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Low prevalence of copy number variation in *pfmdr1* and *pfpm2* in *Plasmodium falciparum* isolates from southern Angola

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Abstract

Background Malaria is the parasitic disease with the highest global morbidity and mortality. According to estimates from the World Health Organization (WHO), there were around 249 million cases in 2022, with 3.4% occurring in Angola. The emergence and spread of drug-resistant *Plasmodium falciparum* have compromised anti-malarial efficacy and threatens malaria elimination campaigns using artemisinin-based combination therapy (ACT). Increased copy number (CNV) of the *P. falciparum* gene plasmepsin 2 (*pfpm2*) have been reported to confer parasite tolerance to piperaquine (PPQ) and the multidrug resistance-1 (*pfmdr1*), resistance to mefloquine (MEF) and decreased susceptibility to lumefantrine (LUM). PPQ, MEF and LUM are ACT partner drugs. Therefore, CNV detection is a useful tool to track ACT resistance risk. The potential for future treatment failure of artemisinin-based combinations (that include PPQ, LUM and AMQ), due to parasite resistance in the region, emphasizes the need for continued molecular surveillance.

Methods One hundred and nine clinically derived samples were collected at Hospital Central Dr. António Agostinho Neto (HCL) in Lubango, Angola. qPCR targeting the small-subunit 18S rRNA gene was used to confirm *P. falciparum* infection. Copy number estimates were determined using a SYBR green-based quantitative PCR assay.

Results Overall, this study revealed a low number of resistance CNVs present in the parasite population at Lubango, for the genes *pfmdr1* and *pfpm2*. Of the 102 samples successfully analysed for *pfpm2* 10 (9.8%) carried increased CNV and 9/101 (8.9%) carried increased CNV of *pfmdr1*.

Conclusions This study provides, for the first time, evidence for the presence of CNVs in the *pfpm2* and *pfmdr1* genes in *P. falciparum* isolates from southern Angola.

Keywords *Plasmodium falciparum*, *pfpm2*, *pfmdr1*, Lumefantrine, Piperaquine, Lubango, Angola

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Background

Malaria is a vector-borne disease caused by *Plasmodium* protozoa and transmitted through the bite of infected female mosquitoes from the genus *Anopheles*. According to the World Health Organization (WHO), the great majority of malaria cases and deaths, attributed to *Plasmodium falciparum*, occur in sub-Saharan Africa, with approximately 249 million cases and over 608.000 deaths reported in 2022 [1].

Angola, a country in Western coast of Southern Africa, recorded more than 8.3 million malaria cases in 2022. Incidence and mortality rate due to malaria was 25–55% higher in 2022 than in 2015 in the country [2].

Malaria burden in Angola is divided into three distinct zones based on climate: high transmission year-round in the north, mesoendemic in the center, and seasonal malaria (unstable) in the south, where Lubango (the capital of Huíla province, see Fig. 1) is located [3]. The country began recommending ACT (Artemisinin-based Combination Therapy) as first-line treatment for uncomplicated malaria in 2005. From 2006, ACT was introduced, and currently, the first-line treatment combinations in Angola are artemether-lumefantrine (AL), artesunate-amodiaquine (ASAQ) and dihydroartemisinin-piperaquine (DHA-PPQ) [4]. After administration, artemisinin derivatives are converted to DHA, which is primarily responsible for the drug's anti-malarial activity [5].

According to the WHO, treatment failures due to parasite resistance remains < 10% in Africa [6] and the resistance to DHA-PPQ has not yet emerged in Africa. However, there are some cases of clinical failures with DHA-PPQ in Asia [7–10]. In Angola, AL has a reported (in 2024) adequate clinical and parasitological response of treatment of uncomplicated malaria of 88.0% to 94.4%, ASAQ 91.0 to 100%, artesunate-pyronaridine (ASPY) 99.6% (depending on the geographical region) and for DHA-PPQ 98.3% [11].

Plasmodium falciparum partial resistant parasites to artemisinin derivatives were first identified in 2008 in Southeast Asia [12–14]. Since then, resistant parasites have been detected in Africa, posing a threat to the current efforts to control this disease in Africa [15–17].

Decreased parasite susceptibility to lumefantrine (LUM) can be modulated by *pfmdr1* (*P. falciparum* multidrug drug resistance gene 1) [18–20]. *pfmdr1* codes for a digestive vacuole transmembrane protein that allows the importation of solutes into the parasite's digestive vacuole [18]. Single nucleotide polymorphisms and increased copy number in *pfmdr1* have been associated with resistance to MEF and potentially LUM decreased susceptibility. Treatment with AL selects for the *pfmdr1* haplotype N86/184F/D1246, while DHA-PPQ treatment, although at a lower level than ASAQ, selects for the opposite haplotype 86Y/Y184/Y1246 [20, 21]. Increased *pfmdr1* copy number (carrying the SNPs N86/184F) has been associated with decreased susceptibly to lumefantrine [22]. And It has been hypothesized that increased *pfmdr1* copy number is associated with enhanced accumulation of

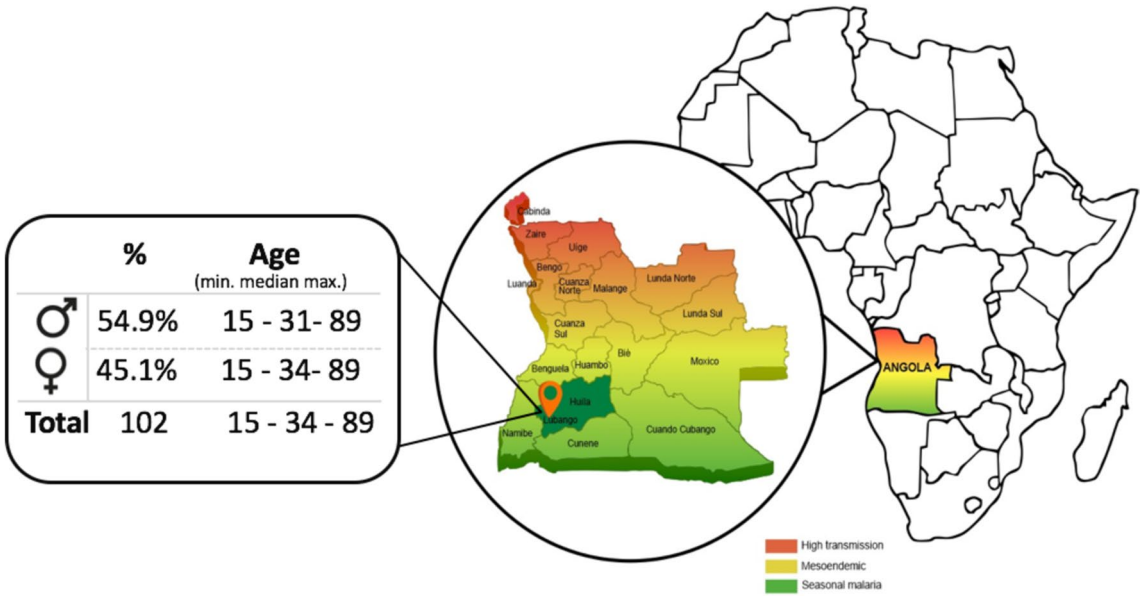

Fig. 1 Summary of sample processing and map of Angola highlighting study site. Pin, Lubango city and malaria incidence areas. Huíla province in dark green

PPQ in the digestive vacuole (DV), leading to increased sensitization to PPQ [18]. The increased copy number of another *P. falciparum* gene, the plasmepsin 2 gene (*pfpm2*) has been associated with clinical treatment failures with DHA-PPQ, in Southeast Asia [19, 23, 24]. Plasmepsin 2, a food vacuole enzyme and is considered a major piperaquine target [23, 25]. The apparent opposing selection pressures of LUM on *pfmdr1* suggest that DHA-PPQ may be a good choice of partner drug in areas where AL was previously largely used [8–10, 19, 23, 24]. Although some authors [23, 25, 26] consider *pfpm2* amplification sufficient to confer PPQ resistance without additional gene mutations (e.g., *P. falciparum* chloroquine resistance transporter; *pfcrt*), others argue that plasmepsin amplification alone is not enough. It may facilitate the selection of mutations in other *P. falciparum* genes, such as *pfcrt*, *pfmdr1* or *P. falciparum* exonuclease (*pfexo*), which could, in turn, enhance PPQ resistance levels [27, 28]. In fact, increased CNV in *pfmdr1* and *pfpm2* were observed in certain African countries [23], even before significant changes in parasite clearance time (the phenotypic manifestation of artemisinin derivative tolerance) [17] or mutations in the *pfK13* gene (which encodes the Kelch 13 protein responsible for *P. falciparum*'s reduced susceptibility to artemisinin derivatives) were detected [29–31].

The close relationship between A/T track-hairpin sequences in the genome of *P. falciparum* leads to the use of microhomology-mediated pathways, which are prone to errors. These events seem to lead to a greater generation of CNVs and contribute to the adaptability of this parasite under selective pressure (namely drugs) [32]. Although CNVs often entail a significant fitness cost [33] there are evidences that CNV formation, is the initial stage in *P. falciparum* that leads to the accumulation of SNPs that confer resistance [34, 35]. These findings prompted the present study to assess whether the increased copy number of genes that *pfmdr1* and *pfpm2* are already present in parasites currently circulating in Angola.

Methods

Ethics statement

Blood samples were collected following individual oral consent and signed informed written consent from all participants or their guardians. Ethical clearance for the work was obtained from: Comité de Ética Independente da Faculdade de Medicina da Universidade Agostinho Neto and by the Hospital Central Dr. António Agostinho Neto (HCL) institutional ethics review committee (deliberation N°08/2020, on 14 November 2020).

Study site

Patients were enrolled at the Emergency Department (ED) of the Hospital Central Dr. António Agostinho Neto of Lubango (hereafter designated HCL), Huíla province, Angola (Fig. 1) from February to June 2023. The HCL is a tertiary-level, regional institution located in the capital of Huíla province. It treats adult patients (> 16 years old) referred from all 14 municipalities in the province and from three other provinces in the southern region of Angola: Cuando-Cubango, Cunene, Namibe, as well as some neighbouring municipalities in Huambo province, totalling a population of approximately 6,500,000 inhabitants. HCL does not provide care for children under 16 years of age or pregnant women. In Huíla province alone, there are around 2.5 million inhabitants (10% Angola's population) at risk of contracting malaria. Malaria transmission in Huíla province is classified as unstable meso-endemic, with periods of low transmission during the months of May to December, and epidemic peaks during the rainy season, mainly in the months of January to April [3, 36].

Samples

Patients presenting at the emergency room of HCL were chosen for the study based on the inclusion criteria: Rapid Diagnostic Test (RDT) or thick smear by optical microscopy (OM) positive for *Plasmodium* spp., and consenting to participate. A total of 109 participants with malaria positive RDT or positive thick smear were recruited after consent and provided 200 µL of blood samples on filter papers (WHA10534320). All dried blood spot samples were then stored at − 20 °C until they were used for genotyping. To confirm presence of *P. falciparum* infection, parasite genomic DNA from dried blood spots was extracted using the Chelex method [37] and stored at − 20 °C. qPCR reactions targeting the 18S rRNA gene were conducted for confirmation, as described elsewhere [38] with modifications. Briefly, forward primer 5′-TATTGCTTTTGAGAGGTTTTGTTACTTTG-3′ and reverse primer ACCTCTGACATCTGAATACGAATGC and the probe FAM-ACGGGTAGTCATGATTGAGTT-MGB-BHQ (Integrated DNA Technologies-IDT) were used. PCR reaction mixture consisted of 7.5 µL of 2X NZYSupreme qPCR Probe master mix (NZYTECH, Portugal), 600 nM of each primer and 200 nM of probe, 2µL of genomic DNA and water up to 15 µL. Negative controls (H_20) were include in each PCR run. PCR conditions: 3 min a 95 °C, followed by 40 cycles a 95 °C for 5 s and a 58 °C for 40 s. Each sample was tested in triplicate in Bioer´s Line-Gene 9600 real_time PCR detection System™ (Biosan,SIA). All reactions were performed

with positive controls (DNA from *P. falciparum* 3D7 (MRA-102).

Assessment of *pfpm2* and *pfmdr1* copy numbers was conducted through SYBR Green I quantitative PCR. The *pfpm2* gene (PF3D7_1408000) and *pfmdr1* (PF3D7_0523000) were evaluated using the single copy gene *pfBtubulin* (*pfβtub;* PF3D7_1008700) as reference gene, as previously described [24, 39, 40]. Primers were designed (supplementary material Apendix1) inside the *pfpm2* and *pfmdr1* genes and as a reference gene, we used the single copy gene *pfβtub* (PF3D7_1008700). qPCR reactions were carried out in 15 µL volumes within a 96-well plate (Nerbe plus, BioPortugal) on a NZYSupreme qPCR SYBR Green Master mix (2X) (NZYTECH, Portugal). For each reaction, 200 nM of each primer, and 1.5 µL of genomic DNA was used. Negative controls (H_20) were include in each qPCR run for each gene. PCR cycling conditions: initial 5 min at 95°C, followed by 45 of 15s at 95°C and 1 min at 60 °C on a Real-Time PCR system Bioer Line-Gene 9600 Real Time PCR detection System™ (Biosan,SIA). Relative copy number was calculated on the basis of the $2^{-\Delta\Delta Ct}$ method for relative quantification. ΔΔCt was calculated as ($Ct_{\text{taget gene in sample}}$—$Ct_{pfBtub\text{in sample}}$)—($Ct_{\text{target gene in 3D7}}$—$Ct_{pfBtub\text{in 3D7}}$), sample is DNA from each patient and 3D7 is the calibration control of genomic 3D7 (MRA-102) DNA, with one copy of all genes (*pfBtub*, *pfpm2* and *pfmdr1*)[41]. DNA from an isolate IPC_6261 (MR4-1284) with 3 copies of *pfpm2* [41] and DNA from Dd2 (MRA-150) as a reference for multiple copies (3) of *pfmdr1* [22, 42–44] were used as an internal plate control. Single copy *vs* multiple copies of *pfpm2* and *pfmdr1*, were defined as copy number < 1.5 and ≥ 1.5 respectively [39, 45].

Amplification efficiencies of *pfpm2* and *pfmdr1* were similar to *pfBtub* (supplementary material). Assays were repeated if one of the following three results was obtained: standard variation of triplicate > 0.38 [46] Ct values > 40. Specificities of *pfpm2*, *pfmdr1* and pfbtub amplification curves were evaluated by visualizing the respective melt curves.

Results

Patients and samples

Of the enrolled 109 clinical samples, with malaria positive by Rapid Diagnostic Test (RDT) or thick smear (parasitaemia ranging between 64 and 580,120 parasites/µl blood with a median of 3120 parasites/µl blood) enrolled, 7 (6,4%) were qPCR negative for 18S from *P. falciparum*. Hence, were removed from further analysis. The remaining 102 DNA samples corresponded to patients that had between 15 and 89 years of age (with a median age 33.5 years), 54.9% (56/102) were males and 45.1% (46/102) females (Fig. 1).

Samples revealed a low level of CNVs both for *pfpm2* and *pfmdr1*

Three different laboratory-adapted parasite lines with known *pfpm2* and *pfmdr1* copy numbers were used as controls, in order to evaluate the accuracy of the assay. As expected, IPC_6261 (MRA 1284) and Dd2 (MRA 150) displayed a median copy number for *pfpm2* of 3.00 (range 2.98–3.40) and *pfmdr1* a median copy number of 2.90 (2.80–3.22) [41, 47, 48]; Fig. 2). Each sample was simultaneously analyzed with IPC_6261 for *pfpm2* and with Dd2 for *pfmdr1*. All 102 samples were successfully analyzed for *pfpm2* and 101 for *pfmdr1* (inserted table of Fig. 2). Using a threshold copy number of ≥ 1.5 as evidence of gene amplification, 10 samples carried more than one copy of *pfpm2* and 9 carried more than one copy of *pfmdr1*, representing 9.8% and 8,9%, respectively (inserted table of Fig. 2). Only two samples, carried simultaneously increased copy number for both genes.

Discussion

Given the potential risk of spreading ACT-resistant parasites in Africa, it is essential to conduct regional analyses to monitor the circulating parasites. This will improve the development of new therapeutic strategies to enhance the efficacy of anti-malarial drugs. Our study determined the copy number variations of *pfpm2* and *pfmdr1* in field isolates from Hospital Central of Lubango (HCL). Lubango is the capital of the Huíla province located in the south

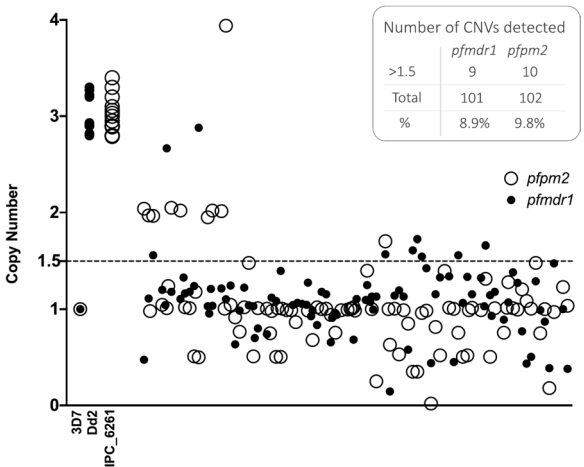

Fig. 2 Copy number of *pfmdr1* and *pfpm2* in *P. falciparum* samples from Lubango. The x-axis represents the each sample, while the y-axis shows the CNV (copy number variation) of each sample for each gene.Inserted table, number of samples with increased copy number of each gene; 3D7 reference strain (*pfmdr1* black circle is superimposed to the clear circle corresponding to *pfpm2*), Dd2 and IPC_6261, control strains; black circles, mean CNV value for *pfmdr1* in each sample; clear circles, mean CNV value for *pfpm2* in each sample

of Angola (Fig. 1). The examination of *pfmdr1* and *pfpm2* CNVs in Lubango (Huíla) indicated a low occurrence of CNV 9.8% (10/102) and 8.9% (9/101) respectively. Nevertheless, because HCL is one of the largest hospitals in southern Angola, serving the population of Huíla province as well as the neighbouring provinces of Namibe and Cunene, this study offers valuable insights into the parasite populations throughout the southern region of Angola. Theu objective was not to generalize these findings to the entire country but to determine whether these mutations are already present.

Regarding *pfmdr1*, 4 studies have been published addressing CNV in Angola. One study from Zaire and Uíge [49] that found no amplification of *pfmdr1*, and two other studies [50, 51], from Benguela, Lunda Sul, and Zaire that also reported absence of elevated copy number fo *pfmdr1*. Another study from Luanda [52] reported that 14.1% (13/92) of the samples carried increased *pfmdr1* copy numbers. Results presented here revealed a lower prevalence of *pfmdr1* CNV (8.9%; with a 95% CI ranging from 3.4 to 14.5%) compared to the 14% (95% CI ranging from 6.9 to 21%) previously reported from Luanda [52]. Luanda, the capital city, with significant international links, is located over 500 km north of Lubango by road. This marginal difference may reflect geographic diversity in *pfmdr1* distribution, or may be due to low sample size (ours n = 101 and [52] n = 93). Furthermore, samples from Luanda study [52] were collected nearly 10 years ago (collected in 2011–2013), during which time various therapies have been used in the region, potentially influencing parasite selection. Regarding the other studies from Angola (samples from Lunda Sul, Zaire, Benguela and Uígre, were collected in between 2013–2015 [51]) this results contrast with the absence of amplification of *pfmdr1* reported by the authors. In this case, geographic diversity may help explain the differences of CNV prevalence in Lunda Sul and Zaire. Both Lunda Sul and Zaire are located in the northern part of the country, more than 1,200 km by road from Lubango in Huíla. However, this is not the case for Benguela, which borders Huíla province. During which it is conceivable that there may have been changes in the genetic pattern of the parasitic populations in these provinces.

Nevertheless, this findings of low CNV prevalence of *pfmdr1*, align with other studies in *P. falciparum* populations from sub-Saharan Africa [47, 48, 53, 54] where the amplification of *pfmdr1* is also low but present.

Additionally, the AL combination has been consistently administered in Lubango, which could potentially have led to the selection of parasites exhibiting *pfmdr1* amplification (increased mRNA expression levels of *pfmdr1* are highly correlated with decreased sensitivity to LUM and DHA [25, 55]. Although *pfmdr1* amplification alone does not fully account for reduced susceptibility to LUM and DHA, *pfmdr1* copy number variation (CNV) remains a key marker for tracking drug-resistant parasites, particularly concerning resistance to mefloquine.

Regarding *pfpm2*, there is only one published study from Angola it includes samples from the provinces of Benguela, Lunda Sul, and Zaire (samples collected in 2017) [50]. That study reports the absence of CNV of *pfpm2*. The assessment of *pfpm2* CNVs in Lubango revealed a low frequency (Figs. 1 and 2) of CNV in *pfpm2*, which is consistent with other reports from sub-Saharan Africa. In these region, *P. falciparum* isolates have shown varying frequencies of CNVs in the *pfpm2* gene ranging from 10.0% in Mali to 33.9% in Uganda [45] and 1.1% to 5% from Mozambique [42, 56, 57] or 2.0% from Kenya [58]. The low frequency of *pfpm2* CNV in Lubango (Fig. 2) is consistent with the relatively low rates of DHA-PPQ use in the province (Huíla; personal communication of Professor Lina Antunes, Director of HCL) and in sub-Saharan Africa overall [27, 59, 60]. Although *pfpm2* amplification is recognized as sufficient to confer piperaquine resistance [23, 25, 26] some studies argue that amplification of plasmepsins alone is not sufficient, but may facilitate the selection of mutations in other genes, such as *pfcrt* (SNPs like F145I [59] G367C [60] C350R [27]), thereby potentiating piperaquine resistance levels [27]. In fact, CNVs in *Plasmodium* seem to eventually be lost in favor of SNPs [61–63]. *Pfcrt* has not yet genotyped in this samples, and to the best of current knowledge, there is no available information on piperaquine susceptibility and *pfcrt* SNPs in Angola. In contrast with *pfpm2*, the increased CNV of *pfmdr1* has been established as the most useful indicator of multidrug-resistant malaria strains, influencing parasite responses to mefloquine, LUM, and DHA [25, 55, 64]. The detection of PPQ/LUM-resistance molecular markers (*pfpm2*/*pfmdr1* CNV) in Angola, even if at a lower frequency compared to Southeast Asia (SEA), highlights the need for constant surveillance. Additionally, in cases where a patient harbours multiple *P. falciparum* clones (in Africa the presence of multiple clones in the same individual is not uncommon) some with a single copy and others with multiple copies, the overall CNV could be underestimated. Mixed infections can obscure the presence of clones with higher copy numbers, resulting in a lower average CNV. This is especially important because evidence from SEA suggests that PPQ resistance developed multiple times on different parasite genetic backgrounds [23]. Hence, as DHA-PPQ use in Angola becomes more extensive, the risk of selecting PPQ-resistant parasites increases, posing a threat to the efficacy of the therapy combination. Therefore, with adequate drug pressure, resistant isolates may spread throughout Angola, as was observed in the past in

Southeast Asia. Despite the limited sample size and limited geographical focus, on patients from the southern provinces of Angola, the present study demonstrates for the first time that these mutations exist within the local parasite population.

Conclusion

Evidence is provided, for the fist time, of the presence of multiple copies of *pfpm2* and *pfmdr1* in currently circulating *P. falciparum* parasites in Lubango, the southern Angola. The study supports the continued need for molecular surveillance, ideally countrywide, to improve the detection of ACT resistance before it becomes widespread.

Abbreviations

ACT	Artemisinin-based combination therapy
AMQ	Amodiaquine
CNV	Increased copy number
DHA	Dihydroartemisinin
HCL	Hospital Central Dr. António Agostinho Neto in Lubango
LUM	Lumefantrine
PPQ	Piperaquine
SEA	Southeast Asia
WHO	World Health Organisation

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Author contributions

D.D. processed samples and prepared the original draft of the text; A.D. analyzed data; F.M., E.S., E.T., E.D, F.S. sample collection and processing; L.V. patient enrrolment supervision and manuscript revision; M.L.A. designed the research study and patient enrrolment supervision; F.N. designed the research study, sample processing supervision, curated the data and edited the final text; All authors reviewed the manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

DNA samples were collected following individual oral consent and signed informed written consent from all participants or their guardians. Ethical clearance for the work was obtained from: Comité de Ética Independente da Faculdade de Medicina da Universidade Agostinho Neto and by the Hospital Central Dr. António Agostinho Neto (HCL) institutional ethics review committee (deliberation Nº08/2020, on 14 November 2020).

Competing interests

The authors declare no competing interests.

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