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Molecular markers of antimalarial drug resistance in *pfk13*, *pfmdr1*, *pfdhfr*, and *pfdhps* genes of *Plasmodium falciparum* in a rural municipality in Cubal, Benguela Province, Angola (2022–2023)

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Abstract

Background Malaria remains a major cause of morbidity and mortality in Angola, where *Plasmodium falciparum* accounts for most infections. The widespread use of artemether–lumefantrine (AL) as first-line treatment and sulfadoxine–pyrimethamine (SP) for intermittent preventive treatment in pregnancy (IPTp) exerts selective pressure on local parasite populations. Molecular surveillance of drug resistance markers is essential to monitor susceptibility and anticipate changes that may influence treatment and prevention strategies. This study analyzed *pfk13*, *pfmdr1*, *pfdhfr*, and *pfdhps* polymorphisms in *P. falciparum* isolates from Cubal, Benguela province, to assess parasite genetic evolution under AL and SP pressure.

Methods A cross-sectional study was conducted between 2022 and 2023 at Hospital Nossa Senhora da Paz (Cubal). A total of 139 real-time PCR–confirmed *P. falciparum* infections were included. Dried blood spot samples underwent sequencing of the genes involved in resistance. Mutation and haplotype frequencies associated with antimalarial resistance were determined.

Results Among 120 *pfk13* sequences, mutations were detected in 12 isolates (10%), including five nonsynonymous variants, but none corresponded to validated or candidate markers of artemisinin resistance. In *pfmdr1* (100 sequences), Y184F was the most prevalent mutation (43%), N86Y was rare (1%), and D1246Y was not detected. Seven additional *pfmdr1* variants were identified.

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Of the 101 isolates correctly sequenced for *pfdhfr/pfdhps*, 77.2% carried the triple *pfdhfr* N51I/C59R/S108N mutation. In *pfdhps*, A437G was nearly fixed (98%), K540E showed moderate prevalence (23.8%), and A581G was absent. The most common *pfdhps* haplotypes were ISGKAA (64.4%) and ISGEAA (22.8%). Combined *pfdhfr/pfdhps* profiles classified 58.4% of isolates as "partially resistant" (IRNG) and 17.8% as "fully resistant" (IRNGE), with no "super-resistant" haplotypes. Additional single mutations not associated with SP resistance were also detected.

Conclusions This study provides an updated molecular profile of antimalarial resistance in a rural area of Angola. No validated or candidate *pfk13* markers of artemisinin resistance were identified. However, the predominance of *pfmdr1* haplotypes linked to reduced lumefantrine susceptibility raises concerns regarding AL efficacy. Although SP remains suitable for IPTp, the presence of the "fully resistant" haplotype (IRNGE) of *pfdhfr/pfdhps* highlight potential risks to long-term effectiveness. These findings reinforce the need for integrated molecular surveillance and periodic therapeutic efficacy studies to guide malaria control policies in rural Angola.

Keywords *Plasmodium falciparum*, Antimalarial resistance, *pfk13*, *pfmdr1*, *pfdhfr/pfdhps*, Artemether-lumefantrine, Sulfadoxine-pyrimethamine, Treatment, Chemoprevention, Angola

Introduction

Malaria remains a major global public health challenge, disproportionately affecting sub-Saharan Africa. In 2024, the World Health Organization (WHO) African Region accounted for approximately 94% of global malaria cases and 95% of malaria-related deaths [1]. Angola, located in Central Africa, reported nearly 10 million cases and 16,500 deaths in 2024, ranking sixth in terms of incidence and eighth in terms of morbidity caused by malaria worldwide [1]. Despite advances in malaria control policies, the disease remains a leading cause of morbidity and mortality in the country [2]. In 2022, 30% of all patients seeking healthcare were diagnosed with malaria, 5% of whom were diagnosed with severe malaria [3]. In 2011, malaria was responsible for 35% of outpatient visits, 40% of prenatal mortality, 25% of maternal mortality, and 35% of child mortality [4]; these are the most recent national data available.

The WHO strategy for malaria control emphasizes prompt and accurate diagnosis, timely treatment, and effective case management, complemented by preventive measures, such as vector control and chemoprophylaxis in vulnerable populations [5]. Currently, the first-line treatment for uncomplicated *Plasmodium falciparum* malaria consists of artemisinin-based combination therapies (ACTs), while intravenous artesunate is recommended for severe cases [6]. Sulfadoxine-pyrimethamine (SP) is currently used as preventive therapy in areas with moderate-to-high *P. falciparum* transmission [7]. SP is recommended for pregnant women as intermittent preventive treatment in pregnancy (IPTp) [1]. In infants, it is administered as intermittent preventive treatment (IPTi), while in children aged 3–59 months, it is combined with amodiaquine (SP + AQ) for seasonal malaria chemoprevention (SMC) [8, 9]. Initially recommended for areas of highly seasonal malaria transmission in the African Sahel region, SMC is now progressively being extended to other endemic regions, where it has also demonstrated substantial effectiveness [8, 9].

The emergence of *P. falciparum* resistance to antimalarial drugs poses a major threat to malaria elimination [10]. Several molecular markers in the *P. falciparum* genome have been associated with resistance to artemisinins and partner drugs in ACTs [11]. Among them, the propeller domain of the *pfk13* gene has been identified as the primary marker of artemisinin resistance [12, 13]. Clinically, this phenotype is defined by delayed parasite clearance after artesunate or ACT administration [14]. In 2020, the WHO reported more than 260 nonsynonymous mutations in *pfk13* [15], of which 13 have been validated and 9 proposed as candidate resistance markers [16]. In Africa, and particularly in the southern and central regions, increasing evidence indicates the independent emergence of both validated and candidate *pfk13* resistance markers. Validated mutations such as R561H and the C469Y have been reported in the Democratic Republic of Congo (DRC) [17, 18] and M476I in Equatorial Guinea [19]. Moreover, a recent study conducted in Zambia have reported the presence of the candidate resistance marker P441L [20]. Additionally, the A578S mutation, the most frequently observed across the continent and reported in Central African countries [21], does not appear to be directly associated with delayed clearance according to some studies [22]. In Angola, available evidence indicates a predominance of wild-type *pfk13* parasites, suggesting maintained susceptibility to artemisinins [23]; however, isolated reports of validated resistance markers, including R539T and P553L, have been described in imported Angolan isolates [19, 24, 25]. Moreover, isolated cases of delayed parasite clearance and occasional ACTs treatment failure have been reported in different provinces of Angola [23, 26–29].

The *P. falciparum* multidrug resistance 1 gene (*pfmdr1*), which encodes P-glycoprotein homolog 1 (Pgh-1) [30],

harbors key polymorphisms—N86Y, Y184F, S1034C, N1042D, and D1246Y—associated with resistance to chloroquine, quinine, amodiaquine, lumefantrine, mefloquine, and piperaquine [31, 32]. The N86Y mutation is associated with resistance to chloroquine and amodiaquine, although it increases sensitivity to lumefantrine [32]. Y184F acts as a modulatory polymorphism that influences resistance when combined with other mutations; for example, the YFY haplotype (codons 86, 184, and 1246) is associated with reduced sensitivity to amodiaquine [33]. Geographically, N86Y and Y184F are more frequent in Asian and African isolates, whereas S1034C, N1042D, and D1246Y are more common in South America [32]. In Angola, a near-complete predominance of the wild-type N86 allele has been reported, a genetic background associated with reduced susceptibility to lumefantrine [23]. On the other hand, the *pfmdr1* D1246Y mutation has recently been detected in countries in West and Central Africa, including Guinea-Bissau, the DRC, and Angola [34].

Mutations in *pfdhfr* and *pfdhps* are linked to reduced sensitivity to SP [35]. In *pfdhfr*, substitutions C50R, N51I, C59R, S108N, and I164L confer pyrimethamine resistance, while in *pfdhps*, mutations S436A, A437G, K540E, A581G, and A613S/T are associated with sulfadoxine resistance [36]. The triple mutant *pfdhfr* haplotype (C**IRN**I) confers high-level pyrimethamine resistance, further intensified by the quadruple mutant (C**IRNL**) [37]. Regarding sulfadoxine resistance, the A437G mutation plays a central role, either alone or in combination with K540E; however, the triple mutant haplotype 437G/540E/581G is more strongly associated with high-level sulfadoxine resistance [38, 39]. The *pfdhps* I431V mutation, first described in 2009 [40], has been shown through crystallographic modeling to potentially reduce sulfadoxine binding and efficacy [41]. Initially detected in Nigeria, it often co-occurs with mutations 436A, 437G, 581G, and 613S [40–42], and displays a mutually exclusive distribution with K540E [38]. Until 2022, the WHO advised against the use of SP in chemoprevention strategies when the prevalence of K540E exceeded 50% for IPTi and ≥ 95% for IPTp [43, 44]; however, recent evidence suggests SP remains effective even in high-prevalence settings [45]. In contrast, areas where A581G prevalence is ≥ 10% may not be suitable for IPTp interventions [46]. In Angola and neighboring countries such as the DRC, resistance to SP is characterized by the near fixation of *pfdhfr* mutations (N51I, C59R, and S108N) and a high prevalence of the *pfdhps* A437G mutation, while critical high-level resistance markers such as *pfdhps* K540E and A581G remain at low to moderate frequencies [47, 48].

In Angola, *P. falciparum* accounts for more than 90% of malaria cases across multiple provinces [49–51]. ACTs were adopted as first-line treatment for uncomplicated malaria in 2004, with nationwide implementation of artemether-lumefantrine (AL) completed between 2007 and 2008 [52]. Benguela province, located in the central-western region of the country, reported a malaria transmission rate of 181 cases per 1000 inhabitants in 2018 [53]. In a study conducted between May and July 2022 in the rural municipality of Cubal, Benguela province, malaria prevalence in febrile patients reached 61.5% using a combined diagnostic approach based on field techniques (microscopy and rapid diagnostic test [RDT]) and real-time PCR. In that study, *P. falciparum* was identified in 100% of infections, followed by *P. malariae* (13.95%) and *P. ovale* (6.98%), detected as coinfections with *P. falciparum* [49]. The Hospital Nossa Senhora da Paz (HNSP) of Cubal—a referral hospital that treats around 22,000 patients annually and recorded a rate of 8.3% of deaths caused by malaria between 2009 and 2013—has used AL as the standard first-line treatment for uncomplicated malaria since 2012, following earlier protocols based on oral quinine plus clindamycin [54]. AL is the standard first-line treatment for uncomplicated malaria in Cubal and is therefore considered the main source of antimalarial selective pressure in this municipality. Similarly, national malaria control policies recommend the use of SP for IPTp across all transmission areas [2]. Despite having increased in recent years, IPTp coverage remains low in Angola, where only 31% of pregnant women received ≥ 3 doses in 2023–2024 [55]. On the other hand, IPTi or SMC campaigns targeting children have not yet been implemented in Angola [47], including Benguela province [56]. Moreover, no studies assessing the feasibility or effectiveness of these interventions have been conducted in rural areas, such as Cubal.

This study aimed to determine the frequency of mutations in key resistance-associated genes (*pfk13*, *pfmdr1*, *pfdhfr*, and *pfdhps*) and to characterize the molecular resistance profile related to AL and SP in the municipality of Cubal, Benguela province.

Material and methods

Study design

A cross-sectional, descriptive, convenience-based study was conducted between May and July 2022 and May 2023 at the HNSP of Cubal, in the east-central area of Benguela province, Angola (Fig. 1). Cubal has an estimated population of 300,000 inhabitants, is classified as a mesoendemic malaria area, and has a tropical climate with a low-transmission dry season (June–October) and a high-transmission rainy season (November–May) [50, 53, 54].

A total of 139 dried blood spot (DBS) samples, in which *P. falciparum* infection had been confirmed by real-time

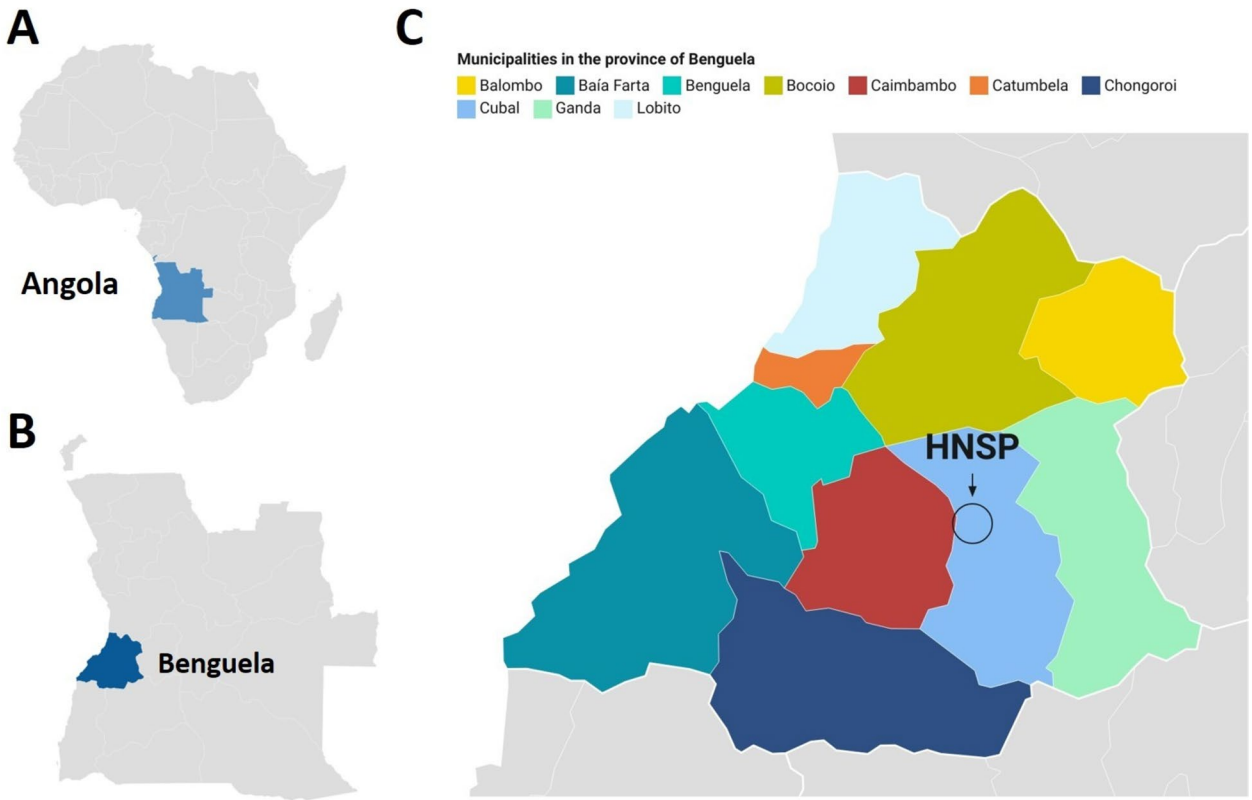

Fig. 1 Geographical location of the Hospital Nossa Senhora da Paz (HNSP). **A** Map of the African continent with Angola highlighted. **B** Map of Angola with Benguela province highlighted. **C** Municipalities of Benguela province, indicating the location of HNSP in the municipality of Cubal. The map was created using the free software Datawrapper (https://app.datawrapper.de/select/map) and Microsoft PowerPoint

PCR at the clinical laboratories of Vall d'Hebron University Hospital (HUVH, Barcelona, Spain), were analyzed [49]. The samples were obtained from febrile patients with suspected malaria who presented to the emergency department of the HNSP. Sampling was conducted in two campaigns: 86 samples were collected between May and July 2022, and 53 samples in May 2023. These sampling periods coincide with the transition from the end of the rainy season to the onset of the dry season, allowing the assessment of resistance marker dynamics during a phase of changing transmission intensity. Patients of all ages were included. The samples were sent to the HUVH for further molecular analysis. All samples were transported, stored, and processed under identical conditions, regardless of the sampling period.

Sample size calculation

The adequacy of the sample size was assessed using the standard formula for estimating proportions in cross-sectional studies, assuming a confidence level of 95%. The expected prevalences for resistance markers were obtained from data published in the study by Sitali et al. conducted in the Zambia [10]. A margin of error of 5% was applied for low- and moderate-prevalence mutations, while a wider margin of 7–9% was considered acceptable for high-prevalence markers, given the study's objective of identifying dominant or quasi-fixed resistance patterns. Under these assumptions, the number of samples successfully genotyped for each gene was sufficient to reliably characterize the molecular resistance profile in the study area.

Sample collection, diagnosis, and DNA extraction

Samples collected between May and July 2022 were obtained from venous blood by venipuncture, with 10 µL spotted onto each circle of Whatman 903™ filter paper cards (GE Healthcare Life Sciences, Cardiff, UK) for DBS preparation [49]. In May 2023, samples were obtained from capillary blood by finger prick, with whole circles of filter paper inoculated directly (each circle corresponding to 50 µL of blood). All filter paper cards were air dried for 24 h at room temperature and stored at 4 °C until shipment to the HUVH, where they were subsequently preserved at − 20 °C until molecular analysis [49]. Despite differences in sample collection, a comparable DBS volume (~ 20 µL) was used for DNA extraction in both

campaigns, ensuring result comparability; moreover, the analyses were based on nucleotide sequences, which are independent of the collection and extraction methods.

Field diagnosis of malaria (microscopy and RDT [STANDARD™ Q Malaria Pf/Pan Ag test, SD Biosensor, Republic of Korea, targeting *Pf*HRP2 and panmalarial pLDH]), DNA extraction from approximately 20 µL of blood per sample (obtained from two 10 µL spots in the first survey or from half a spot in the second), molecular detection of *Plasmodium* spp., and confirmation of *P. falciparum* infection by real-time PCR were performed following the procedures described by Mediavilla et al. [49].

Amplification, sequencing, and gene analysis

Amplification of the *pfk13*, *pfmdr1*, and *pfdhfr*/*pfdhps* genes was performed according to the protocols described by Ariey et al. [13], Li et al. [57], and Pearce et al. [58], respectively, with minor modifications. The primers used and PCR conditions for each reaction are detailed in Table S1 (Additional file 1). All PCR reactions were performed using SimpliAmp thermocyclers (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). Amplicons from each genetic region were visualized on 1.5% agarose gels stained with CondaSafe Stain (Condalab, Madrid, Spain). The amplified samples for each gene were purified using Illustra™ Exoprostar™ 1 Step Kit (Avantor Science Central, Radnor, USA). They were sequenced using Sanger technology with the forward and reverse primers from the nested PCRs corresponding to each genetic fragment, following the protocol described by Molina-de la Fuente et al. [59].

Polymorphism analysis was performed on the obtained sequences using BioEdit v7.7.1 and compared to the reference sequence of the 3D7 clone. For *pfk13*, all detected mutations were evaluated and assessed for their correspondence to validated or candidate variants associated with artemisinin partial resistance, according to the WHO list [16]. For the other genes, all detected mutations were recorded, with the analysis focusing on standard resistance-associated codons in *pfmdr1*, *pfdhfr*, and *pfdhps*, as described by Martín-Ramírez et al. [60]. Samples in which the electropherograms showed peaks corresponding to different bases in the same position were classified as polyclonal infections.

SP resistance was classified according to the scheme proposed by Naidoo et al. [39]: "partially resistant", defined by the combination of *pfdhfr* 51I/59R/108N with *pfdhps* 437G (alone or together with 436A), corresponding to the **IRNG** haplotype; "fully resistant", when *pfdhfr* 51I/59R/108N is combined with *pfdhps* 437G/540E, corresponding to the **IRNGE** haplotype; and "super-resistant", defined by *pfdhfr* 51I/59R/108N associated with *pfdhps* 437G/540E/581G and/or the *pfdhfr* 164L mutation, corresponding to the **IRNGEG** haplotype [39].

When describing the different haplotypes for the *pfmdr1* and *pfdhfr*/*pfdhps* genes, if a single nucleotide polymorphism (SNP) was identified within a polyclonal infection—i.e., a mixed infection involving both wild-type and mutant *P. falciparum* populations—the haplotype considered for analysis was that of the mutant type. This approach was adopted because mutant alleles are commonly associated with antimalarial resistance and, in practice, such infections are likely to behave as drug-resistant.

Statistical analysis

Mutation and haplotype prevalences for each gene were calculated as the proportion of samples carrying the mutation relative to the total number of successfully genotyped samples. Qualitative variables were expressed as percentages and absolute frequencies, while quantitative variables were described using the mean or median and the standard deviation (SD) or interquartile range (IQR), depending on their distribution.

Results

Characteristics of the study population

A total of 139 participants positive for *P. falciparum* by real-time PCR were included in the study, of whom 78 (56.1%) were women, 42/139 (30.2%) were minors (< 18 years), and the median age was 9 years (IQR = 2–20.5).

Malaria diagnosis

Among the 139 PCR-confirmed malaria cases, 97/139 (69.8%) were positive by microscopy and 116/139 (83.5%) by RDT. The median parasite density determined by microscopy was 3,331 (IQR = 674–16,928) parasites/µL. Molecular species identification by real-time PCR showed that 117/139 (84.2%) were single *P. falciparum* infections, 14/139 (10.1%) were mixed infections with *P. falciparum* + *P. malariae*, 6/139 (4.3%) were mixed infections with *P. falciparum* + *P. ovale*, and 2/139 (1.4%) were triple infections with all three species.

Molecular markers analysis

pfk13

A total of 120/139 (86.3%) samples were successfully sequenced for *pfk13*. All sequences were submitted to the GenBank database (accession numbers: PX469096—PX469215). SNPs were detected in 12 (10%) of the samples, of which 6/12 (50%) were synonymous mutations. None of the SNPs corresponded to WHO-validated or candidate mutations associated with partial artemisinin resistance [16]. However, 5/12 (41.7%) were located

within five codons of regions where mutations are known to confer resistance. In addition, 9/12 (75%) of the SNPs were observed in mixed infections together with wild-type *P. falciparum* populations (Additional file 2). Table 1 summarizes the mutations identified in the *pfk13* sequences, along with their main characteristics.

pfmdr1

A total of 100/139 (71.9%) *pfmdr1* samples were successfully amplified and sequenced. All sequences were submitted to the GenBank database (accession numbers: PX469216—PX469315 and PX469316—PX469415). Among the markers associated with resistance to antimalarial drugs, the wild-type N86 allele was detected in all isolates. The only sample in which the N86Y mutation was observed was found in the form of a polyclonal infection together with the wild-type, and the Y184F mutation in 43/100 (43%) samples, of which 20/43 (46.5%) were mixed infections with the wild-type (Table 2; Additional file 3). The wild-type haplotype NEYSNVD was found in 57/100 (57%) isolates, the single mutant haplotype NE**F**SNVD in 42/100 (42%), and the double mutant haplotype **Y**E**F**SNVD in only 1/100 (1%) cases (Fig. 2).

pfdhfr/pfdhps

A total of 101/139 (72.7%) samples were successfully sequenced for both *pfdhfr* and *pfdhps*. All sequences were submitted to the GenBank database (accession numbers: PX469416—PX469516 and PX469517—PX469617). In *pfdhfr*, the N51I and S108N mutations were detected in 101/101 (100%) isolates, while C59R was found in 78/101 (77.2%), and I164L was not detected in any sample (Fig. 3). The C59R mutation appeared as a mixed infection with the wild-type in 15/78 (19.2%) cases, and N51I did so in only one case. An additional mutation, L56R, not associated with pyrimethamine resistance, was detected in one sample as a polyclonal infection (Additional file 4). The triple mutant haplotype *pfdhfr* **IRN**I was observed in 78/101 (77.2%) isolates, while the double mutant haplotype **ICN**I was present in 23/101 (22.8%) (Table 3).

In *pfdhps*, the most frequent mutation was A437G, found in 99/101 (98%) isolates, followed by K540E in 24/101 (23.8%) and S436A in 11/101 (10.9%). Mutations I431V, A581G, and A613S were not detected in any isolate (Fig. 3). S436A occurred as a mixed infection with the wild-type in 5/11 (45.5%) cases, K540E in 7/24 (29.2%), and A437G in 2/99 (2%). The D598N mutation, not associated with sulfadoxine resistance, was observed in one isolate as a polyclonal infection (Additional file 5). The mutant haplotype IS**G**KAA was the most prevalent, detected in 65/101 (64.4%) isolates, followed by IS**GE**AA in 23/101 (22.8%), I**AG**-KAA in 10/101 (9.9%), the wild-type ISAKAA in 2/101 (2%), and the triple mutant I**AGE**AA in a single isolate (0.99%) (Table 3).

Table 1 Mutations detected in *pfk13* (n = 120)

Codon Nº	Wild-type Codon	Wild-type amino acid	Mutant codon	Mutant Amino acid	Mutation	Synonymous/ Non-synonymous	Near region implying resistance (< 5 codons) [a]	Nº of isolates n (%)	Polyclonal infection Wild-type/ mutated n (%) [b]
459	TCG	Serine	TC**A**	Serine	S459S	Yes	Yes (**N458Y**)	1 (0.8)	0 (0)
511	TAT	Tyrosine	TA**C**	Tyrosine	Y511Y	Yes	Yes (*R515K*)	1 (0.8)	1 (100)
573	ACC	Threonine	A**G**C	Serine	T573S	No	Yes (**P574L**)	1 (0.8)	1 (100)
578	GCT	Alanine	GC**C**	Alanine	A578A	Yes	Yes (**P574L**/**C580Y**)	1 (0.8)	1 (100)
581	GTT	Valine	G**C**T	Alanine	V581A	No	Yes (**C580Y**)	1 (0.8)	1 (100)
589	GTC	Valine	**A**TC	Isoleucine	V589I	No	No	1 (0.8)	0 (0)
613	CAA	Glutamine	**G**AA	Glutamate	Q613E	No	No	2 (1.7)	2 (100)
613	CAA	Glutamine	CA**G**	Glutamine	Q613Q	Yes	No	1 (0.8)	0 (0)
627	GCT	Alanine	GC**C**	Alanine	A627A	Yes	No	1 (0.8)	1 (100)
645	AAC	Asparagine	AA**T**	Asparagine	N645N	Yes	No	1 (0.8)	1 (100)
695	TCA	Serine	**A**CA	Threonine	S695T	No	No	1 (0.8)	1 (100)

[a] Validated mutations (bold) or candidate mutations (italics) whose affected codons are located within five positions of the identified SNPs

[b] Number of mutations detected in coinfection with the wild-type allele at the same position (polyclonal infections). The proportion of polyclonal infections relative to the total number of isolates carrying each mutation is shown in parentheses

Table 2 Frequency of SNPs identified in *pfmdr1* (n = 100)

	Mutation	Nº of isolates n (%)	Polyclonal infection Wild-type/ mutated n (%) [a]
Resistant mutations	N86Y	1 (1)	1 (100)
	E130K	0 (0)	–
	Y184F	43 (43)	20 (46.5)
	S1034C	0 (0)	–
	N1042D	0 (0)	–
	V1109I	0 (0)	–
	D1246Y	0 (0)	–
Non-resistant mutations	Y87C	1 (1)	1 (100)
	F103L	1 (1)	0 (0)
	D156N	1 (1)	1 (100)
	Q161Q	1 (1)	1 (100)
	N213S	1 (1)	1 (100)
	T1069T	6 (6)	2 (33.3)
	G1113D	2 (2)	1 (50)
	P1142P	2 (2)	2 (100)
	N1148N	1 (1)	1 (100)
	F1194L	1 (1)	0 (0)
	N1227N	2 (2)	2 (100)
	D1236D	1 (1)	1 (100)
	L1260L	1 (1)	1 (100)

[a] Number of mutations detected in coinfection with the wild-type allele at the same position (polyclonal infections). The proportion of polyclonal infections relative to the total number of isolates carrying each mutation is shown in parentheses

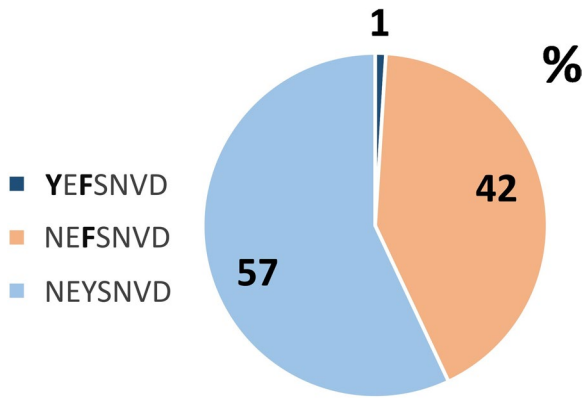

Fig. 2 Frequency of *pfmdr1* haplotypes (n = 100). The graph shows the proportions of the three *pfmdr1* haplotypes identified in the sequences analyzed: wild-type haplotype (NEYSNVD), single mutant haplotype (NEFSNVD), and double mutant haplotype (YEFSNVD). Each letter corresponds to an amino acid at the positions associated with resistance (86, 130, 184, 1034, 1042, 1109, and 1246)

Table 3 shows the combined *pfdhfr* and *pfdhps* haplotypes used for SP resistance classification. A total of 18/101 (17.8%) isolates were classified as "fully resistant" (**IRNGE**) and 59/101 (58.4%) as "partially resistant" (**IRNG**). No "super-resistant" haplotypes (**IRNGEG**) were identified. Only one sample presented the *pfdhfr* **IRN**I haplotype without any associated *pfdhps* mutations (**IRN**IISAKAA).

Discussion

Malaria, caused predominantly by *P. falciparum*, remains a major public health problem in Angola, where treatment of uncomplicated cases relies mainly on ACTs, particularly AL and artesunate-amodiaquine (ASAQ) [2, 23]. SP is also used for IPTp [2, 47]. Given the limited and geographically fragmented data on molecular markers of antimalarial resistance in Angola—especially in rural areas of Benguela province—this study provides new molecular evidence on resistance patterns to ACTs and SP through the analysis of *pfk13*, *pfmdr1*, *pfdhfr*, and *pfdhps* markers, in the rural municipality of Cubal, Benguela.

Since the adoption of ACTs (AL, Coartem®, Novartis, Basel) as first-line treatment in Angola between 2004–2008 [52], concerns have been raised about the potential selection of *pfk13* mutations under sustained drug pressure. Previous reports have identified *pfk13* mutations not linked to artemisinin resistance in the provinces of Zaire [26, 29], Lunda Sul [26], and Huambo [61]. Imported cases from Angola to China have revealed the presence of *pfk13* mutations, including the WHO-validated resistance markers R539T and P553L [19, 24, 25], as well as other variants not currently associated with artemisinin resistance [62, 63]. In the present study, five nonsynonymous *pfk13* mutations were identified, none of which correspond to validated or candidate markers of artemisinin resistance. Notably, mutation V589I and Q613E—also described in imported cases from Angola [19, 24, 25, 63]—were observed. Moreover, the detection of these nonsynonymous variants, in combination with reports of declining AL efficacy in Angola [23, 26, 29, 34, 64], emphasizes the importance for further studies to assess the potential association of SNPs, including T573S, V581A, and Q613E, with artemisinin partial resistance, which has not yet been investigated for these markers. By contrast, the A578S mutation—widespread across Africa but not linked to resistance, present in countries such as DRC, Republic of Congo, Equatorial Guinea, and Gabon [21]—was absent in our cohort, despite being reported in other Angolan provinces [26, 29, 61]. Interestingly, a synonymous substitution at the same position (A578A) was identified in a Cubal isolate, suggesting that this locus may be particularly prone to genetic modifications.

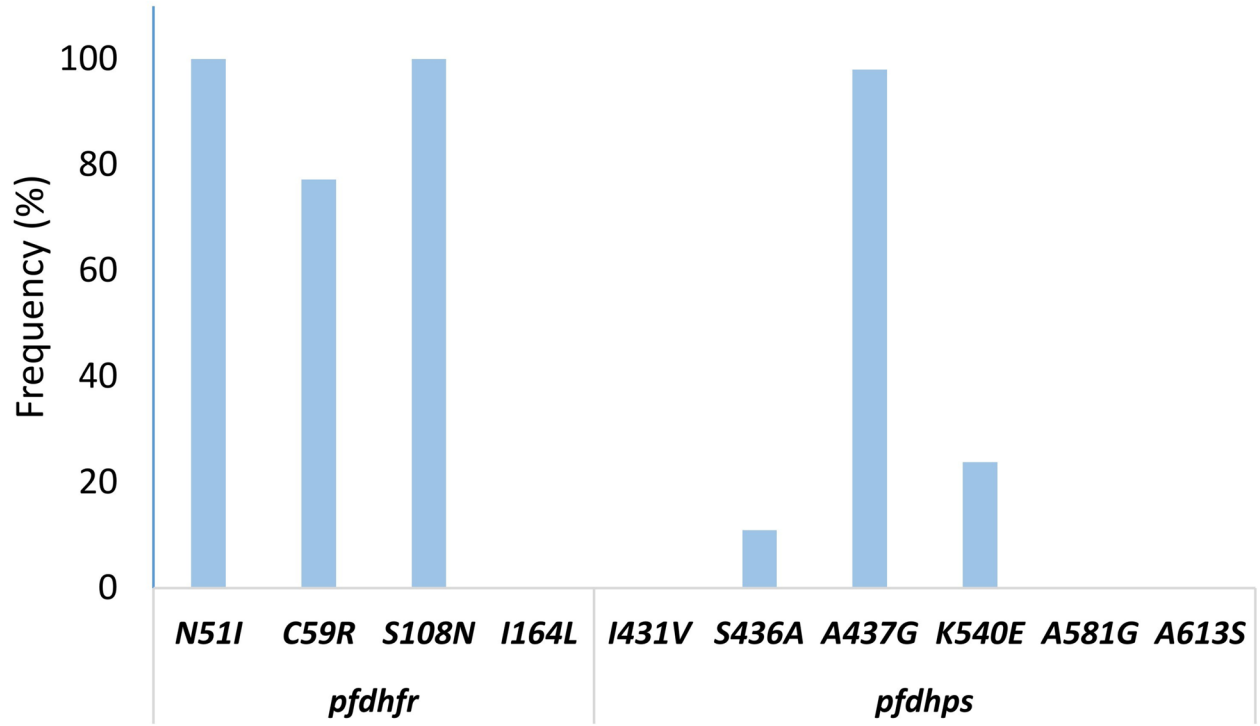

Fig. 3 Frequency of SNPs identified in *pfdhfr* and *pfdhps*. The graph shows the proportion of isolates carrying molecular markers associated with pyrimethamine resistance (*pfdhfr*: N51I, C59R, S108N, and I164L) and sulfadoxine resistance (*pfdhps*: I431V, S436A, A437G, K540E, A581G, and A613S). Numbers in parentheses indicate the number of isolates with each mutation relative to the total number of sequences analyzed (n = 101)

Table 3 Frequency of combined *pfdhfr/pfdhps* haplotypes (n = 101)

Haplotypes [a]	Type of resistance [b]	No of isolates n (%)
IRNI-I**AGE**AA	Fully	1 (1)
IRNI-IS**GE**AA	Fully	17 (16.8)
IRNI-I**AG**KAA	Partial	8 (7.9)
IRNI-IS**G**KAA	Partial	51 (50.5)
IRNI-ISAKAA	–	1 (1)
I**CN**I-IS**GE**AA	–	6 (5.9)
I**CN**I-I**AG**KAA	–	2 (2)
I**CN**I-IS**G**KAA	–	14 (13.9)
I**CN**I-ISAKAA	–	1 (1)

[a] Each letter indicates the amino acid present in the sequences at the corresponding positions (*pfdhfr*: codons 51, 59, 108, and 164; *pfdhps*: codons 431, 436, 437, 540, 581, and 613). Bold letters represent mutations, while non-bold letters correspond to the wild-type

[b] Type of sulfadoxine–pyrimethamine (SP) resistance according to the classification established by Naidoo et al.[39]

In this study, *pfmdr1* analysis—relevant for susceptibility to lumefantrine and amodiaquine [32]—showed a very low prevalence of N86Y (1%), a high prevalence of Y184F (43%), and no mutations at codon 1246. Accordingly, the dominant haplotypes were NFD and NYD, which have previously been associated with AL treatment failures in Angola [23, 26, 56]. In Cubal, the high frequency of Y184F combined with wild-type N86 and D1246 suggests selection of AL-tolerant parasites, consistent with reduced lumefantrine susceptibility reported in other provinces [23, 26, 64]. At the same time, these haplotypes are associated with increased sensitivity to amodiaquine, in line with the favorable performance of ASAQ in Angola [26, 64]. Nevertheless, despite this advantage, AL has been prioritized over ASAQ due to the latter's adverse effects and poor adherence in real-world practice [65]. The genetic profile observed highlights the need for therapeutic efficacy studies in rural settings such as Cubal—ideally conducted every two years—to assess AL performance in relation to the local *pfmdr1* background.

SP was withdrawn as first-line malaria treatment in Angola in 2006 due to widespread resistance and is currently used exclusively for chemoprevention, mainly in IPTp [47]. In Cubal, fixation of the triple *pfdhfr* mutant **IRN**I was observed, together with a very high prevalence of *pfdhps* A437G (98.0%) and a moderate prevalence of K540E (23.8%). No A581G mutations or "super-resistant" haplotypes were detected, indicating

that SP remains a viable preventive option in this rural setting. These findings are consistent with data from Benguela, Lunda Sul, and Zaire (2019), where K540E prevalence was low (10–14%) and A581G was either absent (Benguela, Lunda Sul) or < 5% (Zaire) [47]. Both values remain well below WHO thresholds (≥ 95% for K540E and ≥ 10% for A581G) that would compromise IPTp efficacy [44, 46]. Earlier surveys in Luanda and Lubango (2007–2011) reported a K540E prevalence of 6–11.7% and isolated cases of A581G (7%) [66–68]. The temporal increase in K540E prevalence from 2007 to the present suggests that ongoing SP use for IPTp may be exerting selective pressure favoring "fully resistant" parasites. If this trend continues and combines with the emergence of A581G—already reported in other provinces—the long-term effectiveness of SP-based preventive interventions in Angola could be compromised. For now, however, the absence of "super-resistant" haplotypes in Cubal supports the continued use of SP in maternal prevention programs.

The situation in Angola contrasts with that in East Africa, where the emergence of "super-resistant" parasites—particularly those carrying A581G at prevalences exceeding 40%—has severely compromised IPTp-SP efficacy, as in Uganda and Tanzania [39]. In comparison, Angola has a profile more similar to that of West and Central African countries. In Cubal, while the majority of isolates carried "partially resistant" haplotypes (58.4%), "fully resistant" parasites were detected at a significant frequency (17.8%). This places Angola between the scenarios in Nigeria or Ghana, where total resistance is rare and K540E is nearly absent [60, 69], and Equatorial Guinea, where partial resistance coexists with emerging foci of fully or even super-resistant haplotypes [36].

The molecular profile observed also suggests that SP-based chemoprevention could be considered for other strategies, such as IPTi or SMC in children, particularly in combination with amodiaquine, given the absence of *pfmdr1* resistance markers to this drug. Evidence from countries in the Sahel [8], and others such as South Sudan [9] has shown that SMC with SP + AQ significantly reduces childhood malaria, even in settings with moderate SP resistance, suggesting its potential potential applicability in Angola. However, continued SP use exerts selective pressure on *P. falciparum* [38]. The presence of a substantial proportion of "fully resistant" isolates in Cubal highlights the need for strengthened surveillance. Regular monitoring of molecular resistance markers, complemented by therapeutic efficacy studies, would be essential to detect early increases in key mutations such as K540E, A581G, or *pfdhfr* I164L and to ensure the sustained effectiveness of SP-based preventive interventions [39].

Limitations of this study

A limitation of this study is the absence of correlation analyses between the molecular markers detected and in vivo or in vitro phenotypes. Such analyses could contribute to a better understanding of the potential functional role of the *pfk13* mutations identified as markers of artemisinin resistance in Angola, as well as provide additional insight into lumefantrine response in the study area and its possible association with the *pfmdr1* genotypes detected. Future studies integrating molecular findings with therapeutic efficacy data will be necessary to better assess the clinical relevance of the variants identified. In addition, the variation in *pfmdr1* copy number, a factor associated with susceptibility to AL and potential influence on treatment efficacy [27], was not analyzed due to methodological and resource limitations. Polyclonal infections were operationally defined based on the presence of double peaks in Sanger sequencing electropherograms, as no genotyping or multiplicity of infection analyses were performed, which may have led to an underestimation of within-host parasite diversity. Another limitation was that participant recruitment took place at the end of the rainy season and the beginning of the dry season, which may have influenced parasite diversity and the observed prevalence of resistance markers; therefore, conducting similar studies during the high-transmission season is recommended to better capture seasonal variation.

Conclusions

This study provides updated information on the genetic profile of *P. falciparum* with respect to molecular markers associated with antimalarial drug resistance in the rural municipality of Cubal, Benguela province, Angola. The absence of validated or candidate *pfk13* markers of artemisinin resistance suggests that partial resistance has not yet emerged in this setting, while the patterns observed in *pfmdr1* and in the *pfdhfr*/*pfdhps* genes reflect sustained selective pressure by the antimalarial drugs currently used in the country.

The molecular profiles identified are consistent with a context in which AL, used as first-line curative treatment, and SP, currently deployed for chemoprevention in pregnant women, remain operationally effective, although early warning signals warrant continued vigilance. In this context, the resistance profile observed for SP supports its continued use in IPTp and suggests that the implementation of additional chemoprevention strategies, such as IPTi and/or SMC in children, should be considered in Angola. Overall, these findings emphasize the selective pressure exerted by AL and SP on local *P. falciparum* populations and highlight the

importance of sustained, integrated molecular surveillance, complemented by periodic therapeutic efficacy studies, to detect early changes in drug susceptibility and support timely, evidence-based malaria control and prevention strategies in rural Angola.

Abbreviations

ACTs	Artemisinin-based combination therapies
AL	Artemether-lumefantrine
ASAQ	Artesunate-amodiaquine
DBS	Dried blood spot
DRC	Democratic Republic of Congo
HNSP	Hospital Nossa Senhora da Paz
HUVH	Vall d'Hebron University Hospital
IQR	Interquartile range
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnancy
Pgh-1	P-glycoprotein homolog 1
pfmdr1	*P. falciparum* multidrug resistance gene
RDT	Rapid diagnostic test
SD	Standard deviation
SMC	Seasonal malaria chemoprevention
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
SP + AQ	Sulfadoxine-pyrimethamine-amodiaquine
WHO	World Health Organization

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions
Conception and design of the study: AM, IMF, MLARA, IOS, IM, PB, and ES. Recruitment of participants and acquisition of laboratory data at the HNSP in Cubal, Angola: XG, BFS, AN, and JMC. Acquisition of molecular data and laboratory work at the Hospital Universitario Vall d'Hebron in Barcelona, Spain: AM, PMV, AS, and CRM. Drafting the manuscript and analyzing and interpreting the data: AM. Revising the article critically for important intellectual content: IMF, BFS, AS, KF, CRM, JFM, JMC, IOS, PB, and ES. All authors read and approved the final manuscript.

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Data availability
The data supporting the conclusions of this article are included in this manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Vall d'Hebron University Hospital Drug Research Ethics Committee [PR(AG)79/2024] and by the Ethics Committee of the Angolan Ministry of Health (39 C.E/MINSA.INIS/2022). Each participant signed an informed consent form (in the case of minors, parental/guardian consent was obtained) after being informed of the objectives of the study. All procedures were performed according to the ethical guidelines of the 2013 Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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