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Molecular surveillance of *Plasmodium falciparum histidine-rich protein 2/3* gene deletions in Mozambique, 2023

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Abstract

Background Rapid diagnostic tests (RDTs) based on the detection of *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2) are widely used for the diagnostic of *P. falciparum* in Africa. However, deletions of the *pfhrp2* and *pfhrp3* genes can lead to false negative test results and compromise appropriate case management. Due to the high burden of malaria in Mozambique, it is crucial to monitor the potential emergence of parasites with *pfhrp2/3* gene deletions in the country.

Methods The presence of *pfhrp2/3* deletions was assessed during the 2023 high transmission season in 34 health facilities from 9 districts across 6 provinces in Mozambique. Children between 2 and 10 years of age attending the health facility with fever were tested by both the routine HRP2-RDT and a *P. falciparum* lactate dehydrogenase (PfLDH)-RDT, and dried blood spots (DBS) were collected from those testing positive by one or both RDTs. DBS from children with a negative HRP2-RDT but positive PfLDH-RDT were tested for the presence of *pfhrp2/3* deletions by multiplex real time quantitative polymerase chain reaction (qPCR).

Results 3208 children attended the health facilities during the study. 81.6% (2612/3208) participants were positive for at least one malaria RDT and, among them, 0.8% (210/2612) had discrepant RDT results (22 HRP2 − but LDH + and 188 HRP2 + but LDH −). The overall prevalence of suspected false-negative HRP2-RDT results in Mozambique was 0.91% (95% CI 0.58–1.39; 22/2424). *pfhrp2/3* gene deletions were confirmed in 4 cases (1 in Nampula and 3 in Inhambane). Therefore, the prevalence of *P. falciparum* confirmed cases with *pfhrp2/3* gene deletions in the six provinces sampled was 0.16% (95% CI 0.15–2.57; 4/2424), being 0.27% (95% CI 0.01–1.75; 1/367) in Nampula and 0.59% (95% CI 0.15–1.88; 3/503) in Inhambane.

Conclusion *pfhrp2/3* gene deletions were detected in 2 out of 6 provinces surveyed in Mozambique, but at a prevalence far below the 5% threshold recommended for a change in HRP2-based-RDT.

Keywords Molecular surveillance, Diagnostics, RDT, Malaria, *pfhrp2/3* gene deletions, Mozambique

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Background

Despite large efforts to control and eradicate malaria, the disease continues to be responsible for the loss of human life worldwide [1]. *Plasmodium* infections resulted in around 608,000 deaths in 2022, and the global burden of malaria cases reached 249 million. In 2022, Mozambique accounted for 4.2% of global malaria cases, the large majority caused by *Plasmodium falciparum* [2, 3].

Effective and easy access diagnostic tools are critical for adequate case management and treatment, reducing morbidity and mortality from malaria [4]. The World Health Organization (WHO) currently recommends the use of microscopy and rapid diagnostic tests (RDTs) to confirm suspected cases of malaria. RDTs are most commonly used in malaria-endemic areas, where results are needed rapidly, and both availability of microscopes and training of health facility staff is limited [5, 6]. The most commonly used RDTs are based on the detection of histidine-rich protein-2 (HRP2) [5–7]. These RDTs generally have high sensitivity, good heat-stability profiles and their targeted antigens are expressed exclusively in *P. falciparum* [6, 8, 9]. Despite the advantages of this diagnostic tool, false negative results can compromise adequate diagnosis. Common causes of false-negative RDT results are operator errors, inadequate storage conditions or low parasitemia. However, false-negatives can also occur in infections with parasites deleted in *pfhrp2* and *pfhrp3* genes [10–12]. Although these two genes are distant from each other, they are structurally similar and HRP3 can potentially still be detected by HRP2-RDT in parasites lacking HRP2 due to antibody cross-reactivity [8, 13].

The first record of deletions in the *pfhrp2/3* genes in clinical samples was reported in Peru in 2010 [10, 14], followed by multiple reports on this phenomenon in other parts of the world, including Central America [15, 16], Asia [17, 18] and Africa [19–22]. Of note, rapid emergence of parasites with *pfhrp2* gene deletions has been recently reported in some African countries like Eritrea [23], Djibouti [24] or Ethiopia [25]. The WHO recommends countries with any report of *pfhrp2/3* deletions as well as neighbouring countries to conduct surveillance for *pfhrp2/3* deletion, and has published guidelines on surveillance with this purpose [26]. Countries where the prevalence of *pfhrp2/3* gene deletions exceeds 5%, should replace HRP2-based RDTs by a non-HRP2 based diagnostic approach. This is the prevalence at which false negatives due to lack of HRP2 antigen expression are likely to exceed the false negative rate observed using alternative RDTs based on lactate dehydrogenase (LDH) parasite protein [26, 27].

Due to Mozambique's high malaria burden and its significant contribution to global annual malaria cases, it is crucial to monitor the potential rise of *pfhrp2/3* in this country. To date, two studies have been carried out in Mozambique. The first analysed samples collected in 2018 from Cabo Delgado, Zambézia and Maputo, using a bead-based multiplex immunoassay technique to detect presence of circulating HRP2 antigen [22], whereas a second study used PCR to characterize deletions in the districts of Manhiça and Magude (Maputo province) between 2010 and 2016 [20]. Both concluded that prevalence of parasites carrying *pfhrp2/3* deletions was very low (< 1.5%). However, no study has systematically addressed the prevalence of gene deletions and its impact on malaria diagnosis as a primary objective, and up to date data is lacking for most provinces. This study aimed to determine the prevalence of deletions in the *pfhrp2/3* genes causing false negative RDT results amongst all *P. falciparum* symptomatic patients recruited in six Mozambican provinces during the rainy season in 2023, in order to inform the National Malaria Control Programme (NMCP) on RDT policy recommendations.

Methods

Study design

A convenience health facility survey (HFS) was conducted between January and March 2023 in 6 Mozambican provinces (Inhambane, Manica, Nampula, Niassa, Sofala and Zambézia). The number of health facilities (HF) included in each province was 8 in Nampula, 6 in Niassa, 6 in Manica, 6 in Sofala, 4 in Zambézia and 4 in Inhambane, respectively (Fig. 1).

Under the hypothesis that the prevalence of *pfhrp2/3* gene deletion causing false negative RDTs within *P. falciparum* symptomatic patients is below 5%, a sample size of 370 per sampling domain (i.e. province) was targeted based on WHO guidelines for surveillance of *pfhrp2/3* deletions published in 2020 [26]. Assuming a 10% of loss of samples or uninterpretable analysis, target sample size per province was 407 individuals with a confirmed *P. falciparum* infection.

Participant recruitment and sample collection

Children aged between 2 and 10 years of age presenting at outpatients' clinic with fever (axillary temperature > 37.5 °C) or self-reported history of fever in the previous 24 h were invited to take part in the study. Once the child's legal representative agreed to participate by signing a written informed consent, each child was tested for malaria using both the routine HRP2-based RDTs (SD BIOLINE Malaria Antigen *Pf*, Abbott, 05FK50) in Inhambane, Manica and Sofala, or First Response® Malaria Antigen *P. falciparum* HRP2 (Premier Medical, PI13FRC25) in Zambézia, Nampula and Niassa) and the confirmatory PfLDH-based RDT (BIOCREDIT Malaria

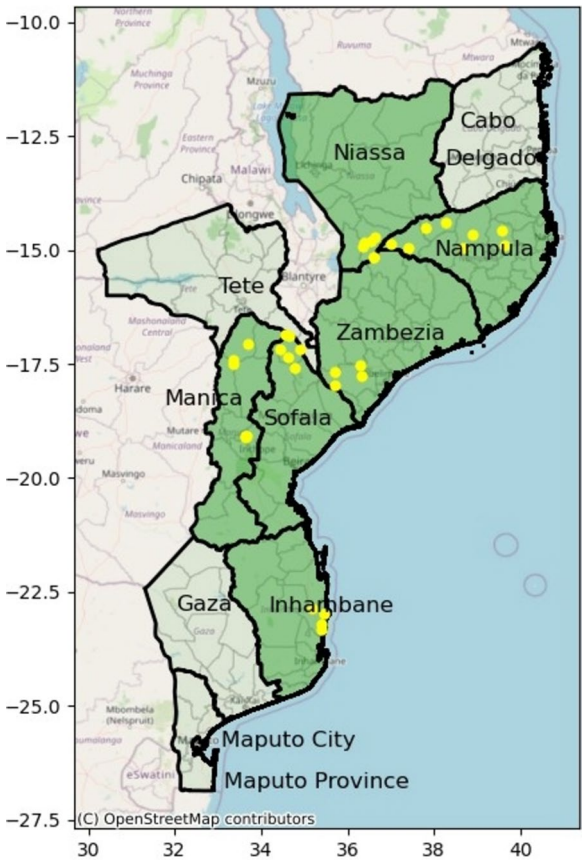

Fig. 1 Map of Mozambique with the study sites. Yellow dots represent health facilities where *P. falciparum* samples were collected. The map was created using R software version 4.2.2

Ag *Pf pLDH,* Rapigen Inc, C14RHG25). Dried blood spots (DBS) were collected from children who tested positive for at least one of the two RDTs. Samples with discrepancies in RDT results (HRP2 −/PfLDH + and HRP2 + / PfLDH −), were selected for molecular characterization.

Molecular analyses

DNA was extracted from a 5 mm DBS punch using a Tween-Chelex based method as previously described [28]. Briefly, the method consisted in washing punched DBS disks with Phosphate-buffered saline (to balance the salt concentration around the cells, preventing osmosis), and finally adding Chelex (Merck) at a concentration of 20%, followed by an incubation at 95 °C for 10 min. The supernatants containing DNA were then transferred to 1.5 ml Eppendorf tubes to be stored at − 20 °C.

The parasite density of discrepant samples was estimated using the *pf18S* ribosomal qPCR reaction as previously described [29]. Parasite densities were quantified from the Ct values by extrapolation to an standard curve composed of six (1:10) dilutions of *P. falciparum* laboratory strain 3D7 from 100,000 to 1 parasite/µL. Deletions in *pfhrp2* and *pfhrp3* were analysed by adapting the qPCR method by Grignard et al. for the multiplex detection of *pfhrp2*, *pfhrp3*, *pfldh* (parasite DNA control) and human *tubulin (humtubb;* DNA extraction control*)* [30]. Briefly, reactions were set up using TaqMan Multiplex Master Mix (Thermo Fisher), previously published primers and probes at custom concentrations (Supplementary Table 1), and run in a 7500 Real- Time PCR Instrument (Applied Biosystems™) under the following cycling conditions: 20 s at 95 °C, 45cycles of 3 s at 95 °C and 33 s at 60 °C. Results were visualized and analysed using the 7500Real-Time SDS software version 2.3 (Applied Biosystems). Laboratory strains with known different deletion profiles were used to validate the assay: 3D7 (no deletions), Dd2 (*pfhrp2*-deleted), and HB3 (*pfhrp3*-deleted; see Supplementary Fig. 1). Sample results were considered valid if amplification in *humtubb (*Ct < 30) and *pfldh* (Ct < 35) targets was detected. Samples without amplification (or Ct > = 40) in *pfhrp2* or *pfhrp3* were considered fully deleted for one or both genes. To estimate presence of infections with mixed *pfhrp2/3* deleted and non-deleted clones we used ΔΔCt relative method quantification as described in [30], normalizing by total DNA quantity and using 3D7 as calibrator.

Definitions and statistical analyses

The prevalence of suspected false-negative HRP2-RDT results was calculated as the number of HRP2-RDT + / PfLDH-RDT — patients divided by the total number of *P. falciparum* confirmed cases (i.e. PfLDH-RDT + patients). The prevalence of suspected false-negative HRP2-RDT results caused by *pfhrp2/3* gene deletions was calculated as the number of samples with *pfhrp2/3* gene deletions divided by the total number of *P. falciparum* confirmed cases [31].

All calculations were done at the provincial level with 95% confidence intervals (CI) estimated for all point estimates. Areas of residence had been pre-categorized as rural or urban based on definitions that consider distance from large towns or cities, access to infrastructure and connectivity.

Ethical considerations

The study protocol obtaining ethical clearance by the National Bioethics Committee for Health of Mozambique (Ref: 131/CNBS/2021; October 2021).

Results

Malaria RDT positivity and patient characteristics

Overall, 3208 children aged 2–10 years were tested with both the HRP2-based RDT and the confirmatory PfLDH-based RDT across six provinces (Fig. 2). A total of 2612

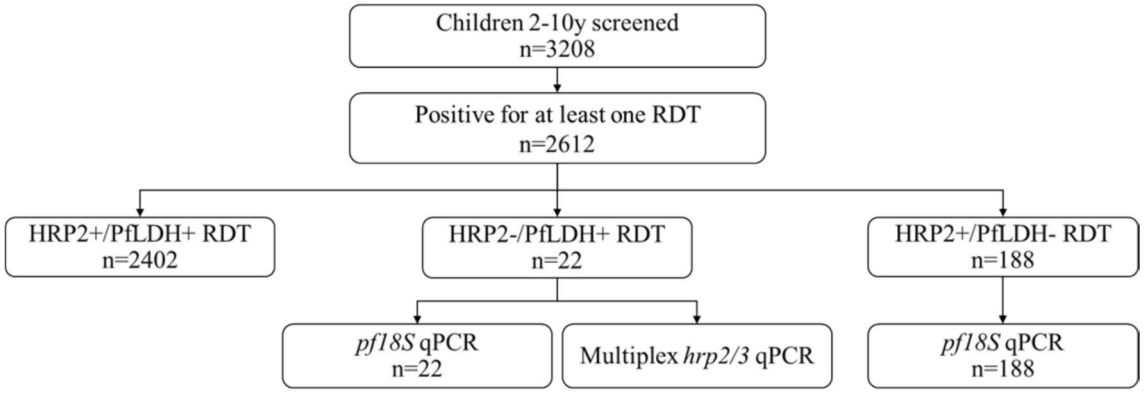

Fig. 2 Survey flow chart and sample selection for molecular characterization

of the participants (81.4%) were positive for at least one malaria RDT (i.e. HRP2 + or PfLDH +), and DBS were collected. By RDT type, positivity rate among febrile individuals was 80.7% (2590/3208) using routine HRP2-RDT testing, and 75.6% (2424/3208) using PfLDH-based RDT. The children had a median age of 5.2 years and 51% (1329/2612) were female. Most of the study participants (84%) were recruited in rural areas (Table 1).

Overall, 8% (210/2612) of study participants who were positive by any RDT had discrepant RDT results, most of them (188/210, 89,5%) due to a positive HRP2 but negative PfLDH result. qPCR analysis of these samples confirmed 87/188 (46.27%) positives for *P. falciparum* DNA, with a median parasite density of 406.1 parasites/µL (interquartile range [IQR] 24.4, 11,890.4).

Prevalence of false-negative HRP2-based RDT and *pfhrp2/3* gene deletions

For subsequent analysis on *pfhrp2/3* deletion surveillance, patients positive with PfLDH-RDT positive result (n = 2424) were considered as confirmed malaria cases. Twenty-two of these participants (0.91%;95% CI 0.15–1.38) had negative HRP2 RDT result (Fig. 2). The prevalence of suspected false-negative HRP2-RDT per province ranged from 0.47% (95% CI 0.08–1.89; 2/422) in Zambézia to 1.68%, 95% CI 0.67–3.70; 6/367) in Nampula (Table 2).

Out of the 22 HRP2 −/PfLDH + samples analysed by qPCR, eight had no detectable parasite DNA (despite human DNA presence confirming adequate DNA extraction) and 3 samples had an invalid Ct result in the multiplex qPCR (Table 3). Among 11 valid samples, *pfhrp2* and/or *pfhrp3* genes were not amplified in 4 cases from Nampula and Inhambane (1 *pfhrp2* deletion, 2 *pfhrp3* deletions and 1 *pfhrp2/3* double deletion). Considering confirmed RDT tests, the prevalence of confirmed uncomplicated *P. falciparum* infections with *pfhrp2/3* gene deletions was 0.27% (95% CI 0.01–1.75; 1/367) in Nampula, 0.59% (95% CI 0.15–1.88; 3/503) in Inhambane and not detected in the other four other provinces. The overall prevalence for the six provinces sampled was 0.16% (95% CI 0.15–2.57; 4/2424). Median parasite densities in infections with *pfhrp2/3* deletions were 1419.6 parasites/µl (IQR 61.9, 41,690.2). Another six samples presented evidence of mixed *pfhrp2/3* deleted and non-deleted parasites, with 4 of them showing a reduction in

Table 1 Characteristics of study participants with a positive malaria RDT result in six provinces of Mozambique, 2023

	Overall N = 2612	Inhambane N = 537	Manica N = 417	Sofala N = 344	Zambézia N = 449	Nampula N = 431	Niassa N = 434
Sex, n (%)							
Female	1329 (51%)	285 (53%)	211 (51%)	184 (53%)	235 (52%)	218 (51%)	196 (45%)
Male	1283 (49%)	252 (47%)	206 (49%)	160 (47%)	214 (48%)	213 (49%)	238 (55%)
Age (years), median (IQR)	5.2 (3.5, 7.4)	6.1 (4.0, 8.3)	5.4 (3.7, 7.4)	5.6 (3.9, 7.1)	5.1 (3.5, 7.3)	4.2 (2.8, 6.3)	5.1 (3.3, 7.0)
Residence, n (%)							
Rural	2184 (84%)	367 (68%)	326 (78%)	344 (100%)	350 (78%)	431 (100%)	366 (84%)
Urban	428 (16%)	170 (32%)	91 (22%)	0 (0%)	99 (22%)	0 (0%)	68 (16%)

IQR inter-quartile range

Table 2 False-negative HRP2-RDT results in six provinces of Mozambique, 2023

Province	Children screened	PfLDH RDT +			Prevalence of suspected false negative
		Total	HRP2 RDT +	HRP2 RDT −	
Niassa	527	404	401	3	0.74% CI (0.19–2.34)
Nampula	527	367	361	6	1.63% CI (0.67–3.70)
Zambézia	511	422	420	2	0.47% CI (0.08–1.89)
Sofala	389	325	322	3	0.92% CI (0.24–2.90)
Manica	520	403	401	2	0.48% CI (0.09–1.98)
Inhambane	734	503	497	6	1.19% CI (0.49–2.71)
Total	3208	2424	2402	22	0.91% CI (0.15–2.57)

Provinces displayed from North to South

CI 95% Confidence interval

Table 3 Molecular analysis of suspected false-negative HRP2 RDT cases in six provinces of Mozambique, 2023

Province	Samples tested	Pf DNA positive	*pfhrp2/3* qPCR					
			Valid	No deletion	*pfhrp2* deletion	*pfhrp3* deletion	Double deletion	Mixed clones*
Niassa	3	0	–	–	–	–	–	–
Nampula	7	3	1	0	0	1	0	0
Zambézia	2	2	2	0	0	0	0	2
Sofala	2	2	2	1	0	0	0	1
Manica	1	1	1	0	0	0	0	1
Inhambane	7	6	5	0	1	1	1	2
Total	22	14	11	1	1	2	1	6

**P. falciparum* infection with evidence of mixed clones with and without *pfhrp2/3* deletions

pfhrp2 as compared to calibrator wild type sample, and 2 a reduction in both *pfhrp2* and *pfhrp3*.

Discussion

This study provides an updated prevalence calculation of *pfhrp2/3* gene deletions in Mozambique, covering 6 provinces and all regions of the country, namely the south, centre and north. This survey found that *P. falcipurum* infections with *pfhrp2/3* gene deletions are rare in Mozambique with only 4 deletions detected in 2 provinces and an estimated prevalence of 0.16% [95% CI 0.15–2.57%]). Given the prevalence remains below the 5% threshold established by the WHO for RDT type replacement [26], results suggest HRP2-based RDTs are currently effective for *P. falciparum* diagnosis in Mozambique.

The low prevalence of *pfhrp2*/3 deletions is similar to results reported in neighbouring countries like Zambia (2009–2017, [32]), Eswatini, (2012–2014, [33]) and Tanzania (2021, [34]) which also suggest that HRP2-based RDTs remain effective tools for malaria diagnosis. In contrast, other African countries like Ethiopia [25] and Eritrea [23], have reported substantial increase in *pfhrp2/3* deletions in recent years, leading to RDT policy changes. In Mozambique, low rates of *pfhrp2/3* deletions had been reported in previous studies [20, 22], although those studies were limited in the number of areas covered and did not follow a standardized study design for the purpose of gene deletion surveillance. The present survey was based on WHO guidelines available on 2020 for both sample size and RDT selection. The PfLDH-based RDT confirmatory test selected (BIOCREDIT Malaria Ag Pf pLDH) has a confirmed acceptable diagnostic performance against both HRP2 expressing and HRP2 non-expressing infections at 200 parasites/µL (*pfhrp2/3* single or double deletions) based on the most recent WHO laboratory assessment [26].

As for the molecular characterization, a multiplex qPCR protocol already used in other *pfhrp2/3* deletion surveys was applied as an alternative to conventional PCR [30, 31]. This method is faster and more cost-effective, and includes a human gene target to inform success of the DNA purification process. Moreover, the method can provide a relative quantification of deleted *pfhrp2/3* parasites in mixed infections (deleted and non-deleted parasites in the same infection). Although these mixed

infections are not considered in the calculation of deletion prevalence according to current WHO guidelines, it provides additional information on the presence of mutated parasites in the population to inform future studies. Importantly, even if all samples with partial deletions and with non-valid *pfhrp2/3* qPCR results had been computed as deletions, prevalence of deletions would still be below < 1.1% in all provinces.

This study also showed that HRP2 + /PfLDH − RDT results discrepancies could occur frequently (7.2% of HRP2 positive test results, 188/2590). These discrepancies could be related to the lower sensitivity of tests based on the PfLDH antigen (46% of the discrepancies were confirmed positive by qPCR), or to false-positives due to detection of HRP2 antigens that are still in the bloodstream despite recent effective parasite clearance [35]. Considering that 54% (101/188) of aforementioned discrepancies tested negative for *P. falciparum* DNA, up to 3.9% (101/2590) of HRP2 routine RDT testing could be false-positive, with fever possibly attributable to non-malaria causes. As for the limitations, firstly, this study was conducted using convenience selection of health facilities and districts rather than a cluster randomized based approach, that would consider all health facilities in the province as eligible and potentially better capture the heterogeneity at the province level [26]. It is worth noting that the sample size targeted [370 samples per domain (province)] was amended by the WHO in 2023 with a recommendation to increase sample size from 370 samples to 600 [36]. Second, this study does not provide estimates of *pfhrp2/3* deletion prevalence among the overall circulating parasite population, as there is a high incidence of multi-clonal infections that can potentially contain both deleted and non-deleted parasites and still give a positive PfHRP2-RDT result. Third, protein expression or phenotypic assays that can confirm conclusively gene deletions as the cause of false-negative RDT results and exclude other factors, especially in the two infections where only *pfhrp3* was deleted, have not been conducted. Finally, this surveillance did not cover adults outpatient visits as it was focused on children—the population at highest malaria risk—to optimize resources.

Conclusions

Pfhrp2/3 gene deletions were detected in 2 out of 6 provinces surveyed in 2023 in Mozambique, with a prevalence of 0.27% (Nampula) and 0.59% (Inhambane), way below the 5% threshold recommended by the WHO to consider a change in RDT type [26]. PfHRP2-based diagnostics are still effective for *P. falciparum* diagnosis in Mozambique. These results were communicated to the National Malaria Control Programme and incorporated into the Integrated Malaria Information Storage System (SIIM). However, given the rapid emergence of *pfhrp2/3* deletions in some African countries and the growing concern, surveillance efforts have to be maintained to prevent the use of ineffective diagnostics.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-024-05230-4.

Additional file 1

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Author contributions
A.M., C.d.S., E.R.V., F.S and B.C: Designed the study protocol; C.d.S., P.d.C., N.N., A.T., H.M., A.H., G.M., S.E., N.C. and B.R.: Conducted the field activities (sample collection); C.d.S., D.T., S.B. and P.C.: Conducted laboratory activities; C.d.S., D.T., P.C., E.R.V., A.P., A.A.D. and A.M.: Analyzed the data; C.d.S., D.T., E.R.V. and A.M.: Wrote the first draft of the manuscript. All authors read, reviewed and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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