repiSalud
Repositorio Institucional de Salud

This is the peer reviewed version of the following article:

MALARIA DIAGNOSIS CHALLENGES AND PFHRP2 AND PFHRP3 GENE DELETIONS USING PREGNANT WOMEN AS SENTINEL POPULATION IN NANORO REGION, BURKINA FASO

Molina – de la Fuente, I., Tahita, M. C., Bérenger, K., Ta Tang, T. H., García, L., González, V., … Berzosa, P. (2024). Malaria diagnosis challenges and pfhrp2 and pfhrp3 gene deletions using pregnant women as sentinel population in Nanoro region, Burkina Faso. Pathogens and Global Health, 118(6), 481–491.

which has been published in final form at:
https://doi.org/10.1080/20477724.2024.2388489

Malaria diagnosis challenges and *pfhrp2* and *pfhrp3* gene deletions using pregnant women as sentinel population in Nanoro region, Burkina Faso

Authors:

Irene Molina – de la Fuente[1,2,3], Marc Christian Tahita[4,5], Kabore Bérenger[4,5], Thuy Huong Ta Tang[2,3], Luz García[2,3], Vicenta González[2,3], Agustín Benito[2,3], Judith M. Hübschen[6], Halidou Tinto[4,5], Pedro Berzosa[2,3]

[1]Department of Biomedicine and Biotechnology, School of Pharmacy, University of Alcalá, Alcalá de Henares, Madrid, Spain; [2]Malaria and Neglected Tropical Diseases Laboratory, National Centre of Tropical Medicine, Institute of Health Carlos III, Madrid, Spain; [3]CIBERINFEC - CIBER Infectious Diseases (ISCIII), Madrid, Spain; [4]Clinical Research Unit of Nanoro, Burkina Faso; [5]Institut de Recherche en Sciences de la Sante/Direction Régionale du Centre-Ouest, Burkina Faso; [6]Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg

Abstract:

Malaria in pregnancy causes adverse consequences and prompt and accurate diagnosis is essential for case management. In malaria endemic countries, diagnosis is mainly based on rapid diagnostic tests (RDT) and microscopy. However, increasing reports of false negatives caused by low parasitemia and *pfhrp2/3* deletions raise concerns about HRP2-based RDT usefulness. This study aimed to assess RDT and microscopy performance and to describe *pfhrp2/3* deletions in a cohort of 418 pregnant women in Burkina Faso. Malaria was diagnosed using RDT and microscopy and blood samples were collected during antenatal care visits. Diagnostic results were compared to PCR as gold standard. *Pfhrp2* and *pfhrp3* deletions were characterized for patients with confirmed *P. falciparum* infection. RDT had better sensitivity (76%) but lower specificity (83%) than microscopy (sensitivity = 57%; specificity = 98%). Low parasitemia (< 150 parasites/µL), especially in multigravidae, was the principal factor causing false negatives by both methods. Moreover, *pfhrp2* deletion frequency among overall false negatives by RDT was 2.6%. Higher frequency of deletions was found among all samples, independently of RDT result, for example around 2% of samples had double deletions meaning that the majority of deletions had no effect on RDT testing. Finally, a relationship was found between *pfhrp2* deletion and lower uterine height, marker of slow fetal growth, during the first trimester. Wider and National surveillance study of deletions is recommended among pregnant women and in Burkina Faso.

Keywords: Malaria, pregnancy, *pfhrp2*, malaria diagnosis

33 **Introduction**

34 Malaria remains a global health issue with 247 million cases resulting in 619,000 deaths in 2021,
35 95% of them in sub-Saharan Africa [1] . Pregnant women and children under five years old are
36 considered as groups at risk. In 2021, about 32% (13.3 million) of pregnant women in the World
37 Health Organization (WHO) African Region were exposed to malaria during pregnancy [1].
38 Malaria in pregnancy (MiP) can have adverse effects on both mothers and their offsprings,
39 including maternal anemia, low birthweight, premature birth and neonatal mortality [2]. Intra
40 uterine growth retardation (IUGR) is also a common consequence of malaria in pregnancy, due
41 to placental insufficiency to provide nutrients to the fetus, and one of its clinical signs is lower
42 uterine height, routinely measure in antenatal care revisions [3]. These adverse effects of
43 malaria infections tend to be more severe in primigravidae, women experiencing their first
44 pregnancy, compared to multigravidae due to lack of specific pregnancy-acquired immunity [4].
45 Pregnant women develop a specific immune response to VAR2CSA, a parasite antigen produced
46 during placental sequestration of parasites [5]. In fact, in areas with stable transmission
47 throughout the year, infected pregnant women are often asymptomatic due to this partial
48 immunity [6]. However, the risk for severe anemia, impaired fetal growth or stillbirth are
49 maintained as infected red blood cells can adhere to placental microvasculature reducing the
50 foeto-maternal blood exchange [7].

51 For the control of MiP, the WHO recommends prompt diagnosis followed by adequate case
52 management, including effective treatment. Moreover, pregnant women are encouraged to
53 follow preventive measures, including the use of long-lasting insecticide-treated nets (LLINs) and
54 intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP) [8].

55 Burkina Faso, a malaria endemic country, accounted for 3.3% of global malaria cases and 3.4%
56 of malaria deaths in 2021 [1]. Malaria is still the main cause of consultations, hospitalizations
57 and deaths in the country [9], and MiP remains high throughout the year, stressing that
58 malaria control is a priority in the country [10].

59 Prompt and accurate diagnosis is the cornerstone of malaria case management. Challenges of
60 diagnosing malaria during pregnancy are related to specific acquired immunity, placental
61 parasite sequestration and the low predictive value of the clinical signs and symptoms
62 suggestive of malaria, especially in endemic areas, because of they are highly unspecific and
63 could have different causes [11,12]. Consequently, all malaria cases should be confirmed either
64 by microscopy or rapid diagnostic tests (RDT), before any treatment [8]. Light microscopy is
65 currently the gold standard for point – of – care diagnosis in endemic areas, but its performance

66 relies on different factors such as skills of microscopists and quality of reagents and equipment
67 [13]. Therefore, good-quality microscopy is particularly hard to maintain in remote areas. In
68 contrast, RDT play an increasing role as point-of-care diagnostic tool, being the principal
69 technique used in malaria screening in Africa [14]. Malaria RDTs present different advantages:
70 they are easy to perform and provide rapid results without the need of electricity, expensive
71 equipment or extensive training. RDTs have, therefore, great potential for rapid and accurate
72 malaria diagnosis in most remote malaria-endemic areas. These tests are based on lateral flow
73 immunochromatography detecting parasite proteins. Histidine-rich protein II (pfHRP2), coded
74 by *pfhrp2* gene, is the predominant specific *Plasmodium falciparum* protein used in pf-RDTs [15].
75 These RDTs could cross-react with the homologous protein pfHRP3, coded by *pfhrp3* gene, this
76 is more common at higher parasite densities. So, if only one of these genes is deleted the RDT
77 could still be positive due to the detection of the other, especially in infections with high
78 parasitemia.

79 Despite the advantages of pfHRP2-based RDT, their usefulness could be threatened due to the
80 appearance of false negatives leading to a lack or delay in treatment. The impact of false
81 negatives is both clinical, as the patient will not receive treatment on time, and collective, as
82 transmission continues. Causes for false negative results include poor storage conditions,
83 interpreting errors, low parasite density, and deletions in the *pfhrp2* and *pfhrp3* genes [16].

84 *Pfhrp2* and *pfhrp3* gene deletions are being reported worldwide [15]. Parasites with these
85 deletions cannot express HRP2/3 proteins. Biological function of these proteins is not yet clear,
86 nor the fitness cost of their deletions with contrary results being reported [17,18].

87 To timely assess the threat of false negative results obtained with HRP2-based RDTs, the WHO
88 is recommending continuous monitoring of these deletions in endemic areas [19]. However,
89 despite the increasing reports of HRP2 deletions in African countries, there is not any proper
90 study, following WHO recommendations, in Burkina Faso. In fact, there was only one recent
91 report of *pfhrp2* and *pfhrp3* deletions in Burkina Faso in asymptomatic children from the
92 Southwest of the country [20], whereas WHO recommends to include general symptomatic
93 population.

94 Taken together, the current diagnostic issues, especially the occurrence of false negatives by
95 RDT, make it essential to assess the challenges for the diagnosis of malaria in pregnancy.

96 Therefore, this study aimed to assess the performance of microscopy and malaria RDTs among
97 pregnant women in Nanoro district (Burkina Faso) and to describe the frequency, distribution,
98 and possible effects of *pfhrp2* and *pfhrp3* deletions on malaria diagnosis in pregnant women.

99 **Methods**

100 **Study area**

101 The study was carried out at the Clinical Research Unit of Nanoro (CRUN), located in the health
102 district of Nanoro, Boulkiemdé province, a rural area placed in the central-western region of
103 Burkina Faso, at 85 kilometers from the capital city, Ouagadougou. Population of Nanoro is
104 growing rapidly, with a high birth rate of 5.56%, a big proportion of young people and literacy
105 rate of around 23% [21,22].

106 Burkina Faso has three climate areas, where Sahel-Sudanian zone, including Boulkiemdé
107 province, has been identified as a region with high risk of seasonal malaria, overlapping with the
108 rainy season (July – October) [23]. Malaria transmission is high in Southwest Burkina Faso, with
109 an entomological inoculation rate of 0.375 infected bites per human per night in 2017 [24].

110 **Sample collection**

111 The present study is an ancillary study of a project (PaMaViT) assessing the characteristics of
112 pregnant women attending antenatal care for the first time. Briefly, all pregnant women
113 attending antenatal care were asked to participate. After obtaining written informed consent, a
114 structured questionnaire was used to collect socio-demographic information, clinical and
115 obstetrical data. Malaria was diagnosed using RDT and microscopy. Additionally, dried blood
116 spots (DBS) were collected for later molecular diagnosis confirmation and genotyping using
117 Whatman 903™ paper grade 3 (GE Healthcare Bio-Sciences Corp.). A total of 418 samples were
118 collected at four different peripheral health centers of the health district of Nanoro (Nanoro =
119 96; Soaw = 84; Kindi = 148 and Pella = 90) from December 2020 to March 2021 (Figure 1). During
120 these months, samples were collected from all the patients that meet inclusion criteria and
121 agree to participate, and differences in sample size among locations respond to different
122 affluence in each health center.

123

124 **Malaria Diagnosis:** for prompt case management, all pregnant women were tested using an
125 HRP2-based malaria RDT (CareStart™ Malaria, Access Bio; product code: RMOM-02571), while
126 microscopy slides were transferred to the central laboratory of the CRUN for reading. All malaria
127 slides were read and quantified according to the local laboratory procedures by two expert
128 microscopists and a third reader was called in case of discrepancies.

129

130 **Molecular study**

131 **1) Malaria diagnosis confirmation**

132 DNA was extracted from the DBS samples using the saponin-chelex method described in Ta-Tang
133 et al., 2022 with the difference of using just one 5 mm diameter-filter paper disc [25]. All samples
134 were analyzed using the nested multiplex PCR for the confirmation and differentiation of
135 *Plasmodium* species [26,27]. The primers used were detailed in supplementary information
136 (Table S1).
137
138 **2) *Pfhrp2* and *pfhrp3* deletion detection:**
139 Confirmed *P. falciparum* positive samples were then analyzed for *pfhrp2* and *pfhrp3*.
140 Amplification of exon 1-2 of both genes and exon 2 of *pfhrp3* was performed using a semi-nested
141 PCR and a conventional PCR for exon 2 of *pfhrp2* [28–30]. PCR amplicons were separated and
142 visualized on a 2% agarose gel stained with Pronasafe (Condalab, Spain). In the absence of
143 amplification for exon 2, results were recorded as potential deletions and the PCR was repeated
144 twice under the same conditions. If amplification never occurred, the sample was quantified by
145 qPCR [26] to ensure enough quality in the sample. If the qPCR was positive, the result was
146 considered as a deletion event. The 3D7 clone of *P. falciparum* was used as a positive control, as
147 it has intact *pfhrp2* and *pfhrp3* genes; clone Dd2 as a negative control for the *pfhrp2* gene
148 amplification since it does not have this gene; and clone HB3 was used as a negative control for
149 the *pfhrp3* gene amplification.
150 Moreover, other two single-copy genes (*pfdhfr* and *pfdhps*) were amplified in all samples to
151 ensure enough parasitemia.
152
153 **Data analysis and definitions**
154 All data were managed using Excel before exporting to R software v4.0.0 for statistical analysis.
155 The specificity, sensitivity, and positive and negative predictive values of microscopy and RDT
156 were calculated considering PCR as gold standard and considering a malaria prevalence
157 determined from the samples used in this study. Cohen's Kappa index was also calculated to
158 assess the agreement between each of these tests and PCR diagnosis. Deletion frequency was
159 calculated by dividing confirmed deletions by all PCR- confirmed *P. falciparum* positive samples
160 included for analysis. Participants were categorized as primigravidae for their first pregnancy;
161 and multigravidae if they had more than one pregnancy.
162 Three–way ANOVA test was used to study the association between uterine height and *pfhrp2*
163 deletion using categorized data according to trimester of pregnancy and gestity, referring to
164 number of pregnancies.

Chi-square test was applied to assess the association between gestity and malaria infection or *pfhrp2* gene deletion. All analyses used a 95% confidence level, calculated using exact method for prevalences, and a p-value < 0.05 for statistical significance.

Results

Malaria prevalence

According to the molecular species identification, two plasmodium species were identified among the 418 samples with 199 *P. falciparum*, 2 *P. vivax and* one mixed infection (*P. vivax* + *P. falciparum*). 215 samples were negative for *Plasmodium* and one sample were excluded due to poor conservation.

The prevalence of malaria infection using PCR as reference method was 48.4% (43.6 – 53.4) and *P. falciparum* malaria infection was 48.0% (43.1 – 52.9). *P. falciparum* infection prevalence varied according to gestational age and gestity.

P. falciparum malaria prevalence among primigravidae was higher (58.7%) compared to multigravidae (34.6%) (p < 0.001) (Supplementary material: Table S2). Also, there were more *P. falciparum* infections in the second and third trimesters (around 50%) compared to the first trimester (34.67%) (p < 0.005) (Figure 2) (Table S2).

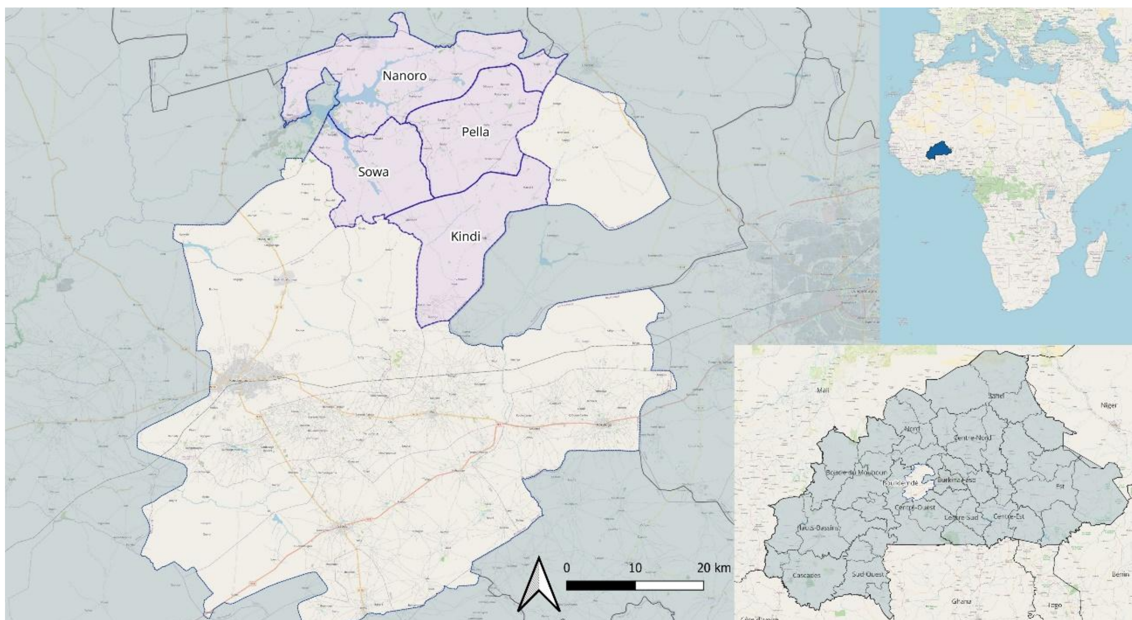
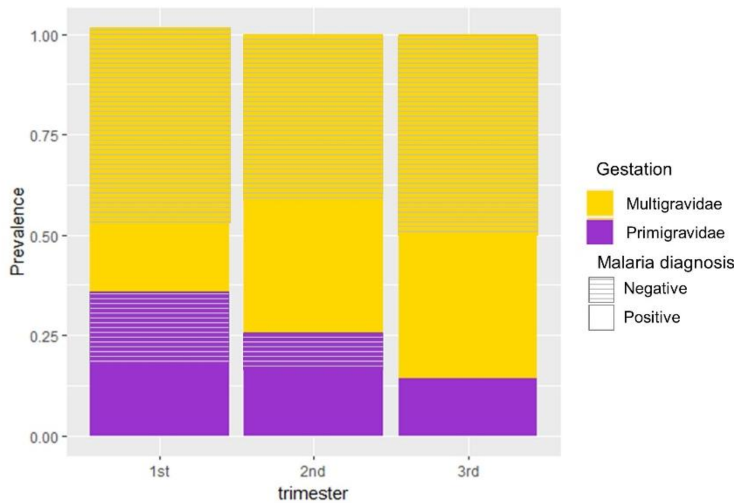


Figure 1. Map of study areas. The four areas colored in purple were those included in the study.

188



189

190 Figure 2. Prevalence of malaria infection according to the trimester of pregnancy and number of
191 pregnancies. Y axis represents malaria prevalence and X axis the pregnancy trimester. The
192 different colors represent the contribution of each gestation group and strip pattern the malaria
193 diagnosis.

194

195 **Performance of microscopy and malaria RDTs using PCR as gold standard**

196 Assessing the performance of the diagnostic methods, we observed a higher specificity of 98.1%
197 (95.8 – 99.4) and positive predictive value (96.5) for microscopy, but a higher sensitivity of 76.0%
198 (70.5 – 80.9) for RDT (Table 1). The agreement between microscopy and RDT using PCR as a gold
199 standard was moderate with a Cohen's Kappa coefficient of 0.55 and 0.59, respectively.

200

201 **Table 1**. Sensitivity, specificity and positive/ negative predictive values for microscopy and RDT
202 using PCR as gold standard.

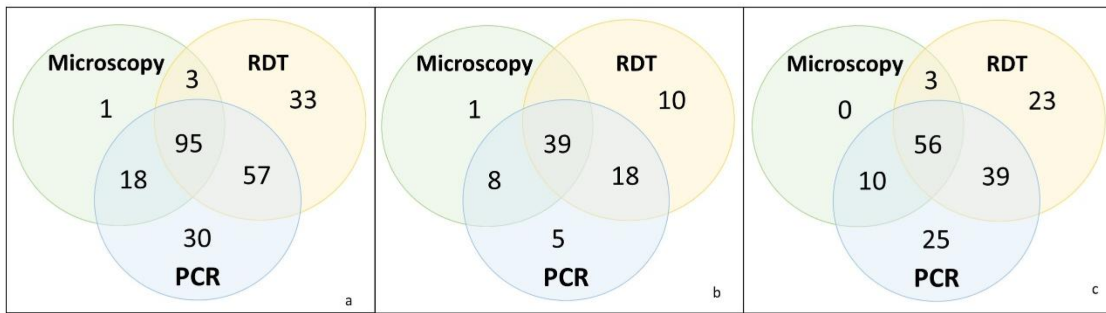
	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Microscopy	56.5 (50.4 – 62.4)	98.1 (95.8 – 99.4)	96.5 (92.4 – 98.4)	71.1 (68.3 – 73.8)
RDT	76.0 (70.5 – 80.9)	83.3 (78.5 – 87.3)	80.6 (76.2 – 84.3)	79.1 (75.4 – 82.4)

203 PPV= positive predictive values; NPV= negative predictive values.

204

205 Slightly better agreement of results with PCR was observed for primigravidae in both diagnostic
206 tests (Figure 3). The better agreement of positive results with RDTs, could be related to the
207 observed higher parasite densities in primigravidae than in multigravidae (Figure 4).

208

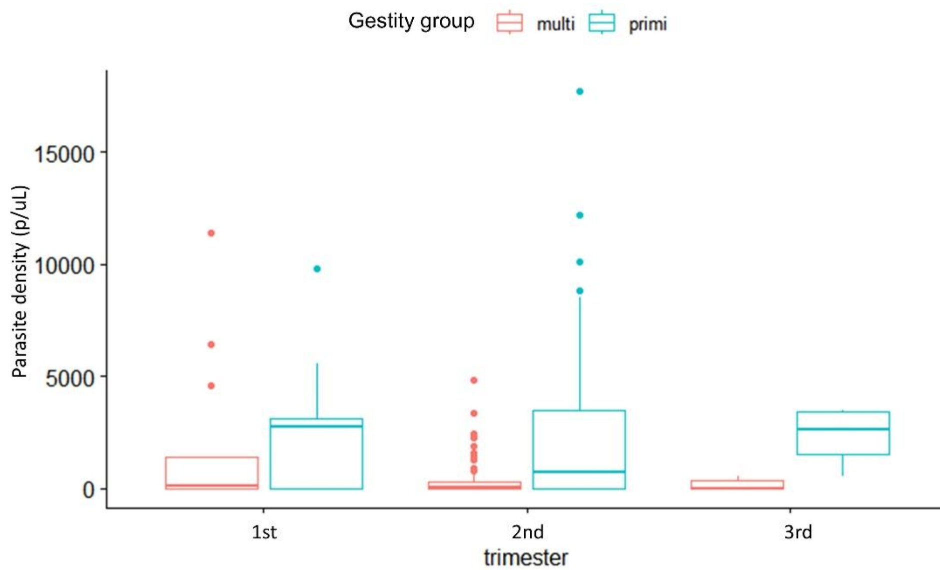


209

210 Figure 3. Venn diagrams of the distribution of positive *P. falciparum* results according to the
211 diagnostic test used for all samples (a), among primigravidae (b) and among multigravidae (c).

212

213



214

215

216 Figure 4. Parasite density by microscopy according to the pregnancy trimester and gestity (multi:
217 multigravidae, and primi: primigravidae).

218 When considering only discordant microscopy - RDT results, 19 samples were negative by RDT
219 but positive by microscopy, among them 18 were confirmed as *P. falciparum* by PCR. The
220 prevalence of suspected false - negative HRP2 RDT (n = 19) among the confirmed positives by
221 microscopy (N = 117) was 16.24% (10.07 – 24.19) (19/117).

222

223 **Deletions in *pfhrp2* and *pfhrp3* genes**

224 The deletion frequency of *pfhrp2* genes in all positive *P. falciparum* samples (200) was 12.50%
225 (8.26 - 17.90) (25/200), independently of the previous RDT result. However, the majority of
226 samples with deletion in *pfhrp2* had low parasitaemia, only three were above 150 parasites/uL,
227 which is considered below the limit of detection for RDTs [31], and 15 were under 150
228 parasites/uL. Single-*pfhrp2* deletions were the most common deletions in all villages (Table 2).
229 There were 15 samples with deletion in *pfhrp3* and 4 samples with *pfhrp2* & *pfhrp3* double
230 deletion (Table 2).

231 To study the possible impact of deletions in RDT as point of care diagnosis, the frequency of
232 deletion was calculated among confirmed *P. falciparum* infections with discordant results,
233 positives by microscopy but negatives by HRP2-RDT (n = 114). The frequency of false negatives
234 by HRP2-RDT with deletion in *pfhrp2* was 2.63% (0.55 – 7.50) (3/114) and with *pfhrp3* deleted
235 was 2.63% (0.55 – 7.50) (3/114).There was one HRP2-RDT false negative sample with double
236 deletion (0.88%; 0.02 – 4.8) (Figure 5).

237 All three samples with deletions in *pfhrp2* and false negatives by RDT (2 from Nanoro and 1 from
238 Soaw) had a parasite density under 150 parasites /uL concordant with the limit of detection for
239 the majority of RDTs. The two samples with single deletions in *pfhrp3* and negative RDT result
240 were from Nanoro and from Pella, and they had parasite densities under 100 parasites /uL (66,
241 39 & 10 parasites/uL).

242 Combining the results from the suspected RDT false negatives (n = 18) and confirmed deletions
243 (n= 3), the positive predictive value of false-negative HRP2-RDT results for *pfhrp2/3* gene
244 deletions in Burkina Faso was 16.67% (3.58 – 41.42).
245 Comparing deletions associated with false negatives in RDT with samples with deletions but RDT
246 positive result, only 11% of samples with *pfhrp2* deletions had a false negative RDT (Figure 5).
247 Similar geographical distribution of deletions among both RDT positives and RDT false negatives
248 has been observed; for example, Nanoro was the health facility with higher frequency of *pfhrp2*
249 deletions independently of RDT result.

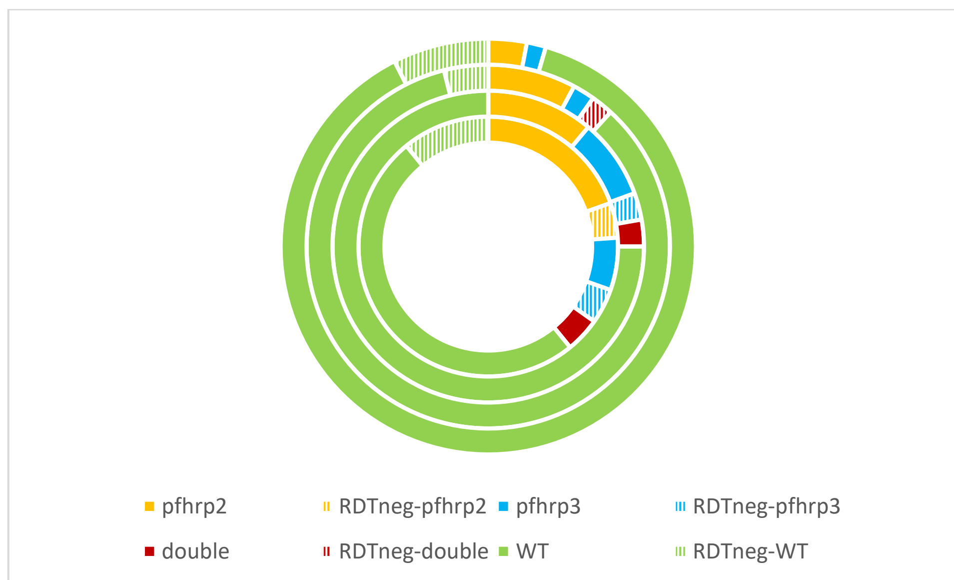
250 **Table 2**. Combination of *pfhpr2* and *pfhrp3* deletions by location.

Location	Single - *Pfhrp2* deletion	Single - *Pfhrp3* deletion	Double deletion	Wild – haplotype (no deletions)

	N	F (95% CI)	N	F (95% CI)	N	F (95% CI)	N	F (95% CI)
Total	21	10.5 (6.6 – 15.6)	11	5.5 (2.9 – 9.6)	4	2 (0.6 – 5.0)	164	82.0 (75.9 – 87.1)
Kindi	2	3.0 (0.4 – 10.4)	1	1.5 (0.0 – 8.0)	0	0.0	64	95.5 (87.5 – 99.1)
Nanoro	11	30.6 (16.4 – 48.1)	5	13.9 (4.7 – 29.5)	2	5.6 (0.7 – 18.7)	28	77.8 (60.9 – 89.9)
Pella	4	11.1 (3.1 – 26.1)	4	11.1 (3.1 – 26.1)	1	2.8 (0.1 – 14.5)	27	75 (57.8 – 87.9)
Soaw	4	7.8 (2.2 – 18.9)	1	2.0 (0.1 – 10.5)	1	2.0 (0.1 – 10.5)	45	88.2 (76.1 – 95.6)

251 F = frequency

252



253

254 Figure 5. Frequency of deletions including discordant results by region (Order from out to inside:
255 Kindi, Soaw, Pella and Nanoro). Frequency of samples with single deletions in pfhrp2 (orange)
256 or pfhrp3 (blue), double deletions (dark red) and no deletions (green) differing between RDT
257 positive (solid filling) and RDT negative samples (stripped filling) are shown.

258

259 **Association between *pfhrp2* deletions, pregnancy characteristics and malaria symptoms**

260 Lower uterine height was observed in pregnant women infected with *pfhrp2* deleted - *P.*
261 *falciparum* in the first trimester, considering both all women (Figure 6a) and after adjusted for
262 primigravidae and multigravidae (Figure 6b). But there were no differences in uterine height
263 between malaria infected women with *pfhrp2* present and non-infected women (Figure 6).
264 However, because of the low sample size the association between lower uterine height and
265 *pfhrp2* deletion could not be demonstrated (Supplementary material Table S3).

Overall, multigravidae women showed higher frequency of *pfhrp2* deletions (16.15%, 10.28 – 23.63) than primigravidae women (5.71%, 1.58 – 13.99) (Table 3). However, this difference is only statistically significant in the second trimester of pregnancy. Five women presented fever and none of them had *pfhrp2* and *pfhrp3* deletions.

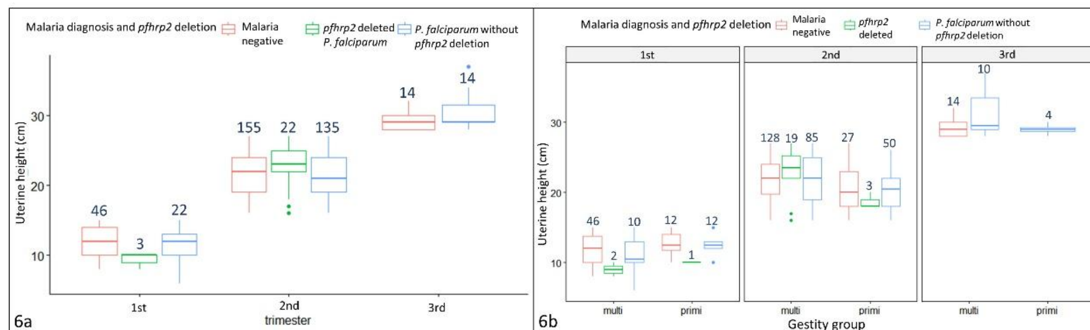


Figure 6. Relation between uterine height and *pfhrp2* deletions by trimester of pregnancy (a) and by number of pregnancies and trimester of pregnancy (b). Multi: multigravidae; Primi: primigravidae. Numbers over each box represent sample size in that group.

Table 3. Frequency of deletions according to trimester of pregnancy and number of pregnancies

	Total	N. Malaria	*pfhrp2* - deletions		*pfhrp3* - deletions	
			N	Frequency (%)	N	Frequency (%)
Trimester of pregnancy						
1st trimester	75	26	3	11.54	1	3.85
2nd trimester	312	132	22	16.67	14	10.61
3rd trimester	28	12	0		0	
Number of pregnancies						
Primigravidae	109	64	4	6.25	6	9,38
Multigravidae	306	106	21	19.81	9	8,49

Discussion

Performance of malaria diagnostic tools, microscopy and RDTs, among pregnant women in endemic settings was evaluated using PCR as gold standard. RDT and microscopy showed moderate agreement when using PCR as gold standard for pregnant women. Microscopy had higher specificity whereas RDT had higher sensitivity. Furthermore, *pfhrp2* and *pfhrp3* deletions were described in pregnant women in Burkina Faso for the first time.

Prompt and accurate malaria diagnosis in pregnant women is an essential step to avoid adverse events for mother and fetus [32]. Following "test – and – treat" strategies, all malaria cases should be confirmed by RDT or microscopy before any treatment. While this strategy prevents the overuse of Artemisinin based Combination Therapy (ACTs) and thus the spread of resistance, false negative test results will lead to a lack or delay of treatment. So high accuracy of diagnosis is the first step for malaria case management. Moreover, in the context of this study, positive malaria diagnosis will determine if the pregnant woman need ACT to threat the malaria infection, but if there was a false negative the women will receive SP instead of malaria treatment. If the infection is not timely treated, this could have fatal consequences not only for her and her pregnancy but will also likely facilitate the spread of SP resistances.

RDT showed better sensitivity than microscopy for pregnant women, that agrees with previous reports in asymptomatic pregnant women [11]. The low sensitivity of microscopy could be related to the high expertise requirements to ensure microscopic diagnosis, especially for infections with low parasitemia [33,34]. So, although *pfhrp2* deletions are detected in Nanoro, RDT will still detect more true malaria cases than microscopy. RDT specificity could decrease because false positives are usually consequence of slow PfHRP2 clearance [35]. Taking into account the easier management of RDT compared with microscopy and their high agreement, RDT could be a great point-of-care diagnosis for pregnant women in Nanoro. Interestingly, the accuracy of microscopy for asymptomatic pregnant women in Nanoro was maintained but accuracy of HRP2-RDT have decreased since the last report in 2011 (sensitivity = 97%; specificity = 65.7%) although principal malaria transmission characteristics were also maintained [36].

Malaria RDT results can be influenced by several factors, including the persistence of the HRP2 antigens, which could cause false positives, or other factors such as low parasitemia and *pfhrp2/3* deletions that could cause false negatives. It would be highly interesting to understand why there were parasites with double deletions and positives by RDT, one possible explanation is the persistence of these proteins from past infections [37].

This study reported that the majority of false negatives by RDT had parasitemia under 150 parasites/uL, the limit of detection of conventional RDT [38]. So, this low parasitemia may be

313 the principal cause of false negatives by RDT, especially in the cases of *pfhrp2* intact
314 independently of *pfhrp3* status. Low parasitemia is specially relevant for malaria diagnosis
315 during pregnancy, as specific pregnancy-acquired immunity usually leads to a lower parasite
316 density, especially in high – to moderate burden settings [39]. Multigravidae usually show a
317 higher level of acquired immunity than primigravidae, who usually have higher parasite
318 densities, which explains the better accuracy in diagnosis for primigravidae. However, apart
319 from acquired immunity there were other non-immune factors that influence parasite densities
320 and, consequently, the diagnosis but they are not assessed in this study [7].

321 Different efforts are being implemented to improve the sensitivity of current RDT, such as the
322 development of new ultrasensitive RDT. In fact, in Nanoro region it has been planned to move
323 from conventional RDT to ultrasensitive RDT [40]. However, these new ultrasensitive RDTs based
324 on pfHRP2 and pfHRP3 are also threatened by these gene deletions. This should be considered
325 in the design of public health strategies.

326 Our study reports the presence of *pfhrp2* and *pfhrp3* deletions for the first time in pregnant
327 women in Burkina Faso. It is worth to study further if pregnant women could be used as a
328 representative group due to their better accessibility [7]. However, there was only one previous
329 report on *pfhrp2* and *pfhrp3* deletions in Burkina Faso (4.4%). But it was carried out in another
330 area and in asymptomatic children, therefore deletions frequency cannot be compared [20].

331 Detected frequency of *pfhrp2* deletions was higher than previous reports in other West African
332 countries, such as Gabon and Nigeria, where reported frequency was under 5% [41]. However,
333 the frequency of deletions among false negative RDT was lower (2.6%) than reports in other
334 West African countries, such as Mali [42] and Ghana [43], where deletions frequency causing
335 false negative results was over 40%. Those differences could be due to different epidemiological
336 setting including use of HRP2 – RDT, or other factors such as malaria transmission prevalence.
337 Thus, high multiplicity of infection (MOI), common in high malaria transmission areas as Burkina
338 Faso, has been related to lower detection likelihood [44,45]. Hence, multiplicity of infection
339 could be influencing the results as, according to previous studies, pregnancy is related with
340 higher MOI, then we may expect more frequency of deletions in general population [46,47]. It
341 is important to highlight that frequency of *pfhrp2* deletions among discordant results between
342 RDT and microscopy was low but continuous monitoring of *pfhrp2* and *pfhrp3* deletions in large-
343 scale national surveillance studies following WHO guidelines is highly recommended [19].

344 In this study, *pfhrp2* deletions were more common than *pfhrp3* deletions, and only 4 samples
345 had both deletions. This could explain the reported high frequency of RDT positives with *pfhrp2*

346 single deletions, because *pfhrp3* could be cross-reacting with HRP2 – RDT and compensating the
347 lack of *pfhrp2* [48]. As parasitemia level could be influencing this cross-reaction, it is important
348 to highlight that the majority of these samples, with positive RDT and *pfhrp3* intact but *pfhrp2*
349 deleted, had parasitemia over 200 parasites/uL.

350 Finally, to our knowledge this is the first study of *pfhrp2* deletions in infected pregnant women.
351 Although PfHRP2 function is still unknown, it has been described as potential mediator in
352 placental sequestration as it is expressed in *P. falciparum*-infected erythrocytes [49]. More
353 severe outcomes are expected if more parasites are sequestered, so lack of PfHRP2 could be
354 expected with less parasite sequestration [49]. Contrastingly, our study found relation between
355 low uterine height during the first trimester of pregnancy and *pfhrp2* deletions. This could be
356 explained by the absence of PfHRP2, as other surface proteins, difficulties host immune
357 response, increasing sequestration. Also, this hypothesis of less detectable parasites is
358 apparently independent of acquired immunity, as the association was maintained for
359 primigravidae and multigravidae [50]. Moreover, the effect is only observed in the first trimester
360 of pregnancy, where patients' characteristics may have more impact on uterine height, but
361 infection could also influence fetus development. However, due to low number of samples and
362 lack of follow up to associate it with low birth weight, it is unclear if the association is reliable or
363 a consequence of other variables. To date, biological function of HRP2 is still unclear, so more
364 research is needed to clarify any additional clinical implication of this deletion for pregnant
365 women.

366 This study presents several limitations, first of all the results cannot be extrapolated nationwide
367 as it represents only a region in a specific province. Also, the study only includes pregnant
368 women and, although it has been demonstrated that pregnant women can be used as a
369 representative subpopulation [7], studies in the general population are recommended to
370 confirm the findings.

371 **Conclusion**

372 Low parasitemia is the main challenge for diagnostic accuracy by RDT and microscopy. RDT had
373 higher sensitivity and microscopy higher specificity, being the combination of both the best
374 option for ensuring diagnosis accuracy. Presence of *pfhrp2* and *pfhrp3* deletions has been
375 confirmed in pregnant women in Burkina Faso, but their impact in RDT results is still limited.
376 HRP2-based RDT are still effective in diagnosing malaria infection in Nanoro region, Burkina
377 Faso. *Pfhrp2* deletions could have some clinical impact on intrauterine growth. *Pfhrp2* and

378 *pfhrp3* deletions monitoring studies are recommended in order to timely assess the threats for
379 efficacy of HRP2 - RDT.

380 **Disclosure statement**

381 The authors report that there are no competing interests to declare.

382 **Ethics approval**

383 This study was approved by the Review Board of the Institut de Recherche en Science de la
384 Santé/ Direction Régionale de l'Ouest (IRSS-DRO), Burkina Faso (N/REF. A15-2020 CEIRES).

385 **Funding**

386 This work was supported by the National Centre of Tropical Medicine (Carlos III Institute of
387 Health – Spain) under Grant TRPY447/21; Foundation Mérieux through the small grant Program
388 and Luxembourg Ministry of Foreign and European Affairs ("Microbiology for Development"
389 grant). IMF received a research fellowship (FPU-UAH2019) from the University of Alcalá (Spain)
390 that enabled her to develop this study.

391 **Acknowledgment**

392 We would like to extend our sincere gratitude to all women who participated in this study.
393 Sincere thanks to the entire medical, laboratory and administrative staffs at Nanoro, Soaw, Pella
394 and Kindi.

395

396

397 **References**

398 1. World malaria report 2022 [Internet]. [cited 2023 Jul 10]. Available from:
399 https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022

400 2. Bakken L, Iversen PO. The impact of malaria during pregnancy on low birth weight in East-
401 Africa: a topical review. Malaria Journal. 2021;20:348.

402 3. Davison BB, Cogswell FB, Baskin GB, Falkenstein KP, Henson EW, Krogstad DJ. Placental
403 changes associated with fetal outcome in the Plasmodium coatneyi/rhesus monkey model of
404 malaria in pregnancy. Am J Trop Med Hyg. 2000;63:158–73.

405 4. Ampomah P, Stevenson L, Ofori MF, Barfod L, Hviid L. Kinetics of B cell responses to
406 Plasmodium falciparum erythrocyte membrane protein 1 in Ghanaian women naturally
407 exposed to malaria parasites. J Immunol. 2014;192:5236–44.

408 5. Tomlinson A, Semblat J-P, Gamain B, Chêne A. VAR2CSA-Mediated Host Defense Evasion of
409 Plasmodium falciparum Infected Erythrocytes in Placental Malaria. Front Immunol.
410 2020;11:624126.

411 6. Aitken EH, Damelang T, Ortega-Pajares A, Alemu A, Hasang W, Dini S, et al. Developing a
412 multivariate prediction model of antibody features associated with protection of malaria-
413 infected pregnant women from placental malaria. Elife. 2021;10:e65776.

414 7. Mayor A, Menéndez C, Walker PGT. Targeting Pregnant Women for Malaria Surveillance.
415 Trends in Parasitology. 2019;35:677–86.

416 8. WHO Guidelines for malaria [Internet]. [cited 2023 Dec 18]. Available from:
417 https://www.who.int/publications/i/item/guidelines-for-malaria

418 9. INSD. Annuaire Statistique National 2019 [Internet]. 2019. Available from:
419 https://www.insd.bf/sites/default/files/2021-12/Annuaire_Statistique_National_2019.pdf

420 10. Rouamba T, Samadoulougou S, Ouédraogo M, Hien H, Tinto H, Kirakoya-Samadoulougou F.
421 Asymptomatic malaria and anaemia among pregnant women during high and low malaria
422 transmission seasons in Burkina Faso: household-based cross-sectional surveys in Burkina
423 Faso, 2013 and 2017. Malaria Journal. 2021;20:211.

424 11. Kattenberg JH, Ochodo EA, Boer KR, Schallig HD, Mens PF, Leeflang MM. Systematic review
425 and meta-analysis: rapid diagnostic tests versus placental histology, microscopy and PCR for
426 malaria in pregnant women. Malaria Journal. 2011;10:321.

427 12. Tahita MC, Tinto H, Menten J, Ouedraogo J-B, Guiguemde RT, van Geertruyden JP, et al.
428 Clinical signs and symptoms cannot reliably predict Plasmodium falciparum malaria infection in
429 pregnant women living in an area of high seasonal transmission. Malar J. 2013;12:464.

430 13. Afriyie SO, Addison TK, Gebre Y, Mutala A-H, Antwi KB, Abbas DA, et al. Accuracy of
431 diagnosis among clinical malaria patients: comparing microscopy, RDT and a highly sensitive
432 quantitative PCR looking at the implications for submicroscopic infections. Malaria Journal
433 [Internet]. 2023 [cited 2023 Dec 18];22. Available from:
434 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9985253/

435 14. Maketa Tevuzula V, Kabalu J, Kabena M, Luzolo F, Muhindo-Mavoko M-M, Schallig HDFH,
436 et al. Comparison of intermittent screening (using ultra-sensitive malaria rapid diagnostic test)
437 and treatment (using a newly registered antimalarial pyronaridine-artesunate-PYRAMAX®) to
438 standard intermittent preventive treatment with sulfadoxine-pyrimethamine for the
439 prevention of malaria in pregnant women living in endemic areas: ULTRAPYRAPREG. Trials
440 [Internet]. 2022 [cited 2023 Dec 18];23. Available from:
441 https://pubmed.ncbi.nlm.nih.gov/36443882/

442 15. Molina-de la Fuente I, Pastor A, Herrador Z, Benito A, Berzosa P. Impact of Plasmodium
443 falciparum pfhrp2 and pfhrp3 gene deletions on malaria control worldwide: a systematic
444 review and meta-analysis. Malar J. 2021;20:276.

445 16. Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, et al. Plasmodium
446 falciparum parasites lacking histidine-rich protein 2 and 3: a review and recommendations for
447 accurate reporting. Malar J. 2014;13:283.

448 17. Nair S, Li X, Nkhoma SC, Anderson T. Fitness Costs of pfhrp2 and pfhrp3 Deletions
449 Underlying Diagnostic Evasion in Malaria Parasites. J Infect Dis. 2022;226:1637–45.

450 18. Sepúlveda N, Phelan J, Diez-Benavente E, Campino S, Clark TG, Hopkins H, et al. Global
451 analysis of Plasmodium falciparum histidine-rich protein-2 (pfhrp2) and pfhrp3 gene deletions
452 using whole-genome sequencing data and meta-analysis. Infect Genet Evol. 2018;62:211–9.

453 19. Organization WH. Master protocol for surveillance of pfhrp2/3 deletions and biobanking to
454 support future research [Internet]. World Health Organization; 2020 [cited 2023 Dec 28].
455 Available from: https://iris.who.int/handle/10665/331197

456 20. Tarama CW, Soré H, Siribié M, Débé S, Kinda R, Nonkani WG, et al. Assessing the histidine-
457 rich protein 2/3 gene deletion in Plasmodium falciparum isolates from Burkina Faso. Malaria
458 Journal. 2023;22:363.

459 21. Derra K, Rouamba E, Kazienga A, Ouedraogo S, Tahita MC, Sorgho H, et al. Profile: Nanoro
460 Health and Demographic Surveillance System. Int J Epidemiol. 2012;41:1293–301.

461 22. Noori N, Derra K, Valea I, Oron AP, Welgo A, Rouamba T, et al. Patterns of child mortality in
462 rural area of Burkina Faso: evidence from the Nanoro health and demographic surveillance
463 system (HDSS). BMC Public Health. 2021;21:1425.

464 23. Sangaré I, Ouattara CA, Soma DD, Soma D, Assogba BS, Namountougou M, et al. Spatial-
465 temporal pattern of malaria in Burkina Faso from 2013 to 2020. Parasite Epidemiol Control.
466 2022;18:e00261.

467 24. Soma DD, Zogo BM, Somé A, Tchiekoi BN, Hien DF de S, Pooda HS, et al. Anopheles
468 bionomics, insecticide resistance and malaria transmission in southwest Burkina Faso: A pre-
469 intervention study. PLoS One. 2020;15:e0236920.

470 25. Ta-Tang T-H, Berzosa P, Rubio JM, Romay-Barja M, Ncogo P, Agudo D, et al. Evaluation of
471 LAMP for the diagnosis of *Loa loa* infection in dried blood spots compared to PCR-based assays
472 and microscopy. Mem Inst Oswaldo Cruz. 2022;116:e210210.

473 26. Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired
474 human infection with Plasmodium cynomolgi. Malar J. 2014;13:68.

475 27. Rubio JM, Post RJ, van Leeuwen WMD, Henry MC, Lindergard G, Hommel M. Alternative
476 polymerase chain reaction method to identify Plasmodium species in human blood samples:
477 the semi-nested multiplex malaria PCR (SnM-PCR). Trans R Soc Trop Med Hyg. 2002;96 Suppl
478 1:S199-204.

479 28. Molina - de la Fuente I, Yimar M, García L, González V, Amor A, Anegagrie M, et al. Deletion
480 patterns, genetic variability and protein structure of pfhrp2 and pfhrp3: implications for
481 malaria rapid diagnostic test in Amhara region, Ethiopia. Malar J. 2022;21:287.

482 29. Gamboa D, Ho M-F, Bendezu J, Torres K, Chiodini PL, Barnwell JW, et al. A Large Proportion
483 of P. falciparum Isolates in the Amazon Region of Peru Lack pfhrp2 and pfhrp3: Implications for
484 Malaria Rapid Diagnostic Tests. PLoS One. 2010;5:e8091.

485 30. Bharti PK, Chandel HS, Ahmad A, Krishna S, Udhayakumar V, Singh N. Prevalence of pfhrp2
486 and/or pfhrp3 Gene Deletion in Plasmodium falciparum Population in Eight Highly Endemic
487 States in India. PLoS One. 2016;11:e0157949.

488 31. Yimam Y, Mohebali M, Abbaszadeh Afshar MJ. Comparison of diagnostic performance
489 between conventional and ultrasensitive rapid diagnostic tests for diagnosis of malaria: A
490 systematic review and meta-analysis. PLoS One. 2022;17:e0263770.

491 32. Briand V, Cottrell G, Tuike Ndam N, Martiáñez-Vendrell X, Vianou B, Mama A, et al.
492 Prevalence and clinical impact of malaria infections detected with a highly sensitive HRP2 rapid
493 diagnostic test in Beninese pregnant women. Malaria Journal. 2020;19:188.

494 33. Koliopoulos P, Kayange N, Jensen C, Gröndahl B, Eichmann J, Daniel T, et al. Challenges in
495 Diagnosing and Treating Acutely Febrile Children with Suspected Malaria at Health Care
496 Facilities in the Lake Mwanza Region of Tanzania. Am J Trop Med Hyg. 2024;110:202–8.

497 34. Garba MN, Dème AB, Diongue K, Diédhiou Y, Mbaye AM, Dia NdM, et al. Quality
498 assessment of malaria microscopic diagnosis at the Aristide Le Dantec University Hospital of
499 Dakar, Senegal, in 2020. BMC Res Notes. 2024;17:68.

500 35. Plucinski MM, Dimbu PR, Fortes F, Murphy SC, Smith NT, Cruz KR, et al. Malaria Parasite
501 Density in Individuals with Different Rapid Diagnostic Test Results and Concentrations of HRP2
502 Antigen. Am J Trop Med Hyg. 2019;100:1202–3.

503 36. Kattenberg JH, Tahita CM, Versteeg IAJ, Tinto H, Traoré Coulibaly M, D'Alessandro U, et al.
504 Evaluation of antigen detection tests, microscopy, and polymerase chain reaction for diagnosis
505 of malaria in peripheral blood in asymptomatic pregnant women in Nanoro, Burkina Faso. Am J
506 Trop Med Hyg. 2012;87:251–6.

507 37. Mayxay M, Pukrittayakamee S, Chotivanich K, Looareesuwan S, White NJ. Persistence of
508 Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. Trans R Soc
509 Trop Med Hyg. 2001;95:179–82.

510 38. Ding XC, Incardona S, Serra-Casas E, Charnaud SC, Slater HC, Domingo GJ, et al. Malaria in
511 pregnancy (MiP) studies assessing the clinical performance of highly sensitive rapid diagnostic
512 tests (HS-RDT) for Plasmodium falciparum detection. Malar J. 2023;22:60.

513 39. Matambisso G, Brokhattingen N, Maculuve S, Cisteró P, Mbeve H, Escoda A, et al. Gravidity
514 and malaria trends interact to modify P. falciparum densities and detectability in pregnancy: a
515 3-year prospective multi-site observational study. BMC Med. 2022;20:396.

516 40. Tahita MC, Sondo P, Kabore B, Ilboudo H, Rouamba T, Sanou H, et al. Impact and
517 operational feasibility of adding malaria infection screening using an ultrasensitive RDT for
518 placental and fetal outcomes in an area of high IPTP-SP coverage in Burkina Faso: the ASSER
519 MALARIA pilot study protocol. Pilot Feasibility Stud. 2022;8:221.

520 41. Krueger T, Ikegbunam M, Lissom A, Sandri TL, Ntabi JDM, Djontu JC, et al. Low Prevalence
521 of Plasmodium falciparum Histidine-Rich Protein 2 and 3 Gene Deletions-A Multiregional Study
522 in Central and West Africa. Pathogens. 2023;12:455.

523 42. Koita OA, Doumbo OK, Ouattara A, Tall LK, Konaré A, Diakité M, et al. False-Negative Rapid
524 Diagnostic Tests for Malaria and Deletion of the Histidine-Rich Repeat Region of the hrp2
525 Gene. Am J Trop Med Hyg. 2012;86:194–8.

526 43. Amoah LE, Abuaku B, Bukari AH, Dickson D, Amoako EO, Asumah G, et al. Contribution of P.
527 falciparum parasites with Pfhrp 2 gene deletions to false negative PfHRP 2 based malaria RDT

528 results in Ghana: A nationwide study of symptomatic malaria patients. PLoS One.
529 2020;15:e0238749.

530 44. Molina-de la Fuente I, Benito MJS, Flevaud L, Ousley J, Pasquale HA, Julla A, et al.
531 Plasmodium falciparum pfhrp2 and pfhrp3 Gene Deletions in Malaria-Hyperendemic Region,
532 South Sudan. Emerg Infect Dis. 2023;29:154–9.

533 45. Gatton ML, Chaudhry A, Glenn J, Wilson S, Ah Y, Kong A, et al. Impact of Plasmodium
534 falciparum gene deletions on malaria rapid diagnostic test performance. Malar J. 2020;19:392.

535 46. Lamptey H, Ofori MF, Kusi KA, Adu B, Owusu-Yeboa E, Kyei-Baafour E, et al. The prevalence
536 of submicroscopic Plasmodium falciparum gametocyte carriage and multiplicity of infection in
537 children, pregnant women and adults in a low malaria transmission area in Southern Ghana.
538 Malar J. 2018;17:331.

539 47. Schleiermacher D, Rogier C, Spiegel A, Tall A, Trape JF, Mercereau-Puijalon O. Increased
540 multiplicity of Plasmodium falciparum infections and skewed distribution of individual msp1
541 and msp2 alleles during pregnancy in Ndiop, a Senegalese village with seasonal, mesoendemic
542 malaria. Am J Trop Med Hyg. 2001;64:303–9.

543 48. Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, et al. Effect of sequence variation
544 in Plasmodium falciparum histidine- rich protein 2 on binding of specific monoclonal
545 antibodies: Implications for rapid diagnostic tests for malaria. J Clin Microbiol. 2006;44:2773–
546 8.

547 49. Sharma L, Shukla G. Placental Malaria: A New Insight into the Pathophysiology. Front Med
548 (Lausanne). 2017;4:117.

549 50. Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria.
550 Trends Parasitol. 2004;20:597–603.

551