the great majority occurring in children and pregnant women in Sub-Saharan African (SSA) countries and caused by *P. falciparum* (1).

A significant reduction in malaria prevalence, morbidity and mortality has been observed between 2000 and 2015 globally and in SSA thanks to control strategies. However, decline stalled between 2015–2019. The incidence of clinical cases and deaths increased in 2020–21, due in part to service disruptions from the COVID-19 pandemic. Since then, morbidity has continued to increase while mortality has remained stable. The challenges to sustained success of malaria control include insufficient coverage of interventions, particularly among vulnerable populations and population groups, and biological threats such as resistance to insecticides by malaria vectors, ability to escape Rapid Diagnostic Testing (RDT) and resistance to antimalarial drugs by malaria parasites (1).

Malaria is endemic in South Sudan, with high intensity (>250 case per 1,000 people per year) transmission in the whole country (1,2). In 2021, when this study started, South Sudan accounted for 1.2% of global malaria cases and deaths (3). Transmission is perennial although seasonal variations are observed, with a peak of cases between July and October (2,4). The predominant parasite species is *P. falciparum* which represented 100% of cases

reported to WHO (5); however, the Malaria Indicator Survey previously conducted in 2017 reported the presence also of *P. malariae*, *P. ovale* and *P. vivax* (6). The major vector species are *An. gambiae, An. arabiensis, An. funestus,* while *A. nili* is a secondary vector, although little is known on their distribution and prevalence at subnational level (6).

At the time of the study, the National Malaria Strategic Plan 2014-2021 was in place, launched in 2014 by the Minister of Health of South Sudan with the support of the World Health Organization (WHO). Malaria control interventions included, for prevention, vector control through free of charge distribution to all age groups of long-asting insecticide treated nets (59.3% coverage in 2021), indoor residual spraying of houses and use of larvicides at sites of vector reproduction, as well as chemoprevention through intermittent preventive treatment in pregnancy (IPTp) with Sulfadoxine-Pyrimethamine (SP; <30% coverage in 2021). For case management, Rapid Diagnostic Test (RDT) detecting *P. falciparum* Histidine Rich Protein (HRP; 58.9% of suspected cases tested in 2021), Artemisinin Combination Therapy (ACT) - specifically Artesunate-amodiaquine (ASAQ) - for the treatment of uncomplicated cases and Arthemeter, Artesunate or Quinine monotherapy for severe cases, were provided free of charge to all age groups. The WHO reports inconsistent coverage data of the above interventions (3). Routine monitoring of parasite resistance to antimalarial drugs (1,7,8) and lack of HRP expression caused by *pfhrp2/hrp3* deletion (8–10) is not conducted, nor of vector resistance to insecticides (1,11). Nonetheless, partial resistance to artemisinin has been documented in East African countries neighbouring South Sudan (12–14), and markers of resistance to artemisinin and partner

drugs have been recently reported in South Sudan and South Sudanese refugees (15–17). Also, *pfhrp2/3* deletions have been reported among travellers returning from South Sudan (18) and, recently, among children in South Sudan (19).

The South Sudan Health System (HS) is organized to offer three levels of preventive and curative care: i) community based health care (Boma Health Initiative) provided by a network of village based agents (Boma Health Workers), in a target proportion of 1 every ≈100 inhabitants, whose mandate and tasks are in line with the Integrated Community Case Management approach (e.g. diagnosis and treatment of uncomplicated malaria/diarrhoea/pneumonia in children under 5 years of age, tracking and referral of children under 1 year of age with missing vaccinations, informing and sensitizing local population on the main primary health care topics); ii) primary health care, provided by health care professionals operating in Units (providing outpatients consultations, ante and post-natal care, family planning, routine immunization and management of acute malnutrition for children and pregnant/lactating women) and Centres (Units package of services plus inpatient care, delivery assistance, basic obstetric complications handling); iii) secondary care, provided by healthcare professionals operating in County or State Hospitals (complementing the primary care service package with the comprehensive management of obstetric complications, elective and emergency surgical interventions, severe cases inpatient care). The HS counts about 1980 primary health care facilities, 78 County Hospitals, 10 State Hospitals. Malaria services are integrated at each level of care.

Since 2013, the functioning of the HS relies on the financial and technical support provided by a restricted group of international donors (Health Pooled Fund until 2024; Health System Transformation Project from 2024), and by the implementation support provided by selected Non-Governmental Organisations (NGOs), that work aside County health authorities to implement the policies defined by the National Ministry of Health (at the time of the study, Basic Package for Health and Nutrition Services) and deliver services at each of the above mentioned levels of care.

In this context, the NGO Doctors With Africa CUAMM (CUAMM) coordinated a programme aimed at strengthening malaria response in three Counties of Western Equatoria State, South Sudan, through the improvement in access, utilization and quality of preventive/diagnostic/curative services and their integration into the three levels of care of the HS. The programme was funded by the Italian Agency for Development Cooperation (AICS) in the framework of the Technical Support Spending to Global Fund. A capacity building and research component led by the University of Pisa (UNIPI) was carried out, aiming at enhancing malaria diagnosis and surveillance by narrowing current gaps through training and large-scale epidemiology investigations at three peripheral health centres, located in Mundri East, Mundri West and Mvolo Counties in Western Equatoria State. Training included malaria testing by RDT and microscopy, collection of Dried Blood Spot (DBS) samples, DNA isolation from DBS and *Plasmodium* speciation by qPCR. The epidemiological investigation targeted children less than five years old and pregnant women, a population groups particularly vulnerable to malaria infection, and included the

collection of DBS to enable Next Generation Sequencing (NGS) analysis and genotyping of *Plasmodium* parasites, including markers of drug resistance (20–22).

Methods

Study sites and population

The epidemiological investigation was conducted over a period of six months (November 2021-June 2022) at three Primary Health Care Centres (PHCCs) in Western Equatoria State, South Sudan: Mundri PHCC (Mundri West County), Mvolo PHCC (Mvolo County), and Lakamadi PHCC (Mundri East County). Participants included children under five years of age and pregnant women, recruited during routine outpatient (OPD) or antenatal care (ANC) visits respectively. Participants were recruited regardless of the presence of fever or other clinical signs of uncomplicated disease. Capacity building activities were conducted at the study PHCCs as well as at Lui Hospital in Mundri East and at the National Laboratory of Public Health (NLPH) in Juba.

Capacity building

The study included an essential component of capacity building aimed to improve the quality of both routine malaria services and research activities. At each PHCC, laboratory needs assessment was performed prior to the beginning of the study and all necessary material was provided and installed. A 1-week training course covered biology and control of malaria, study objectives and Standard Operating Procedures (SOPs), malaria diagnosis by RDT (23) and microscopy (24,25). The course included theoretical and practical sessions,

as well as assessments, and was attended by PHCC and County Health Department staff members, CUAMM County Coordinators (study supervisors), and Co-Principal Investigators. Training was followed by on-the-job supervision during the first week of recruitment and monthly during the study. A further 1-day training was conducted at the midpoint of the study. At Lui Hospital, training on malaria microscopy, including on-job supervision and internal/external quality assessment, was conducted at the beginning of the study (3 days) and at the midpoint of the study (2 days). At NLPH, a 1-day training on molecular assays was conducted, including isolation of genomic DNA from Dried Blood Spots (DBS) and Real-Time PCR for *Plasmodium* detection and species identification.

Participant recruitment

Based on PHCC attendance data from 2019, the study aimed to recruit a total of 2000 subjects during a 6-month period, namely 400 in Lakamadi PHCC, 800 in Mundri PHCC and 800 in Mvolo PHCC, with a 1:1 ratio between children and pregnant women.

Recruitment of participants started on 22nd November 2021 and ended on 7th June 2022. Patients attending OPD and ANC services have been informed about the study and invited to participate during routine consultation. For children attending OPD services, eligibility criteria were age between six months and five years, and absence of any conditions requiring emergency care. For women attending ANC services, eligibility criteria included being pregnant at any gestational age, being of any age, having no conditions requiring emergency care, and absence of signs of labour. Patients willing to participate in the study were asked to sign a written informed consent.

Data and sample collection

At recruitment, each participant was assigned a unique study ID/barcode that was reported on the written informed consent and data collection forms, and on biological specimens.

The data collection form was used to register: i) date, PHCC (Lakamadi, Mundri, Mvolo) and department (OPD, ANC) of consultation; ii) personal information (date of birth, sex, number of past pregnancies); iii) axillary temperature (°C); iv) collection (yes/no) of biological specimens (malaria Rapid Diagnostic Test, blood slide, Dried Blood Spot).

Capillary blood samples were obtained in the PHCC laboratory by finger pick according to WHO guidelines for phlebotomy (26). Approximately 150µl of blood were collected per participant using a sterile plastic micropipette. The collected blood was used to perform a malaria RDT (see subsection "Malaria diagnosis by RDT and treatment") and to prepare blood films and DBS. One thick and one thin blood film were prepared for each participant according to WHO guidelines for malaria microscopy (27). One paper (Whatmann 903TM) card was prepared for each participant, with two dried blood spots (DBS) of three drops of capillary blood (~15µl) each. Paper cards were air-dried for four hours and individually stored in sealed plastic bags containing desiccant.

Malaria diagnosis by RDT and treatment

A malaria RDT was performed in the PHCC laboratory for each participant, regardless of the presence of malaria signs or symptoms. The test (Malaria Pf/Pan Antigen Test, Beacon), which detects both *P. falciparum* HRP and *Plasmodium* spp. LDH, was conducted according to the manufacturer's instructions. Notably, the test used in this study differed from the RDT

routinely employed in South Sudan (First Response Malaria Antigen *P. falciparum* HRP2), which detects only *P. falciparum* HRP. Despite training, laboratory staff experienced difficulties in recording individual HRP and LDH results and therefore documented only the overall RDT outcome, classifying samples as positive regardless of whether the HRP band, LDH band, or both bands were detected. In accordance with the guidelines of the NMCP of South Sudan (28) and as per study PHCC routine service delivery, PHCC OPD or ANC clinical staff offered antimalarial treatment with ACT (Artesunate+Amodiaquine) to participants with a positive RDT result and fever (axillary temperature>37.5°C).

DNA isolation

DBS samples were shipped to MalariaGEN Laboratory at the Wellcome Sanger Institute (WSI), Hinxton, UK where they were processed with the SpotMalaria platform (29). Samples were registered and tracked in a secure online database, where location and date of collection were recorded. DNA was extracted from samples using the QIAamp DNA Investigator Kit (QUIAGEN) according to manufacturer's instructions. Parasite DNA was amplified by applying selective whole genome amplification (sWGA) as previously described (30).

Genotyping

Amplicon Next Generation Sequencing (AmpSeq) was performed by Illumina MiSeq and targeted two mitochondrial regions, antimalarial drug resistance related mutations within 10 genes (*mdr1*, *mdr2, dhfr, crt, dhps, kelch13, exo, fd, arps10, plasmepsin2/3;* detailed in Table

S2) in *P. falciparum*, as well as 101 Single Nucleotide Polymorphisms (SNPs) across the *P. falciparum* genome that were selected for their high variation between parasite clones (29). Genotypes were called using the AmpRecon pipeline v1.4.0 (31). Genetic barcodes were constructed by concatenating the 101 SNP alleles (29). Genotyping success in a given sample is dependent on the number of sequencing reads, that, in turn, depends on parasite density; the proportion of successfully genotyped barcode positions, or barcode call rate, can be therefore used as a proxy of parasite density. The drug resistance genotypes and genetic barcode of each sample, together with species identification and estimated complexity of infection (see below), were subsequently assembled in a single tabular file designated as the "Genetic Report Card".

Species identification

Sequencing data targeting conserved regions of the mitochondrial genome was used to identify *Plasmodium* species (29). Initial species classification was performed using Kraken2 (v2.0.7), and results were summarized with krakentools (v1.2), based on a custom database containing mitochondrial sequences from six *Plasmodium* species (Table S1). A species was considered present in a sample if it was assigned at least five reads and constituted at least 1% of total reads. To validate these classifications, both assigned and unassigned reads were re-analyzed using Kraken2 with a more stringent confidence threshold (20% of total reads) against the PlasmoDB database, which includes genomes of 45 *Plasmodium* strains (32). Samples for which a species could be identified were considered *Plasmodium* positive by AmpSeq.

Complexity of infection

A malaria infection can either be sustained by a unique clone (monoclonal infection) or by multiple and genetically different clones (polyclonal infection). The number of *P. falciparum* clones with distinct genotype, or Complexity of Infection (COI), was estimated using THE REAL McCOIL method using the genetic barcode in each sample (33). Polyclonality was defined as proportion of samples with COI >1, excluding samples with undetermined COI, and is reported with Wilson score 95% confidence intervals. Association between polyclonality of parasite populations with the epidemiological factors was assessed by multivariable logistic regression. Odds ratios were adjusted for all other variables included in the model, and the contribution of each factor was assessed using a likelihood-ratio test.

Population structure

Sample-level and barcode SNP-level quality control was performed prior to genetic diversity and population structure analyses. Samples were retained if they were identified as *Pf* with the AmpSeq method, monoclonal (the COI value of 1), and had at least 70 of 100 barcode SNPs successfully called. SNPs were retained if a call was obtained in at least 75% of the samples. Only homozygous calls were used in the computation. Expected heterozygosity (He) was calculated at each SNP as (34):

$$He = \left[\frac{n}{n-1}\right][1 - \sum pi^2]$$

where *n* is the number of homozygous calls at locus and *pi'* is the frequency of the *i*th allele in a given population, and averaged across loci. Confidence intervals were obtained by bootstrapping loci (2,000 replicates, percentile method) and mean He was compared

between parasite populations by two-sided label-permutation test (10,000 permutations) with Holm–Bonferroni correction.

Genetic differentiation between parasite populations was assessed using Wright's fixation index (Fst) with the following formula:

$$Fst = (H_T - H_S)/H_T$$

where H_S is the mean within-population expected heterozygosity and H_T the expected heterozygosity of the pooled sample, each averaged over loci (35). Pairwise Fst value of 0.05 and below was considered indicative of low genetic differentiation between two parasite populations. Additional sample-level pairwise genetic distance was also calculated as the proportion of mismatching alleles across loci at which both samples carried a homozygous call. The resulting distance matrix was used for principal coordinate analysis by classical multidimensional scaling (36).

All statistical analyses and plotting listed in this section were performed in Python v3.9 using open-access libraries (NumPy v1.26.4, pandas v2.2.2, SciPy v1.14.1, statsmodels v0.14.4, Matplotlib v3.8.4).

Drug resistance

Depending on the allele frequency in a given sample, the genotype call at a given SNP was considered as either homozygous, heterozygous or missing. A homozygous genotype required at least 10 high-quality reads supporting a single allele. A heterozygous genotype required at least 5 high-quality reads supporting the minor allele, with the minor allele representing a minimum of 10% of the total reads at that position. Other calls were

categorised as missing. To calculate population-level allele frequencies for drug resistance mutations (Table S2), heterozygous calls were excluded, and the allele frequency was calculated as the proportion of homozygous alternate calls among all homozygous calls (37). Likewise, haplotypes were constructed by concatenating homozygous calls at SNPs within the same gene, and haplotype frequencies were calculated as the proportion of alternate haplotypes among all constructed haplotypes.

Data management and analysis

Data collection forms have been collected monthly from each study PHCC by CUAMM study supervisors, checked for completeness/accuracy and validated. Data from validated forms have been entered into a centralized dataset by two different UNIPI operators and checked for entry errors and discrepancies using EpiData. This clinical dataset was then merged with the dataset containing the genotyping information (Genetic Report Card).

The distribution of study participants by population group, study site, and month of sample collection was calculated. As recruitment began at the end of November 2021, data for November and December were combined and reported as Nov/Dec. Similarly, as recruitment ended at the beginning of June 2022, data for May and June were combined and reported as May/Jun. Age was missing for 43.0% of participants and was therefore excluded from analysis. The proportion of female subjects and the proportion of subjects with fever were calculated and compared between groups using the Likelihood Ratio test.

The results of RDT and AmpSeq were compared using a two-by-two table and by comparing the proportion of positive results between the two methods.

For both methods, the proportion of positive results with its Standard Error (SE) and 95% Confidence Interval (95% CI) was computed and shown using barplots. The association between epidemiological factors (population group, PHCC, month, fever, gravidity) and RDT and AmpSeq positivity (yes/no) was investigated by multivariate logistic regression, and Odds Ratio with its 95% Confidence Interval and P-value was reported.

Among AmpSeq positive samples, the median of barcode call rates with its interquartile range (IQR) was computed and the distribution was shown using boxplots. The association between epidemiological factors and barcode call rate was investigated by multivariate linear regression, and the Beta coefficient with its 95% Confidence Interval and P-value was reported.

Analyses listed in this section have been performed in STATA v.13.

Results dissemination

Preliminary findings including RDT results were shared with County Health Departments, PHCC staff and the National Malaria Control Programme at the end of the epidemiological study in June 2022. Final results including the Genetic Report Card, a guide to the interpretation of the data, and a summary presentation were shared with the National Malaria Control Programme after completion of AmpSeq analysis in September 2024.

Ethical considerations

The study protocol was approved by the Ethical Committee of the South Sudan Ministry of Health (MOH-RERB approval N°: 22/10/11/2021). The decision to participate in the study

was entirely voluntary. Written informed consent has been sought from parents/guardians of children attending OPD visit and from pregnant women attending ANC visits, using forms that have been developed based on a template elaborated by the Global Health Network (38). For subjects unable to read English, an oral translation of the content of the informed consent form has been made into the local language in the presence of an adult scholar serving as a witness. For subjects unable to read and write, consent has been given by fingerprint instead of signature. Confidentiality and anonymity of participants have been guaranteed throughout the study. Data are herein reported in an aggregated way with no information that could allow identification of participants. Shipment of DBS to the WSI was regulated by a Human Material transfer Agreement approved by the South Sudan Ministry of Health.

Results and discussion

A total of 1,873 participants were recruited for which DBS were collected and AmpSeq was performed successfully (Table S3), corresponding to 93.7% of the target study population. Participants included 51.7% children under five years of age and 48.3% pregnant women, closely matching the original 1:1 recruitment target. The distribution across study sites was also close to the planned 1:2:2 ratio, with 20.6% of participants recruited in Lakamadi, 38.0% in Mundri, and 41.4% in Mvolo. Participant recruitment was not consistent over time, with month-to-month fluctuations due to staff shortages (November/December 2021=125, January 2022=108, February 2022=561, March 2022=281, April 2022=478, May/June 2022=320). The sex ratio (50% female) of children did not vary across study sites (P-

value=0.246). The proportion of patients with fever was higher among children (11.6%, CI=9.7–13.8%) than pregnant women (2.6%, CI=1.7–3.8%; P-value<0.001). Among children, febrile patients were less frequent in Lakamadi than in Mundri and Mvolo (P-value=0.004), while no differences between study sites were observed among pregnant women (P-value=0.406).

Plasmodium DNA was detected by AmpSeq in 1,202 of the 1,873 samples that were successfully analysed (64.2%). In the vast majority of positive samples (97.3%), the detected species was *P. falciparum*, either as a mono-infection or in mixed infections with *P. malariae*, *P. ovale wallikeri* and *P. ovale curtisi* (Fig. 1).

Among the 1,735 samples for which both AmpSeq and valid RDT results were available (Table S4), *Plasmodium* infection was detected in 64.7% of samples by AmpSeq compared with 26.5% by RDT (P-value <0.001; 2A). Of the 679 samples with false-negative RDT results, 27 (4.0%) carried non-*falciparum* infections, 49 (7.2%) carried mixed *P. falciparum* infections, and the remaining 603 (88.8%) carried *P. falciparum* mono-infections. Among AmpSeq-positive samples, the proportion of successfully genotyped barcode positions (barcode call rate) was significantly lower in RDT-negative (median=11.9%, IQR=4.0-26.7%) than in RDT-positive (median=92.1%, IQR=34.7-99.0%) samples (P-value<0.0001; Fig. 2B). Together, these findings indicate that, as expected, AmpSeq detected *Plasmodium* infections with higher sensitivity than RDTs and suggest that false-negative RDT results are likely attributable to infections caused by non-*falciparum* species and/or parasite densities below the RDT limit of detection, which are associated with lower AmpSeq barcode call rates.

The COI was estimated in 545 (46.6%) of the 1,170 *P.falciparum* positive samples, which were then used in the polyclonality analysis and 99 SNPs passed the QC ahead of the population structure analysis.

To investigate malaria epidemiology in the study area, metrics of *P. falciparum* infection, the most prevalent species, were calculated and their variation assessed according to population group, study site, month of sample collection, presence of fever, and, among pregnant women, gravidity (i.e. the number of pregnancies). Multivariate regression was used to evaluate variation in the proportion of AmpSeq-positive samples (Table 1, Fig. 3), used as a proxy for infection prevalence, and – among positive samples - in barcode call rate (Table 2, Fig. 4), used as a proxy for parasite density. In the subset of samples for which COI could be estimated, variation was analysed according to the same factors to further investigate differences in transmission intensity. Finally, variation in measures of parasite genetic differentiation, including Fst and pairwise genetic distances, was examined to assess patterns of population structure.

P. falciparum positivity was significantly higher among children under five years of age (69.7%) than among pregnant women (54.9%). Similarly, the barcode call rate was higher in children (32.7%) than in pregnant women (15.8%). These findings indicate that both the risk of infection and parasite density are lower in pregnant women in the study area, consistent with the expected effect of age-dependent immunity to malaria parasites. No significant differences (P-value=0.343) in COI were observed between the children and pregnant

442 women groups, indicating exposure to same level of transmission, as expected (Fig. 5A).
443 Similarly, parasite genetic diversity, measured with mean heterozygosity (*He*), was indifferent
444 in these two groups as *Fst* value was near 0 (Table S6). PCA of pairwise genetic distances
445 further demonstrated no distinct clusters of parasites between the same two groups (Fig.
446 S1).
447
448 Interestingly, *P. falciparum* positivity was significantly lower in Mundri PHCC (48.4%) than in
449 Lakamadi (70.7%) and Mvolo (71.2%) PHCCs, suggesting lower malaria transmission in the
450 Mundri area. To further investigate this hypothesis, analyses were stratified by population
451 group, as infection estimates among children attending the OPD may be influenced by
452 differences in healthcare access, whereas those among pregnant women attending ANC are
453 expected to be less affected by such bias. The differences in positivity between PHCCs were
454 confirmed in both children (Mundri: 56.0%, 95% CI=50.8–61.0%; Lakamadi: 86.7%, 95%
455 CI=81.2–90.8%, P-value<0.001; Mvolo: 74.0%, 95% CI=69.5–78.1%, P-value<0.001) and
456 pregnant women (Mundri: 49.5%, 95% CI=40.1–59.0%; Lakamadi: 58.7%, 95% CI=46.1–
457 70.3%, P-value=0.009; Mvolo: 69.1%, 95% CI=60.8–76.2%, P-value<0.001). Consistent with
458 these findings, the barcode call rate was also significantly lower in Mundri (14.9%) than in
459 Lakamadi (22.8%) and Mvolo (27.7%). Lower transmission in the Mundri area was confirmed
460 by a lower polyclonality (29.4%; 95% CI = 20.8–39.8%; Figure 5A), which was significantly
461 different from Mvolo (45.3%, 95% CI = 37.3–53.6%) but not Lakamadi (40.2%, 95% CI = 30.3–
462 51.1%; Table S5). Nonetheless, the near 0 pairwise *Fst* values between the study sites
463 indicated very low genetic differentiation (Table S6). PCA of pairwise genetic distances

revealed no clear population structure (Figure S1), with samples from different locations clustering closely together. These results collectively suggest extensive gene flow among parasite populations across the Western Equatoria region.

With respect to the month of sample collection, AmpSeq positivity remained relatively stable between November and March (range: 62.8–76.8%), declined in April (47.9%), and returned to previous levels in May–June (67.8%). A similar pattern was observed for barcode call rate, which showed little variation from November–December through February (range: 24.8–31.7%), decreased in March and April (range: 15.3–18.8%), and then increased sharply in May–June (43.6%). These findings suggest that malaria transmission remains relatively stable for several months after the end of the rainy season, reaches its lowest levels during the driest months of the year, and rises rapidly with the onset of the rains (39). Importantly, they also indicate that malaria transmission is sustained throughout the dry season. No statistically significant differences in the proportion of polyclonal infections between months were observed (Figure 5A, Table S5), confirming stable transmission across rainy and dry seasons (39). Similarly, there was no distinct population structure between the low (January-April) and high (May-December) transmission seasons (Fig. S1, Table S6).

AmpSeq positivity did not differ significantly according to the presence of fever (febrile=69.7%; afebrile=61.6%). In contrast, median barcode call rate was significantly higher among febrile (97.0%) than among afebrile (21.8%) subjects. These findings are likely due to the high sensitivity of AmpSeq, which enables the detection of low-density

asymptomatic infections, and by higher parasite densities associated with clinical manifestations (31) that are reflected by the barcode call rate metric.

Among pregnant women attending ANC, AmpSeq positivity and barcode call rate were further evaluated according to gravidity (Table S7, Table S8). AmpSeq positivity was highest during the first pregnancy (60.5%), showed a non-significant decline during the second (51.6%) and third pregnancies (56.3%), and was significantly lower during the fourth or subsequent pregnancies (51.2%). A similar but more evident pattern was observed for barcode call rate, which decreased from 26.2% in the first pregnancy to 15.8% in the second (non-significant trend), 13.9% in the third, and 14.4% in the fourth or subsequent pregnancies. These findings indicate the acquisition of gravidity-dependent immunity to malaria parasites, with multigravidae being less susceptible to infection than primigravidae, in line with previous evidence (40,41). The lack of significant differences in COI according to gravidity (P-value=0.6, Table S7) suggests that the number of distinct parasite clones harboured by infected individuals reflects transmission intensity (42) rather than immunity. The genetic differentiation between the parasites infecting primigravidae and multigravidae was low (*Fst*<0.05, Table S6).

Variation in RDT positivity according to epidemiological factors (Table S9, Fig. S2) differed from the pattern observed for AmpSeq positivity but more closely resembled that of barcode call rate. For example, the proportion of positive RDT result was much higher in febrile (54.8%) than afebrile (23.9%) subjects (P-value<0.001), unlike the proportion of positive

AmpSeq results which was comparable in the two groups, but similarly to the median barcode pass rate which was higher in participants with fever, as previously described. This finding is likely attributable to the lower sensitivity of RDTs, whereby differences in positivity reflect differences in parasite density that fall below or above the RDT limit of detection (43).

Genotyping of loci associated with resistance to antimalarial drugs in *P. falciparum* (Fig. 6, Table S10) revealed the presence of *kelch13* mutations in 1.6% of samples, including A675V (0.3%), a WHO validated marker of artemisinin resistance (44,45). A parasite genetic background based on mutations at multiple genes and that allows for the emergence of *kelch13* mutations has been previously described (45); mutant haplotypes were observed with a 21.6% frequency. Mutant *mdr1* haplotypes associated with resistance to the artemisinin partner drugs amodiaquine, lumefantrine and mefloquine (46–48) were observed in 59.4% of samples. In particular, 86Y and 1246Y mutations associated with increased tolerance to amodiaquine had 9.0% and 7.2% frequency, respectively. The point-break mutation causing *plasmepsin2/3* duplication and resistance to piperaquine a further artemisinin partner drug (49), was observed in 0.5% of samples, while the 414G *exonuclease* mutation associated with resistance to the same drug (50) was not observed. The frequency of *crt* CVIET haplotype associated with chloroquine resistance (51,52) was much lower, 25.0%, likely as a result of reduced drug pressure since chloroquine has not been in use for several decades. Finally, the quintuple mutant *dhfr-dhps* haplotype IRN-GEA associated with mid-level resistance to sulfadoxine-pyrimethamine (SP) (53,54) - used for chemoprevention among pregnant women and, in association with amodiaquine, among children - showed a

67.5% frequency, while the sixtuple mutant haplotype IRN-GEG associated with high-level resistance (53,54) showed a 1.4% frequency.

Few publications have previously reported on the frequency of antimalarial drug resistance markers in South Sudan (Table S10). Two studies were conducted in Western Equatoria State, in 2019-2020, the same period of the study herein presented, in the context of the pilot implementation of Seasonal Malaria Chemoprevention with SPAQ in Yombio County. The first study used Sanger sequencing to detect *pfk13* mutations among children (n=468) with uncomplicated malaria (16). Eight distinct *pfk13* mutations were detected across 11 samples (2.4% overall frequency), although none of them was a WHO-validated marker of artemisinin resistance. The second study used PCR-RFLP to genotype markers of AQ and SP resistance at *mdr1*, *dhfr*, *dhps* loci also among children (n=532) with uncomplicated malaria (15). The *mdr1* N86Y mutation had 18.9% frequency and the D1246Y mutation a 9.5% frequency. The *dhfr-dhps* IRNGEA quintuple mutant was predominant (62.4%), while the IRNGEG sixtuple mutant had a low frequency (4.5%). A further study was conducted in Uganda in 2022, and used molecular inversion probe (MIP) technology to genotype drug resistance markers at *kelch13*, *mdr1*, *dhfr*, *dhps* loci among among asymptomatic children refugees (n=52) from South Sudan (17). The frequency of *kelch13* 675V was 15.4% and that of C469Y was 8.0%. Also, high prevalence of the *mdr1* N86Y mutation (33.3%) was observed. Although the frequency of *dhfr-dhps* mutations was generally high, the frequency of combined haplotypes was not computed. Overall, the frequencies of mutant alleles or haplotypes observed in the present study are comparable to those obtained in a different County from Western

552 Equatoria in the same study period, while they are different (generally lower) than those
553 obtained from refugees in Uganda (Table S10). This is likely due to refugees originating from
554 a different area of South Sudan, or from multiple regions.
555
556 **Conclusions**
557 To our knowledge, this is the first comprehensive AmpSeq-based molecular investigation of
558 malaria parasites conducted in South Sudan, encompassing genetic markers of antimalarial
559 drug resistance and providing evidence to inform the activities of the National Malaria
560 Control Program (NMCP).
561 Findings demonstrated how molecular metrics can be derived from AmpSeq results and
562 usefully employed to investigate malaria epidemiology. In particular, barcodes of
563 concatenated SNP alleles can be used to calculate COI, a useful proxy of transmission
564 intensity, as well as Fst and pairwise genetic distances, metrics of population structure that
565 can provide complementary insights on transmission dynamics, as previously shown (55).
566 Furthermore, barcode call rate, the proportion of successfully genotyped barcode position,
567 was shown for the first time to be a useful proxy of parasite density.
568 In Western Equatoria State, *P. falciparum* transmission was shown to be stable during the
569 dry season, albeit of lower intensity. Also, differences in transmission intensities between
570 Counties could be identified. Such evidence can guide subregional tailoring of control
571 interventions and the strategic allocation of resources.

572 Although *P. falciparum* was the most prevalent species, non-falciparum species were
573 detected in 11.6% of samples. This observation might suggest the adoption of an RDT based
574 on both Pf-HRP and Pan-LDH detection to reduce the occurrence of false negative results.
575 The *kelch13* A675V validated marker of artemisinin resistance was detected for the first time
576 in South Sudan, among other non-synonymous mutations. Despite their current low
577 frequency, along with the high frequency of mutations associated with resistance to
578 amodiaquine, the artemisinin partner drug currently in use in South Sudan for first-line
579 malaria treatment (artesunate-amodiaquine, AS-AQ), this observation warrants the design
580 and implementation of a nationwide molecular surveillance system to monitor the spread of
581 antimalarial resistance markers.
582 Currently, Whole Genome Sequencing of *P. falciparum* positive samples by Illumina MySeq
583 is underway. Results will allow calling of Copy Number Variations such as *hrp2/3* deletions,
584 responsible of false negative results of RDT based on HRP (8–10), as well as *plasmepsin2/3*
585 duplication, which correlates with piperaquine resistance (49,50), and *mdr1* duplication, a
586 correlate of resistance to lumefantrine and mefloquine, further artemisinin partner drugs
587 (56–58). These data will complement AmpSeq findings in guiding NLMP activities.
588 The burden of infection observed both by RDT and AmpSeq among pregnant women, while
589 lower than among children as expected, is noteworthy. Although most infections were
590 asymptomatic, the possibility that they affect pregnancy outcomes cannot be excluded, in
591 particular among primigravidae, where higher parasite densities were observed (59–63). This
592 finding, combined with evidence from routine health facilities data (DHIS2) showing low
593 coverage of ANC, IPTp and ITN among pregnant women in Western Equatoria State,

indicates a need to investigate existing barriers and implement context-adapted interventions to improve access and utilisation of malaria preventive measures by pregnant women (64–66). Such investigation is currently ongoing in partnership with the Ministry of Health. This finding also indicates that malaria testing regardless of symptoms at the first ANC contact could provide an important health benefit to pregnant women, in particular during the first trimester, when chemoprevention with SP is contraindicated.

Barcode analysis revealed no remarkable differences in parasite population structure between children and pregnant women, similarly to findings from a recent study in the Democratic Republic of Congo (67). It has been previously suggested that pregnant women represent an ideal population group to monitor malaria transmission intensity and effectiveness of control interventions as they provide an unbiased estimate of infection prevalence (68–70). The present findings confirm that pregnant women attending ANC are an optimal sentinel population for molecular surveillance, as the collected evidence is representative of the general population (67). Consequently, a surveillance system where data and sample collection are integrated as part of routine ANC services at the first consultation is being designed in collaboration with the NMCP.

Finally, further training at the National Laboratory of Public Health is needed to enhance national capacity in malaria molecular surveillance. Such surveillance will enable the NMCP to monitor the major biological threats to malaria control, evaluate the effectiveness of malaria diagnostic, preventive and treatment tools, and to make counteracting evidence-based decisions.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Review Board of the Ministry of Health of the Republic of South Sudan (22/10-06-2021, renewal 22/10-11-2021). The same board authorised publication of findings (D.03/2024). Every subject enrolled in the study has provided written informed consent to participation.

Availability of data and materials

In accordance with the study protocol approved by the Ethics Committee, data are reported only in aggregated form. The individual-level dataset is not publicly available but may be made available upon reasonable request to the corresponding author.

Competing interests

The authors report there are no competing interests to declare.

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Authors' contributions

MP, AAR, EU, RDP, CA, VW, MO, JL, DO, MW, DL, FL, BP, VDM performed research activities. GJMT, VAA and VDM coordinated the research. GP, CS, LT and VDM designed the research. VDM, EU and VW performed data analysis and VDM wrote the draft of the manuscript. All authors revised and approved the final version of the manuscript.

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References

1. World Health Organization, editor. World malaria report 2025: Addressing the threat of antimalarial drug resistance. Geneva: World Health Organization; 2025.

2. National Malaria Control Programme, editor. South Sudan Malaria Epidemiological Stratification 2025. Juba, South Sudan: Ministry of Health; 2025.

3. World Health Organization, editor. World Malaria Report 2022. Geneva: World Health Organization; 2022. 1 p.

4. National Malaria Control Programme, editor. South Sudan Malaria Indicator Survey 2023, Final Report. Juba, South Sudan: Ministry of Health; 2023.

5. National Malaria Control Programme, editor. South Sudan Malaria Indicator Survey 2017, Final Report. Juba, South Sudan: Ministry of Health; 2017.

6. Haileselassie W, Ejigu A, Alemu T, Workneh S, Habtemichael M, David RE, et al. International border malaria transmission in the Ethiopian district of Lare, Gambella region: implications for malaria spread into South Sudan. Malar J. 2023 Feb 22;22(1):64. doi:10.1186/s12936-023-04479-5

7. Strategy to Respond to Antimalarial Drug Resistance in Africa. Geneva: World Health Organization; 2022. 1 p.

8. Greenwood B. Artemisinin-Resistant and HRP-Negative Malaria Parasites in Africa. N Engl J Med. 2023 Sep 28;389(13):1162–4. doi:10.1056/NEJMp2309142

9. Beshir KB, Parr JB, Cunningham J, Cheng Q, Rogier E. Screening strategies and laboratory assays to support Plasmodium falciparum histidine-rich protein deletion surveillance: where we are and what is needed. Malar J. 2022 Jun 24;21(1):201. doi:10.1186/s12936-022-04226-2

10. World Health Organization, editor. Response plan to pfhrp2 gene deletions. 2nd edn. Geneva: World Health Organization; 2024.

11. Ranson H, Lissenden N. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends Parasitol. 2016 Mar;32(3):187–96. doi:10.1016/j.pt.2015.11.010

12. Balikagala B, Fukuda N, Ikeda M, Katuro OT, Tachibana SI, Yamauchi M, et al. Evidence of Artemisinin-Resistant Malaria in Africa. N Engl J Med. 2021 Sep 23;385(13):1163–71. doi:10.1056/NEJMoa2101746

13. Conrad MD, Asua V, Garg S, Giesbrecht D, Niaré K, Smith S, et al. Evolution of Partial Resistance to Artemisinins in Malaria Parasites in Uganda. N Engl J Med. 2023 Aug 24;389(8):722–32. doi:10.1056/NEJMoa2211803

14. Fola AA, Feleke SM, Mohammed H, Brhane BG, Hennelly CM, Assefa A, et al. Plasmodium falciparum resistant to artemisinin and diagnostics have emerged in Ethiopia. Nat Microbiol. 2023 Aug 28;8(10):1911–9. doi:10.1038/s41564-023-01461-4

15. Molina-de La Fuente I, Sagrado Benito MJ, Lasry E, Ousley J, García L, González V, et al. Seasonal malaria chemoprevention in a context of high presumed sulfadoxine-pyrimethamine resistance:

malaria morbidity and molecular drug resistance profiles in South Sudan. Malar J. 2023 Nov 10;22(1):345. doi:10.1186/s12936-023-04740-x

16. Molina-de La Fuente I, Sagrado Benito MJ, Ousley J, Gisbert FDB, García L, González V, et al. Screening for K13-Propeller Mutations Associated with Artemisinin Resistance in Plasmodium falciparum in Yambio County (Western Equatoria State, South Sudan). Am J Trop Med Hyg. 2023 Nov 1;109(5):1072–6. doi:10.4269/ajtmh.23-0382

17. Tukwasibwe S, Garg S, Katairo T, Asua V, Kagurusi BA, Mboowa G, et al. Varied Prevalence of Antimalarial Drug Resistance Markers in Different Populations of Newly Arrived Refugees in Uganda. J Infect Dis. 2024 Aug 16;230(2):497–504. doi:10.1093/infdis/jiae288

18. Prosser C, Gresty K, Ellis J, Meyer W, Anderson K, Lee R, et al. *Plasmodium falciparum* Histidine-Rich Protein 2 and 3 Gene Deletions in Strains from Nigeria, Sudan, and South Sudan. Emerg Infect Dis. 2021 Feb;27(2):471–9. doi:10.3201/eid2702.191410

19. Molina-de La Fuente I, Benito MJS, Flevaud L, Ousley J, Pasquale HA, Julla A, et al. *Plasmodium falciparum pfhrp2* and *pfhrp3* Gene Deletions in Malaria-Hyperendemic Region, South Sudan. Emerg Infect Dis. 2023 Jan;29(1):154–9. doi:10.3201/eid2901.220775

20. Dalmat R, Naughton B, Kwan-Gett TS, Slyker J, Stuckey EM. Use cases for genetic epidemiology in malaria elimination. Malar J. 2019 Dec;18(1):163. doi:10.1186/s12936-019-2784-0

21. Neafsey DE, Taylor AR, MacInnis BL. Advances and opportunities in malaria population genomics. Nat Rev Genet. 2021 Aug;22(8):502–17. doi:10.1038/s41576-021-00349-5

22. Miotto O. Genetic Epidemiology Use Cases for Malaria Control Programmes: A Methodology [Internet]. Malaria Genomic Epidemiology Network; 2023. Available from: https://malariagen.net/resources/use-cases-for-nmcps/

23. World Health Organization, editor. How to use a Rapid Diagnostic Test, a guide for training at village and clinic level. Geneva: World Health Organization; 2010.

24. World Health Organization, editor. Basic malaria microscopy. Part I. Learner guide. 2nd ed. Geneva: WHO; 2010. 2 p.

25. World Health Organization, editor. Basic malaria microscopy. Part II. Tutor guide. 2nd ed. Geneva: WHO; 2010. 2 p.

26. Dhingra N. WHO guidelines on drawing blood: best practices in phlebotomy. Geneva, Switzerland: Safe Injection Global Network, World Health Organization; 2010. 1 p.

27. World Health Organization, editor. Malaria microscopy quality assurance manual. Version 2. Geneva: World Health Organization; 2016. 1 p.

28. National Malaria Control Programme, editor. South Sudan malaria case management guidelines and training manual. Juba, South Sudan: Ministry of Health; 2015.

29. Jacob CG, Thuy-Nhien N, Mayxay M, Maude RJ, Quang HH, Hongvanthong B, et al. Genetic surveillance in the Greater Mekong subregion and South Asia to support malaria control and elimination. eLife. 2021 Aug 10;10:e62997. doi:10.7554/eLife.62997

30. Oyola SO, Ariani CV, Hamilton WL, Kekre M, Amenga-Etego LN, Ghansah A, et al. Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. Malar J. 2016 Dec;15(1):597. doi:10.1186/s12936-016-1641-7

31. Unlu E. AmpRecon pipeline v1.4.0 [Internet]. Malaria Genomic Epidemiology Network. Available from: https://github.com/malariagen/AmpRecon

32. Lu J, Salzberg S. EuPathDB Eukaryotic Pathogen Database Project [Internet]. Available from: https://ccb.jhu.edu/data/eupathDB/

33. Chang HH, Worby CJ, Yeka A, Nankabirwa J, Kamya MR, Staedke SG, et al. THE REAL McCOIL: A method for the concurrent estimation of the complexity of infection and SNP allele frequency for malaria parasites. Pascual M, editor. PLOS Comput Biol. 2017 Jan 26;13(1):e1005348. doi:10.1371/journal.pcbi.1005348

34. Anderson TJC, Haubold B, Williams JT, Estrada-Franco§ JG, Richardson L, Mollinedo R, et al. Microsatellite Markers Reveal a Spectrum of Population Structures in the Malaria Parasite Plasmodium falciparum. Mol Biol Evol. 2000 Oct 1;17(10):1467–82. doi:10.1093/oxfordjournals.molbev.a026247

35. Nei M. Analysis of Gene Diversity in Subdivided Populations. Proc Natl Acad Sci. 1973 Dec;70(12):3321–3. doi:10.1073/pnas.70.12.3321

36. Gower JC. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika. 1966;53(3–4):325–38. doi:10.1093/biomet/53.3-4.325

37. White NFD, Whitton G, Wasakul V, Amenga-Etego L, Dara A, Andrianaranjaka V, et al. Disparate co-evolution and prevalence of sulfadoxine and pyrimethamine resistance alleles and haplotypes at dhfr and dhps genes across Africa. Sci Rep. 2025 Apr 17;15(1):13222. doi:10.1038/s41598-025-98035-7

38. Global Health Network. Informed consent template [Internet]. Available from: https://globalhealthtrials.tghn.org/articles/downloadable-templates-and-tools-clinical-research/

39. Chen H, Guo J, Zhang Z, Xu CY. Prediction of temperature and precipitation in Sudan and South Sudan by using LARS-WG in future. Theor Appl Climatol. 2013 Aug;113(3–4):363–75. doi:10.1007/s00704-012-0793-9

40. Ataíde R, Mayor A, Rogerson SJ. Malaria, primigravidae, and antibodies: knowledge gained and future perspectives. Trends Parasitol. 2014 Feb;30(2):85–94. doi:10.1016/j.pt.2013.12.007

41. Walker PGT, Griffin JT, Cairns M, Rogerson SJ, Van Eijk AM, Ter Kuile F, et al. A model of parity-dependent immunity to placental malaria. Nat Commun. 2013 Mar 19;4(1):1609. doi:10.1038/ncomms2605

42. Watson OJ, Okell LC, Hellewell J, Slater HC, Unwin HJT, Omedo I, et al. Evaluating the Performance of Malaria Genetics for Inferring Changes in Transmission Intensity Using Transmission Modeling. Leitner T, editor. Mol Biol Evol. 2021 Jan 4;38(1):274–89. doi:10.1093/molbev/msaa225

43. Wu L, Van Den Hoogen LL, Slater H, Walker PGT, Ghani AC, Drakeley CJ, et al. Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. Nature. 2015 Dec;528(7580):S86–93. doi:10.1038/nature16039

44. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature. 2014 Jan;505(7481):50–5. doi:10.1038/nature12876

45. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, et al. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat Genet. 2015 Mar;47(3):226–34. doi:10.1038/ng.3189

46. Nsobya SL, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ. *In Vitro* Sensitivities of *Plasmodium falciparum* to Different Antimalarial Drugs in Uganda. Antimicrob Agents Chemother. 2010 Mar;54(3):1200–6. doi:10.1128/AAC.01412-09

47. Venkatesan M, Gadalla NB, Stepniewska K, Dahal P, Nsanzabana C, Moriera C, et al. Polymorphisms in Plasmodium falciparum Chloroquine Resistance Transporter and Multidrug Resistance 1 Genes: Parasite Risk Factors That Affect Treatment Outcomes for P. falciparum Malaria After Artemether-Lumefantrine and Artesunate-Amodiaquine. Am Soc Trop Med Hyg. 2014 Oct 1;91(4):833–43. doi:10.4269/ajtmh.14-0031

48. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, Uhlemann AC, et al. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat Commun. 2016 May 18;7(1):11553. doi:10.1038/ncomms11553

49. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, et al. A surrogate marker of piperaquine-resistant Plasmodium falciparum malaria: a phenotype–genotype association study. Lancet Infect Dis. 2017 Feb;17(2):174–83. doi:10.1016/S1473-3099(16)30415-7

50. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, et al. Genetic markers associated with dihydroartemisinin–piperaquine failure in Plasmodium falciparum malaria in Cambodia: a genotype–phenotype association study. Lancet Infect Dis. 2017 Feb;17(2):164–73. doi:10.1016/S1473-3099(16)30409-1

51. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the P. falciparum Digestive Vacuole Transmembrane Protein PfCRT and Evidence for Their Role in Chloroquine Resistance. Mol Cell. 2000 Oct;6(4):861–71. doi:10.1016/S1097-2765(05)00077-8

52. Wellems TE, Plowe CV. Chloroquine-Resistant Malaria. J Infect Dis. 2001 Sep 15;184(6):770–6. doi:10.1086/322858

53. Picot S, Olliaro P, De Monbrison F, Bienvenu AL, Price RN, Ringwald P. A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. Malar J. 2009 Dec;8(1):89. doi:10.1186/1475-2875-8-89

54. Van Eijk AM, Stepniewska K, Khairallah C, Rodriguez E, Ahn J, Gutman JR, et al. The impact of sulfadoxine–pyrimethamine resistance on the effectiveness of intermittent preventive treatment for the prevention of malaria in pregnancy in Africa: an updated systematic review and meta-analysis. Lancet Infect Dis. 2025 Dec;25(12):1336–46. doi:10.1016/S1473-3099(25)00219-1

55. Ngoi JM, Oriero EC, Ünlü ES, Thompson K, Ayeke D, Sakyi MLE, et al. Characterising recent antimalarial resistance in West Africa: Insights from amplicon sequencing of 17,384 Plasmodium falciparum infection samples. Wellcome Open Res. 2026 Aug 8;11:515. doi:10.12688/wellcomeopenres.26525.1

56. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature. 2000 Feb;403(6772):906–9. doi:10.1038/35002615

57. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. The Lancet. 2004 Jul;364(9432):438–47. doi:10.1016/S0140-6736(04)16767-6

58. Malmberg M, Ferreira PE, Tarning J, Ursing J, Ngasala B, Björkman A, et al. Plasmodium falciparum Drug Resistance Phenotype as Assessed by Patient Antimalarial Drug Levels and Its Association With pfmdr1 Polymorphisms. J Infect Dis. 2013 Mar 1;207(5):842–7. doi:10.1093/infdis/jis747

59. Cottrell G, Moussiliou A, Luty AJF, Cot M, Fievet N, Massougbodji A, et al. Submicroscopic Plasmodium falciparum Infections Are Associated With Maternal Anemia, Premature Births, and Low Birth Weight. Clin Infect Dis. 2015 May 15;60(10):1481–8. doi:10.1093/cid/civ122

60. Rogerson SJ, Desai M, Mayor A, Sicuri E, Taylor SM, Van Eijk AM. Burden, pathology, and costs of malaria in pregnancy: new developments for an old problem. Lancet Infect Dis. 2018 Apr;18(4):e107–18. doi:10.1016/S1473-3099(18)30066-5

61. Accrombessi M, Yovo E, Fievet N, Cottrell G, Agbota G, Gartner A, et al. Effects of Malaria in the First Trimester of Pregnancy on Poor Maternal and Birth Outcomes in Benin. Clin Infect Dis. 2019 Sep 27;69(8):1385–93. doi:10.1093/cid/ciy1073

62. Satapathy P, Khatib MN, Gaidhane S, Zahiruddin QS, Sharma RK, Rustagi S, et al. Adverse pregnancy outcomes in maternal malarial infection: A systematic review and meta-analysis. New Microbes New Infect. 2024 Dec;62:101474. doi:10.1016/j.nmni.2024.101474

63. Gilder ME, Saito M, Haohankhunnatham W, Ling CL, Gornsawun G, Bancone G, et al. Submicroscopic malaria in pregnancy and associated adverse pregnancy events: A case-cohort study of 4,352 women on the Thailand–Myanmar border. Beeson JG, editor. PLOS Med. 2025 Mar 4;22(3):e1004529. doi:10.1371/journal.pmed.1004529

64. Hill J, Hoyt J, Van Eijk AM, D'Mello-Guyett L, Ter Kuile FO, Steketee R, et al. Factors Affecting the Delivery, Access, and Use of Interventions to Prevent Malaria in Pregnancy in Sub-Saharan Africa: A Systematic Review and Meta-Analysis. Noor AM, editor. PLoS Med. 2013 Jul 23;10(7):e1001488. doi:10.1371/journal.pmed.1001488

65. Koita K, Kayentao K, Worrall E, Van Eijk AM, Hill J. Community-based strategies to increase coverage of intermittent preventive treatment of malaria in pregnancy with sulfadoxine–pyrimethamine in sub-Saharan Africa: a systematic review, meta-analysis, meta-ethnography, and economic assessment. Lancet Glob Health. 2024 Sep;12(9):e1456–69. doi:10.1016/S2214-109X(24)00228-6

66. Kretchy IA, Atobrah D, Adumbire DA, Ankamah S, Adanu T, Badasu DM, et al. Enhancing the uptake of intermittent preventive treatment for malaria in pregnancy: a scoping review of interventions and gender-informed approaches. Malar J. 2025 Feb 18;24(1):49. doi:10.1186/s12936-025-05275-z

67. Onyamboko M, Wasakul V, Bakomba SB, Kayembe DK, Nzambiwishe BK, Ekombolo PE, et al. Pregnant women as a sentinel population for genomic surveillance of malaria in the Democratic Republic of the Congo: a population-based study. Lancet Glob Health. 2025 Mar;13(3):e479–87. doi:10.1016/S2214-109X(24)00497-2

68. Mayor A, Menéndez C, Walker PGT. Targeting Pregnant Women for Malaria Surveillance. Trends Parasitol. 2019 Sep;35(9):677–86. doi:10.1016/j.pt.2019.07.005

69. Pujol A, Brokhattingen N, Matambisso G, Mbeve H, Cisteró P, Escoda A, et al. Detecting temporal and spatial malaria patterns from first antenatal care visits. Nat Commun. 2023 Jul 6;14(1):4004. doi:10.1038/s41467-023-39662-4

70. Da Silva C, Matambisso G, Boene S, Rovira-Vallbona E, Pujol A, Comiche K, et al. *Plasmodium falciparum* molecular surveillance to inform the Mozambican National Malaria Control Programme strategy: protocol. BMJ Open. 2024 Nov;14(11):e092590. doi:10.1136/bmjopen-2024-092590

TABLES

Table 1 *Plasmodium falciparum* AmpSeq positivity

Factor	Category	Pf positivity (%)		Association with Pf positivity		
		Proportion	95% CI	OR	95% CI	P-value
Population group	PW (ref)	54.6	51.4-57.9	.	.	.
	U5	69.7	66.8-72.5	1.9	1.6-2.4	0.000
PHCC	Mundri (ref)	48.4	44.7-52.1	.	.	.
	Lakamadi	70.7	66.0-75.1	2.7	2.0-3.6	0.000
	Mvolo	71.2	67.9-74.3	2.7	2.1-3.4	0.000
Month	Nov-Dec 2021	76.8	68.6-83.4	2.6	1.7-4.2	0.000
	Jan 2022	63.9	54.4-72.4	1.2	0.7-1.8	0.502
	Feb 2022	68.2	64.2-71.9	2.4	1.8-3.1	0.000
	Mar 2022	62.6	56.8-68.1	1.7	1.3-2.4	0.001
	Apr 2022 (ref)	47.9	43.6-52.4	.	.	.
	May-Jun 2022	67.8	62.5-72.7	2.0	1.5-2.8	0.000
Fever	No (ref)	61.6	59.3-63.9	.	.	.
	Yes	69.7	61.3-77.0	1.2	0.8-1.8	0.397
Total		62.4	60.2-64.6	.	.	.

The table shows the proportion (%) of *P. falciparum* positive AmpSeq results and its 95% Confidence Interval (CI) according to population group, PHCC, month and presence of fever. The table also shows the results of multivariate logistic regression analysis (Odds Ratio with its 95% CI, P-value) investigating the association between these different factors with AmpSeq positivity. Legend: Pf, *P. falciparum*; PW, pregnant women; ref, reference category; OR, Odds Ratio.

888 **Table 2** *Plasmodium falciparum* AmpSeq barcode call rate

889

Factor	Category	Pf call rate (%)		Association with Pf call rate		
		Median	IQR	Beta coeff	95% CI	P-value
Population group	PW (ref)	15.8	5.0–50.1	.	.	.
	U5	32.7	8.9–97.0	0.15	0.11 - 0.19	0.000
PHCC	Mundri (ref)	14.9	3.0–78.2	.	.	.
	Lakamadi	22.8	10.9–94.1	0.09	0.03 - 0.15	0.003
	Mvolo	27.7	9.9–91.1	0.09	0.03 - 0.14	0.001
Month	Nov-Dec 2021	26.2	8.7–75.0	0.04	-0.05 - 0.12	0.428
	Jan 2022	31.7	13.4–91.6	0.09	-0.01 - 0.20	0.066
	Feb 2022	24.8	7.7–93.1	0.09	0.03 - 0.15	0.004
	Mar 2022	15.3	4.0–54.2	0.00	-0.08 - 0.07	0.958
	Apr 2022 (ref)	18.8	6.4–66.3	.	.	.
	May-Jun 2022	43.6	9.9–98.5	0.16	0.09 - 0.23	0.000
Fever	No (ref)	21.8	5.9–82.2	.	.	.
	Yes	97.0	20.8–99.0	0.22	0.13 - 0.30	0.000
Total		23.8	6.9-89.4	.	.	.

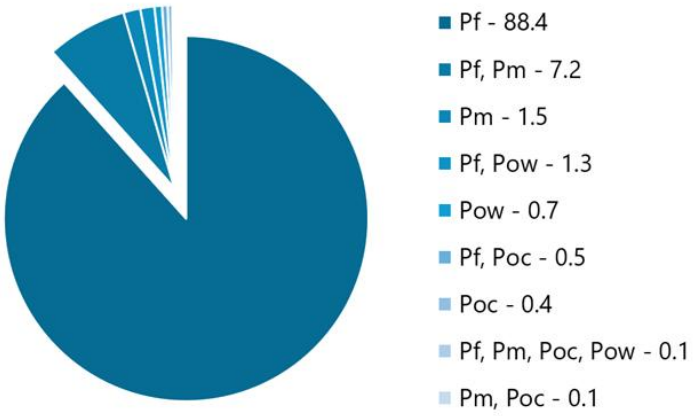
890

891 The table shows the median *P. falciparum* AmpSeq barcode call rate and its InterQuartile
892 Range (IWR) according to population group, PHCC, month and presence of fever. The table
893 also shows the results of multivariate linear regression analysis (Beta coefficient with its 95%
894 CI, P-value) investigating the association between these different factors and AmpSeq call
895 rate. Legend: Pf, *P. falciparum*; PW, pregnant women; ref, reference category; Beta coeff, Beta
896 coefficient.

897

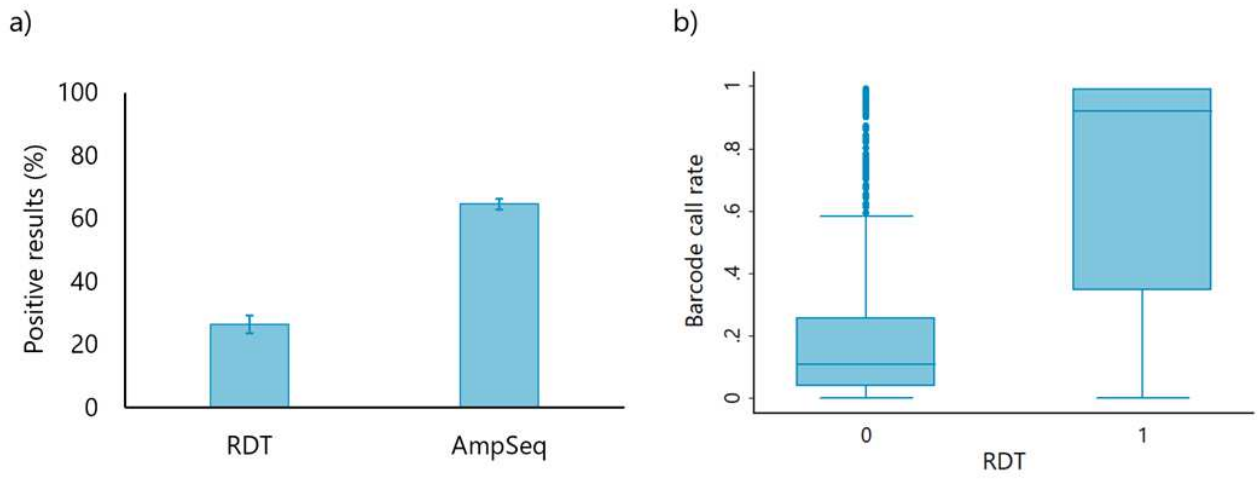
FIGURES

Figure 1 *Plasmodium* species detected by AmpSeq



The pie-chart shows the relative proportion of samples where infection with different Plasmodium species was detected by AmpSeq. Legend: Pf, *P. falciparum*; Pm, *P. malariae*, Poc, *P. ovale curtisi*; Pow, *P. ovale wallikeri*.

907 **Figure 2** Comparison of RDT and AmpSeq results



908

909 Panel a: the barplot show the proportion of samples with a positive result (%) by RDT and

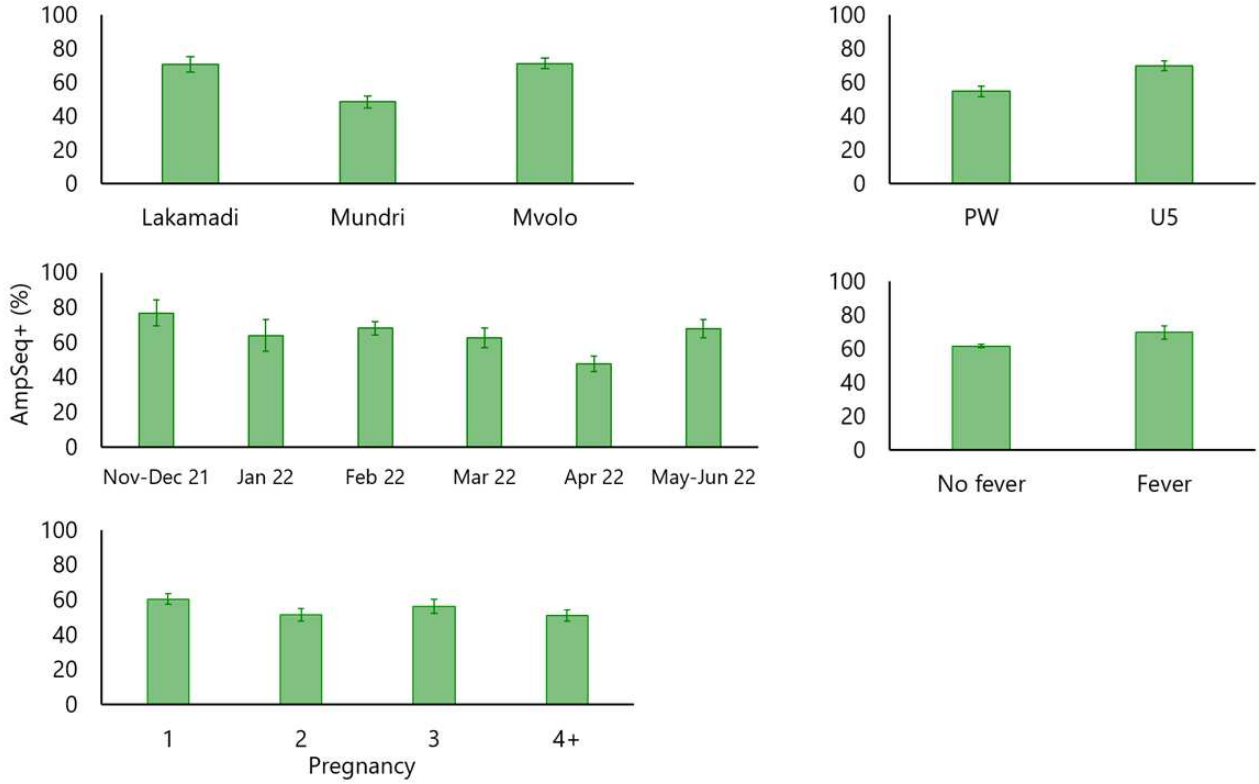
910 AmpSeq detection of Plasmodium infection (panel a). Panel b: the boxplot shows the

911 distribution of barcode call rate among RDT negative (0) and positive (1) samples.

912

913 **Figure 3** *P. falciparum* AmpSeq positivity according to epidemiological factors

914



915

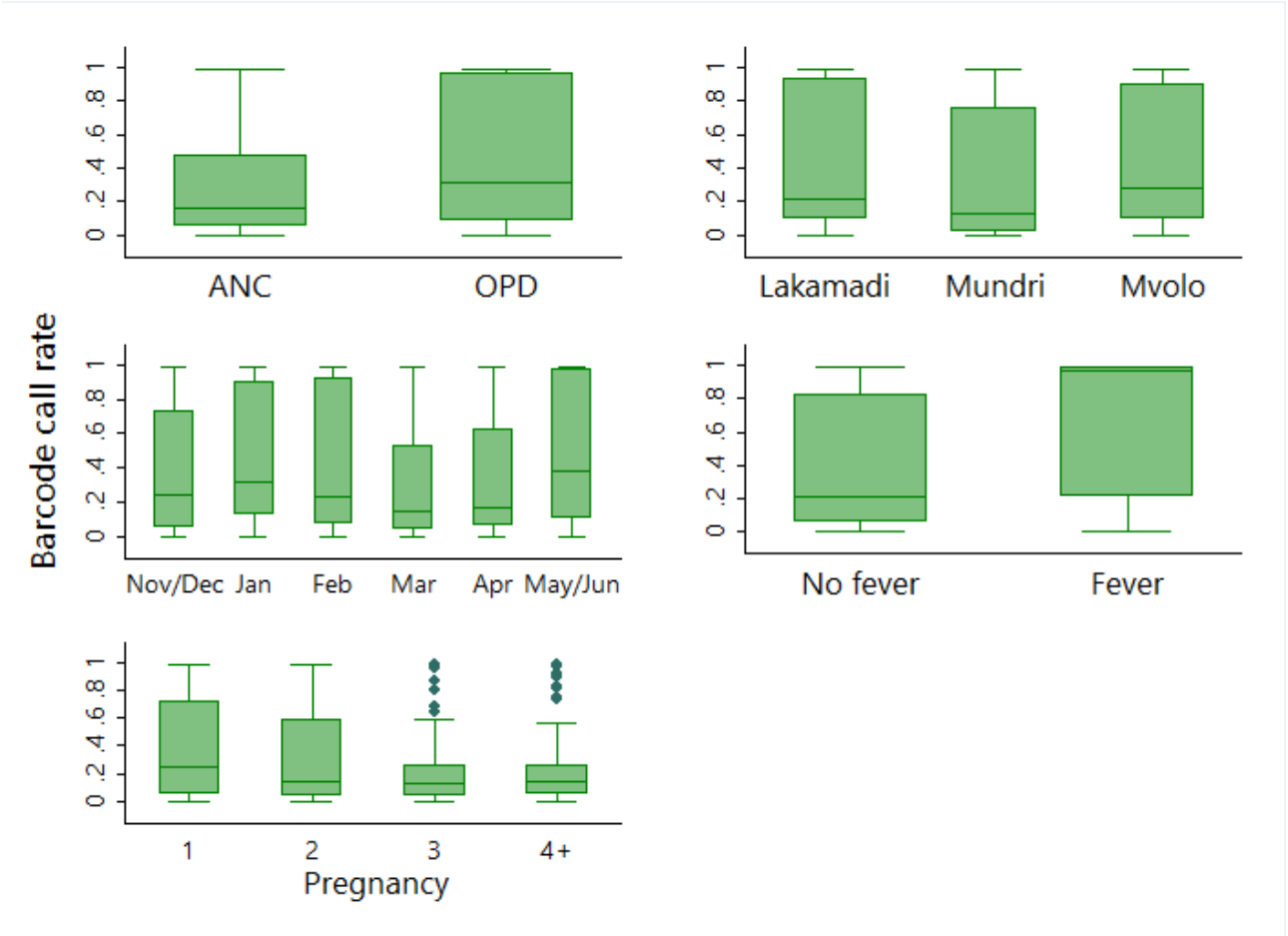
916 The barplots show the proportion of AmpSeq results positive for *P. falciparum* according

917 to study PHCC (panel a), population grop (panel b), months of sample collection (panel c),

918 presence of fever (panel d), and – among pregnant women – gravidity (panel e).

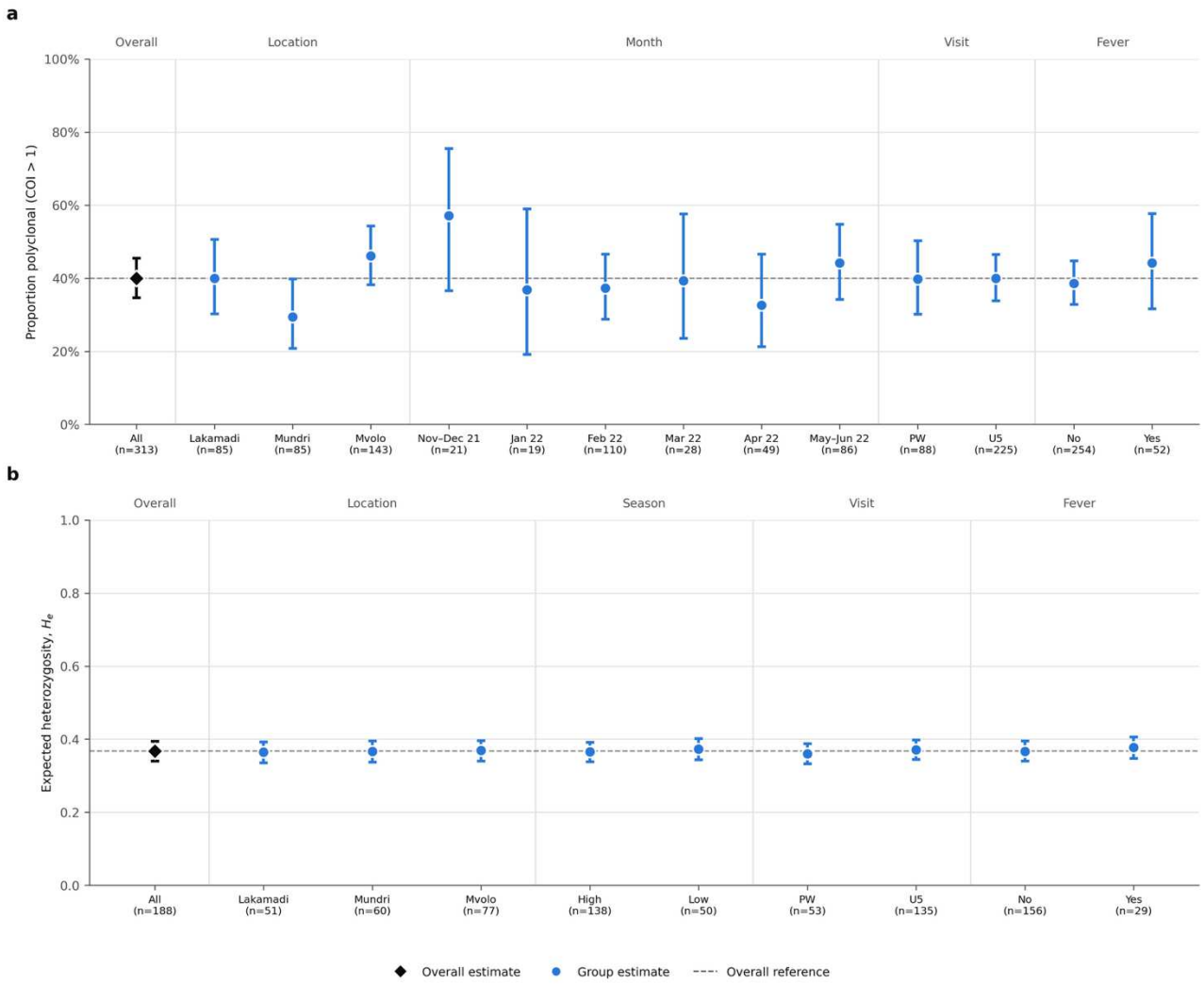
919

Figure 4 *P. falciparum* AmpSeq barcode call rate according to epidemiological factors



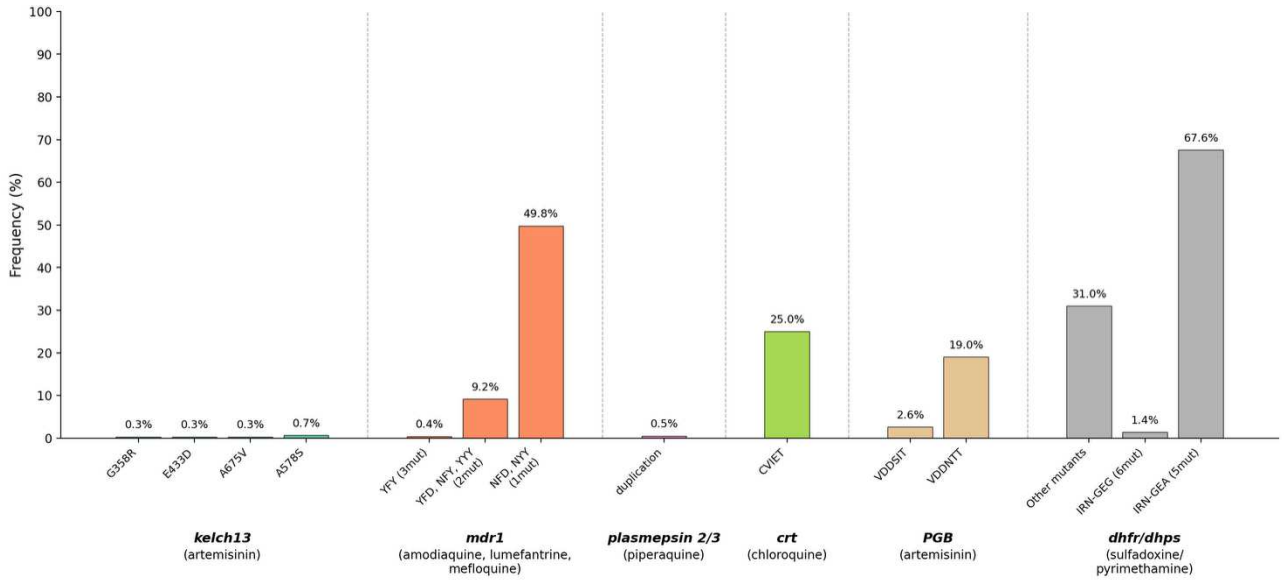
The boxplots show the distribution of *P. falciparum* AmpSeq barcode call rate according to study PHCC (panel a), population grop (panel b), months of sample collection (panel c), presence of fever (panel d), and – among pregnant women – gravidity (panel e).

Figure 5 Polyclonality and expected heterozygosity according to epidemiological factors



The dot-and-whisker plots show the estimated policlonality (panel a) and expected heterozygosity (panel b) according to study PHCC, population grop, month of sample collection, presence of fever.

Figure 6 Mutations associated with resistance to antimalarial drugs



The barplot shows the frequency of mutant alleles or haplotypes at loci associated with resistance to antimalarial drugs.

SUPPLEMENTARY INFORMATION

Table S1 Mitochondrial sequence sources used to classify six *Plasmodium* species

Species	Record Location
P. falciparum	https://plasmodb.org/plasmo/app/record/genomic-sequence/download/Pf3D7_MIT_v3
P. vivax	https://plasmodb.org/plasmo/app/downloads/Current_Release/PvivaxSal1_(contig PVAD80_MIT)
P. knowlesi	https://plasmodb.org/plasmo/app/record/genomic-sequence/download/PKNH_MIT_v2
P. malariae	https://www.ncbi.nlm.nih.gov/nuccore/LT594637
P. ovale curtisi	https://www.ncbi.nlm.nih.gov/nuccore/HQ712052.1
P. ovale wallikeri	https://www.ncbi.nlm.nih.gov/nuccore/HQ712053

The table shows, for each investigated *Plasmodium* species, the link to the database source where mitochondrial sequences were retrieved to conduct Kraken analysis.

Table S2 Genes and corresponding loci associated with resistance to antimalarial drugs that were genotyped with the SpotMalaria platform

P. falciparum target gene	Antimalarial drug	Core aa	Wild Type
pfkelch13	artemisinin	any mutation found in BTB/POZ and propeller domains	WT
exonuclease	piperaquine	415	E
plasmepsin 2/3	piperaquine	N/A (breakpoint mutation)	WT
pfcrt	chloroquine, piperaquine	72, 73, 74, 75, 76	CVMNK
pfmdr1	chloroquine, amodiaquine, lumefantrine, mefloquine	86, 184, 1246	NYD
pfarps10 *ferredoxin* *pfcrt* *pfmdr2*	artemisinin (parasite genetic background, PGB)	127, 128 193 326, 356 484	VDDNIT
pfdhfr	pyrimethamine, cycloguanil	51, 59, 108 (164)	NCS(I)
pfdhps	sulfadoxine	(436) 437, 540, 581 (613)	(S)AKA(A)

The table shows the loci and mutant alleles or haplotypes associated with resistance to antimalarial drugs that were investigated through AmpSeq.

Table S3 Study participants

Population group	PHCC	N	Female ratio	Fever (95% CI)
Children <5 years	Lakamadi	196	0.5	5.4 (2.9–9.8)
	Mundri	368	0.5	14.3 (11.0–18.3)
	Mvolo	404	0.5	11.9 (9.1–15.5)
	Total	968	0.5	11.6 (9.7–13.8)
Pregnant women	Lakamadi	190	1.0	3.7 (1.8–7.5)
	Mundri	343	1.0	1.8 (0.8–3.9)
	Mvolo	372	1.0	2.7 (1.5–5.0)
	Total	905	1.0	2.6 (1.7–3.8)
Total		1873	0.8	7.2 (6.1–8.5)

The table shows the counts (N) of study participants recruited during the entire study period by population group and PHCC, as well ratio of female participants and the proportion of subjects with fever with its 95% Confidence Interval (CI).

969 **Table S4** Comparison of AmpSeq and RDT results

970

RDT	AmpSeq		
	Negative	Positive	Total
Negative	597	679	1276
Positive	16	443	459
Total	613	1122	1735

971

972 The table shows the number of positive and negative results obtained by RDT and AmpSeq.

973

974

975 **Table S5** *P. falciparum* polyclonality

976

Factor	Category	n	Polyclonal (%)	95% CI	OR	95% CI	P-value
Population group	PW (ref)	88	39.8	30.2–50.2	.	.	.
	U5	218	39.4	33.2–46.1	0.86	0.51–1.47	0.585
PHCC	Mundri (ref)	85	29.4	20.8–39.8	.	.	.
	Lakamadi	82	40.2	30.3–51.1	1.54	0.78–3.07	0.216
	Mvolo	139	45.3	37.3–53.6	1.88	1.01–3.49	0.046
Month	Nov–Dec 2021	20	60.0	38.7–78.1	2.45	0.81–7.40	0.114
	Jan 2022	17	35.3	17.3–58.7	0.90	0.27–2.95	0.859
	Feb 2022	106	35.8	27.4–45.3	1.07	0.52–2.24	0.847
	Mar 2022	28	39.3	23.6–57.6	1.05	0.38–2.89	0.923
	Apr 2022 (ref)	49	32.7	21.2–46.6	.	.	.
	May–Jun 2022	86	44.2	34.2–54.7	1.30	0.60–2.80	0.503
Fever	No (ref)	254	38.6	32.8–44.7	.	.	.
	Yes	52	44.2	31.6–57.7	1.33	0.69–2.56	0.393
Gravidity	First pregnancy (ref)	40	37.5	24.2–53.0	.	.	.
	Two or more	40	42.5	28.5–57.8	1.23	0.50–3.02	0.648
Total		306	39.5	34.2–45.1	.	.	.

977

978 The table shows the proportion (%) of polyclonal *P. falciparum* infections and its 95%
979 Confidence Interval (CI) according to population group, PHCC, month, presence of fever and
980 – among pregnant women - gravidity. The table also shows the results of multivariate logistic
981 regression analysis (Odds Ratio with its 95% CI, P-value) investigating the association

between these different factors with policlonality. Legend: Pf, *P. falciparum*; PW, pregnant women; ref, reference category; OR, Odds Ratio.

985 **Table S6** Summary of pairwise Fst values by epidemiological factors

986

Factor	Comparison	N1	N2	Fst	95% CI
Population group	PW vs U5	53	135	0.007	0.005-0.009
PHCC	Lakamadi vs Mundri	51	60	0.012	0.009-0.016
	Lakamadi vs Mvolo	51	77	0.011	0.008-0.014
	Mundri vs Mvolo	60	77	0.009	0.006-0.011
Transmission season	High vs Low	138	50	0.008	0.006-0.010
Fever	Absence vs presence	156	29	0.012	0.008-0.015
Gravidity	One vs over one pregnancy	25	23	0.026	0.018-0.033

987

988 The table shows the Fst value and its 95% Confidence Interval (CI) for the comparison
989 between population groups, study PHCCs, transmission season, patient's fever status, and –
990 for pregnant women – gravidity. N1 and N2 indicate the number of samples in the first and
991 second comparison group, respectively.

992

993

Table S7 *P. falciparum* AmpSeq positivity according to gravidity among pregnant women

Gravidity	Pf positivity (%)		Association with RDT positivity		
	Proportion	95% CI	OR	95% CI	P-value
1 (ref)	60.5	54.5-66.3	.	.	.
2	51.6	44.4-58.8	0.72	0.5-1.1	0.116
3	56.3	48.5-63.9	0.78	0.5-1.2	0.254
>3	51.2	45.0-57.4	0.58	0.4-0.9	0.005

The table shows the proportion (%) of positive AmpSeq and its 95% Confidence Interval (CI) according to the number of pregnancies, together with the results of multivariate logistic regression analysis (Odds Ratio with its 95% CI, P-value) investigating the association between gravidity and AmpSeq positivity, adjusted for study site, month of collection and presence of fever. Legend: Pf, *P. falciparum*; ref, reference category; OR, Odds Ratio.

Table S8 *P. falciparum* AmpSeq barcode call rate according to gravidity among pregnant women

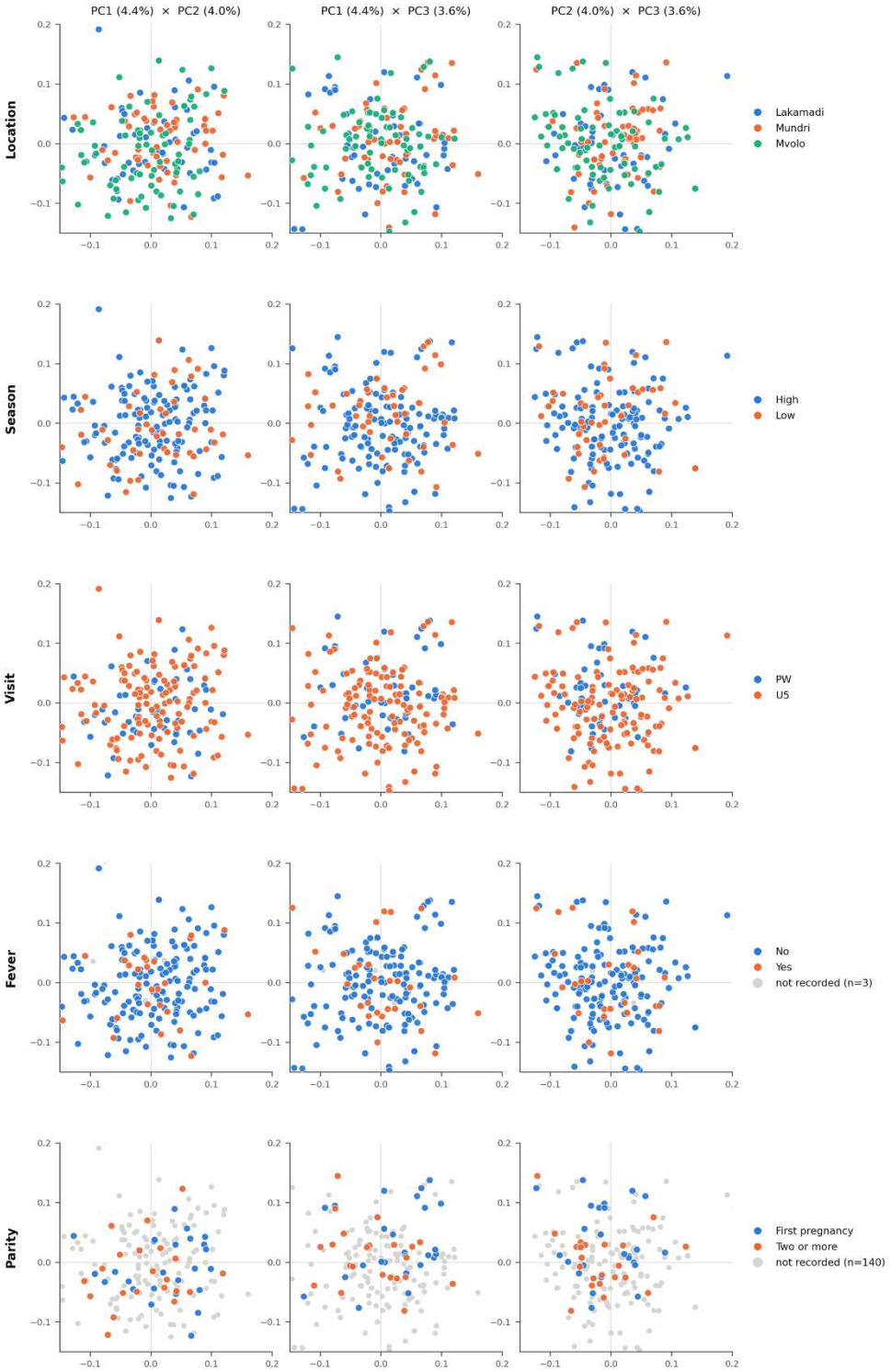
Gravidity	Pf call rate (%)		Association with Pf call rate		
	Median	IQR	Beta coeff	95% CI	P-value
1 (ref)	26.2	5.0-75.5	.	.	.
2	15.8	5.0-67.3	-0.05	-0.13, 0.03	0.266
3	13.9	13.9-27.2	-0.15	-0.24, -0.07	0.000
>3	14.4	5.9-28.2	-0.16	-0.25, -0.08	0.000

The table shows the median (%) AmpSeq barcode call rate and its InterQuartile Range (IQR) according to the number of pregnancies, together with the results of multivariate linear regression analysis (Odds Ratio with its 95% CI, P-value) investigating the association between gravidity and barcode call rate, adjusted for study site, month of collection and presence of fever. Legend: Pf, *P. falciparum*; ref, reference category; OR, Odds Ratio.

1014 **Figure S1** Principal coordinate analysis (PCoA) by epidemiological factors)

1015

1016



The scatter plots show the distribution of samples according to first (PC1) and second (PC2) components in PcoA. For each component, the proportion of explained variability is shown in brackets. Only samples with less than 30% missingness in their genetic barcode and COI=1 were included in the analysis (n=188).

Malaria RDT positivity

The proportion of RDT positive results, or RDT positivity, was 26.4% in the overall study population (Table S9, Figure S2). As expected, RDT positivity was significantly higher among children less than five years old (38.5%) than among pregnant women (13.6%). A significantly lower RDT positivity was observed in Mundri (19.5%) compared to Lakamadi (30.1%) and Mvolo (30.9%). RDT positivity did not vary significantly between November and February (average 30.8%), while it decreased in March and April (average 18.6%) to increase again in May-June (37%). Also, RDT positivity was significantly higher among patients with fever (54.7%) than among those with no fever (23.9%), indicating that febrile subjects are more likely to harbor Plasmodium parasites at a density detectable by RDT. This observation was confirmed both in children (afebrile: 35.2%, febrile: 60.6%; OR=2.88, 95% CI=1.83-4.53; P-value<0.001) and pregnant women (afebrile: 13.2%, febrile: 29.2%; OR=2.48, 95%CI=0.99-6.21; P-value=0.053).

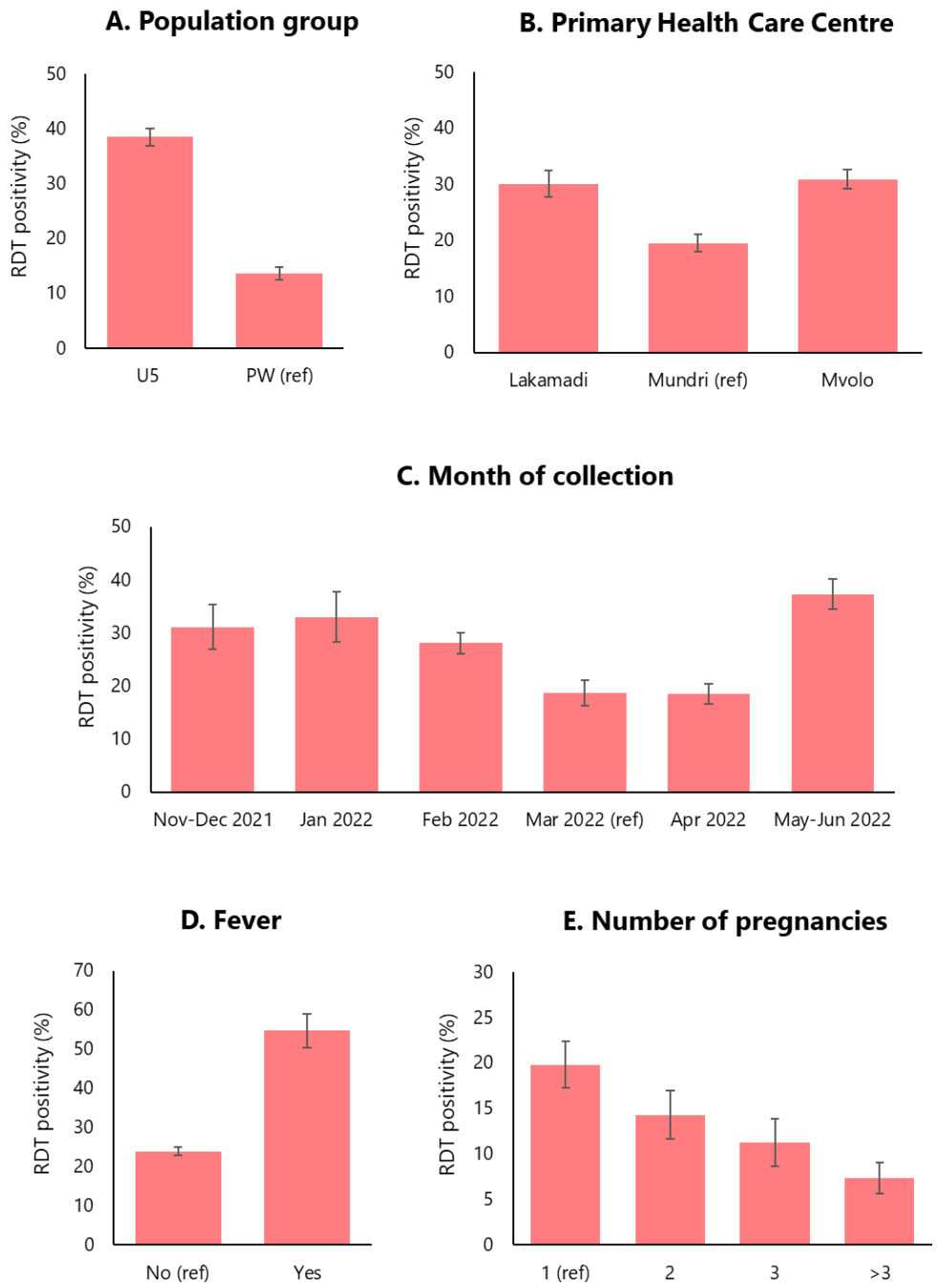
Among pregnant women attending ANC, RDT positivity was further assessed according to their gravidity, i.e. the number of pregnancies. It was observed the RDT positivity showed a significant and linear decrease from 19.8% among women in their first pregnancy to 7.4% in women in their fourth or subsequent pregnancy indicating the acquisition of gravidity-dependent immunity (OR=0.69, 95%CI=0.57-0.84, P-value<0.001).

Table S9 Positivity to malaria Rapid Diagnostic Testing

Factor	Category	RDT positivity		Assocation with RDT positivity		
		Proportion	95% CI	OR	95% CI	P-value
Population group	U5	38.5	35.3-41.7	3.8	2.9-4.8	0.000
	PW (ref)	13.6	11.5-16.1	.	.	.
PHCC	Lakamadi	30.1	25.7-34.9	1.8	1.3-2.5	0.000
	Mundri (ref)	19.5	16.7-22.7	.	.	.
	Mvolo	30.9	27.6-34.4	1.9	1.5-2.6	0.000
Month	Nov-Dec 2021	31.1	23.5-39.9	1.7	1.0-2.8	0.062
	Jan 2022	33.0	24.5-42.8	1.6	0.9-2.7	0.128
	Feb 2022	28.1	24.5-32.1	1.6	1.1-2.4	0.018
	Mar 2022 (ref)	18.7	14.4-23.9	.	.	.
	Apr 2022	18.5	15.1-22.4	1.0	0.6-1.5	0.929
	May-Jun 2022	37.3	32.0-43.0	2.5	1.6-3.8	0.000
Fever	No (ref)	23.9	21.9-26.0	.	.	.
	Yes	54.7	46.0-63.1	2.6	1.7-3.8	0.000
Total		26.40	24.4-28.5	.	.	.

The table shows the proportion (%) of positive RDT and its 95% Confidence Interval (CI) according to population group, PHCC, month and presence of fever. The table also shows the results of multivariate logistic regression analysis (Odds Ratio with its 95% CI, P-value) investigating the association between these different factors with RDT positivity. Legend: PW, pregnant women; ref, reference category; OR, Odds Ratio.

Figure S2 Proportion of positive malaria Rapid Diagnostic Tests



The barplots show the proportion of positive malaria Rapid Diagnostic Tests among children under five years of age and pregnant women (A), recruited in Lakamadi, Mundri

and Mvolo PHCCs (B), during the study period (C), and according to the presence of fever (D) and the number of pregnancies (D). Whiskers represent Standard Errors (SE).

Gene	Drug	Mutant allele /haplotype	Prato 2026 Western Equatoria, South Sudan	Molina-de la Fuente 2023 (16) Western Equatoria, South Sudan	Molina-de la Fuente 2023 (15) Western Equatoria, South Sudan	Tukwasibwe 2024 (17) Adjumani and Kyangwali, Uganda
kelch13	artemisinin	G358R	0.3% (1/293)	.	.	.
		E433D	0.3% (1/293)	.	.	.
		C469Y	0.0% (0/293)	0.0% (0/468)	.	8.0% (4/50)
		A486S	0.0% (0/293)	0.21% (1/468)		
		R561L	0.0% (0/293)	0.2% (1/468)	.	.
		Y519H	0.0% (0/293)	0.21% (1/468)		
		A578S	0.7% (2/293)	0.2% (1/468)	.	.
		E602K	0.0% (0/293)	0.21% (1/468)		
		E605K	0.0% (0/293)	0.21% (1/468)		
		P615S	0.0% (0/293)	0.21% (1/468)		
		S623G	0.0% (0/293)	0.9% (4/468)	.	.
		A675V	0.3% (1/293)	0.0% (0/468)	.	15.4% (8/52)
arps10, ferredoxin, crt, mdr2	artemisinin genetic background	VDDNTT	19.0% (36/189)	.	.	.
		VDDSIT	2.6% (5/189)	.	.	.
mdr1	amodiaquine, chloroquine, lumefantrine, mefloquine	N86Y	9% (35/388)	.	18.9% (101/532)	33.3% (13/39)
		Y184F	43.6% (169/331)	.	.	69.2% (27/39)
		D1246Y	7.2% (28/338)	.	9.5% (51/532)	10.3% (4/39)
		NFD, NYY (1mut)	49.8% (114/229)	.	.	.

		YFD, NFY, YYY (2mut)	9.2% (21/229)	.	.	.
		YFY (3mut)	0.4% (1/229)	.	.	.
plasmpepsin2	piperaquine	duplication (break point mutation)	0.5% (1/209)	.	.	.
exonuclease	piperaquine	E415G	0.0% (0/736)	.	.	.
crt	cloroquine	K76T	25.3% (91/360)	.	21.2% (113/532)	48.7% (19/39)
		CVIET	25.0% (86/344)	.	.	.
	piperaquine	H97Y	4.9% (26/526)	.	.	.
dhfr	pyrimethamine	N51I	99.8% (476/477)	.	97.2% (517/532)	97.3% (38/39)
		C59R	78.4% (312/398)	.	84.0% (447/532)	64.1% (25/39)
		S108N	100.0% (472/472)	.	97.0% (516/532)	100.0% (39/39)
		I164L	0.6% (3/464)	.	0.7% (4/532)	2.6% (1/39)
dhps	sulfadoxine	A437G	91.5% (323/353)	.	81.7% (435/532)	71.1% (27/38)
		K540E	87.0% (255/293)	.	86.5% (460/532)	61.5% (24/39)
		A581G	1.5% (5/339)	.	6.4% (34/532)	5.1% (2/39)
dhps-dhfr	sulfadoxine-pyrimethamine	IRNGEA	67.6% (141/209)	.	62.4% (332/532)	.
		IRNGEG	1.4% (3/209)	.	4.5% (24/532)	.
		Other mutant haplotypes	31.0% (65/209)	.	.	.

Table S10 Frequency of markers of antimalarial drug resistance and comparison with previous studies

The table shows the allele frequencies (%) of mutant alleles or haplotypes, together with the number of samples with the mutation and the total number of succesfully genotyped samples, observed in the present study and in previous reports.