

Highlights

- The *Pfk13* mutation R561H is present in DRC, a WHO-validated marker of resistance.
- The *Pfk13* mutation P441L is present in DRC, a WHO-candidate marker of resistance.
- A regional emergence of *Pfk13* mutations in bordering areas of Rwanda and Uganda.
- *Pfk13* and *Pfdhps* mutations in Eastern DRC seem closely related to East Africa.
- Emergence of partial ACT resistance requires an urgent and coordinated response.

Journal Pre-proof

Identification of the *PfK13* mutations R561H and P441L in Democratic Republic of Congo (DRC)

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Abstract

Background: Partial artemisinin resistance, mediated by *P. falciparum* K13 (*PfK13*) mutations, has been confirmed in certain areas of East Africa which are historically associated with high-level antimalarial resistance. DRC borders these areas in the East.

Objectives: To determine the prevalence of resistance markers in six national malaria control programme (NMCP) surveillance sites; Boende, Kabondo, Kapolowe, Kimpese, Mikalayi and Rutshuru.

Methods: The SNPs in *P. falciparum* genes *PfK13*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfcrt* were assessed using targeted NGS of isolates collected at enrolment in therapeutic efficacy studies.

Results: *PfK13* SNPs were detected in two samples; in Kabondo (R561H) and in Rutshuru (P441L), both areas near Uganda and Rwanda. The *Pfdhps* ISGEGA haplotype, associated with reduced SP chemoprevention efficacy, ranged from 0.8% in Mikalayi (central DRC) to 42.2% in Rutshuru (East DRC).

Conclusions: R561H and P441L observed in eastern DRC are a concern, as they are associated with delayed ACT-clearance and candidate marker of resistance, respectively. This is consistent with previous observations of shared drug resistance profiles in parasites of that region with bordering areas of Rwanda and Uganda. The likely circulation of parasites has important implications for the ongoing surveillance of partial artemisinin-resistant *P. falciparum* and for future efforts to mitigate its dispersal.

Keywords

Democratic Republic of Congo, antimalarial drugs, drug resistance, artemisinin, Pfk13

Introduction (rest max 3500 words)

According to the World Health Organization (WHO), the DRC accounts for 12.3% of global malaria cases and 12.6% of all malaria attributable deaths; in figures this is approximately 30 million cases and almost 80,000 deaths in 2021 [1]. The DRC has implemented a number of control efforts to combat malaria. These include the distribution of insecticide-treated bed nets (ITNs), the use of artemisinin-based combination therapies (ACTs) against uncomplicated malaria (artesunate-amodiaquine [ASAQ] or artemether-lumefantrine [AL]) and intermittent preventive treatment of pregnant women (IPTp) using sulfadoxine-pyrimethamine (SP) [1]. Access to healthcare services remains limited however, particularly in rural and hard-to-reach areas [2] and in addition, the ongoing security concerns in some Eastern provinces of the country makes it difficult to implement control interventions effectively.

The emergence of drug resistance, seen with previous antimalarials, such as chloroquine and SP led to their replacement with ACTs. The ongoing effectiveness of current ACT treatments is crucial to management of clinical malaria. Furthermore, improper usage of antimalarial drugs and guidelines, as well as the usage of substandard or counterfeit drugs, widespread throughout the DRC, may further escalate the emergence of antimalarial drug resistance [3].

Surveillance of antimalarial drug resistance can be monitored by cross-sectional measures of prevalence of molecular markers associated with *P. falciparum* resistance to various antimalarial drugs. Single nucleotide polymorphisms (SNPs) in the *P. falciparum* *PfKelch13* (*PfK13*) gene, the multidrug resistance gene-1 (*Pfmdr1*) and the chloroquine resistance transporter (*Pfcr1*) gene are associated with various levels of *P. falciparum* susceptibility to components of ACTs [4-6]. Similarly, accumulating SNPs in the *P. falciparum* genes *Pfdhfr* and *Pfdhps* are associated with various levels of SP resistance [7,8].

The *PfK13* SNPs associated with delayed parasite clearance time (PCT) after treatment with ACT, are now widespread in South-East Asia [1,9]. Until recently these were not a challenge on the African continent [12]. Validated SNPs occurring in the *PfK13* propeller domain and the BTB/POZ domain, upstream of the propeller domain are used as molecular markers to predict clinical treatment outcome and tracking partial artemisinin-resistant malaria parasites strains and include F446I, N458Y, C469Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y, R622I and A675V; all in the propeller domain [10]. Recent studies have demonstrated the *de novo* emergence and clonal expansion *PfK13* R561H mutation in East African countries, Rwanda, and Tanzania [11,12]. In Uganda, *P. falciparum* parasites with mutations in the A675V or C469Y of the *PfK13* gene were significantly associated with prolonged PCT and increased in frequency from 3.9% in 2015 to 19.8% in 2019 [13].

Moreover, Conrad *et al.* recently reported a dramatic increase in the prevalence of R561H and P441L reaching 23% in 2022 for both SNPs in certain sites of Uganda [14]. Other validated *PfK13* mutations reported in Africa, albeit at low frequency, include F446I, M476I, P553L, P574L, C580Y, C469F/Y, and R622I [15].

To explore trends in the current distribution of these molecular markers in DRC, we analysed *P. falciparum* samples obtained from patients on the day of recruitment (day 0) enrolled in Therapeutic Efficacy Studies (TES) to monitor AL and ASAQ performance conducted by the National Malaria Control Program (NMCP) in sentinel sites of six provinces across the country in 2020-21. These provinces were Kimpese and Rutshuru in 2020 and Kapolowe, Kabondo, Mikalayi and Boende in 2021.

Methods

Study sites and participants

Six sites in the equatorial zone of DRC were included in this retrospective study: Boende, (Tshuapa province), Kabondo (Kisangani, Tshopo province), Mikalayi (Kasai Central province) and Kimpese (Kongo Central province). Based on the 2017-2018 Multiple Indicator Cluster Surveys (MICS) malaria prevalence measured by malaria rapid diagnostic test (mRDT) among children aged 6–59 months was 52.2% in Boende and Kabondo, 45.5% in Mikalayi and 40.0% in Kimpese (the last two sites are near the Angola border). The fifth site, Rutshuru, is located in the mountainous zone next to the border with Rwanda and Uganda in the North Kivu province of eastern DRC, where malaria prevalence measured by

mRDT among children 6–59 months old was 11.4%. The sixth site, Kapolowe, is in the Haut Katanga province in the southern part of the country, next to the border with Zambia, and is in the tropical zone, where malaria prevalence measured by mRDT among children 6–59 months old was 42.7%. The national malaria prevalence measured by mRDT among children 6–59 months old was 38.5% [16].

Children aged 6–59 months with uncomplicated *P. falciparum* malaria infection were recruited at participating health centers. A sample size of 88 children per arm per site was targeted and calculated assuming a 5% drug failure, a 95% confidence level and 5% precision in estimated treatment failure, and the assumption of 20% loss to follow up [17].

Study procedure

Criteria for inclusion were *P. falciparum* infection measured by microscopy with parasite densities between 2,000 and 200,000 trophozoites/ μ l; axillary temperature of $\geq 37.5^{\circ}\text{C}$; ability to take oral medication; ability to adhere to the follow-up procedures of the study; declared consent from a parent or guardian; absence of signs of severe illness, malnutrition, or other illness associated with fever; and absence of past allergic reaction to the study medication following recommendations of WHO [17].

Microscopic blood examination was performed by trained microscopists using thick and thin smears on the same slide to determine parasite densities and species, respectively. For the present study only *P. falciparum* microscopy defined positive samples from children enrolled in the study before provided antimalarial treatment (day 0) was used.

DNA extraction and qPCR diagnosis of microscopy-positive samples

Capillary blood was collected on Whatman (GE Healthcare, Chicago, IL) filter paper for characterization of molecular markers of antimalarial resistance. All *P. falciparum* positive

dried blood spots collected at baseline from the six provinces were shipped to Centre for Medical Parasitology (CMP), University of Copenhagen, in October 2022, where DNA was extracted using the Omega E-Z 96 Tissue DNA Kit (Omega Bio-tek, Norcross, United States), according to manufacturer's instructions.

Target Amplicon Sequencing

A series of nested PCRs amplified regions in genes associated with artemisinin, lumefantrine, amodiaquine, chloroquine and SP resistance (namely genes in *Pfdhfr*, *Pfdhps*, *PfK13*, *Pfmdr1* and *Pfprt*) as described by Nag *et al.*, modified to be run in simplex [18]. Samples were dual indexed by incorporating non-annealing overhangs in the nested PCR, and specific 8-base pair sequences were attached in an index PCR step for unique barcoding of each sample for the Illumina MiSeq flow cell.

After the index step, the samples were purified using beads and then diluted to a final concentration of 4nM. To account for low nucleotide diversity caused by the high AT content in the *P. falciparum* genome, a 5% PhiX library was spiked in (Illumina, California, United States).

Quality control of Illumina reads and analyses of the markers

After sequencing, samples were sorted into folders matching their original extracted DNA plates according to index sequences, the FastQ files were then controlled and trimmed using Galaxy (<http://www.usegalaxy.au>). The individual reads were quality-controlled using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and the QC results were then aggregated using MultiQC. The reads were then trimmed using Trimmomatic with the following parameters: average Phred across 4 bases had to be above 20 (base-calling

probability above 99). The trimmed R1 and R2 files were then combined using the concatenate datasets function, and these files were then ready for further analysis.

The trimmed and combined fastq files were then aligned to the 3D7 reference genome using a Python programme *Assimpler*, as described in [18]. SNPs with more than 50 reads at a given position were considered present and additionally, if more than two nucleotides in the same position were represented with minimum 25% of the total reads, the sample was considered a mixed infection.

Statistical analysis

Statistical analysis was performed using Microsoft® Excel v.16.54 and GraphPad Prism 9 v.9.5.1 by exploring differences in the prevalence of the markers of interest. Prevalence estimates were compared across the six sites using a χ^2 -test and were conducted on a 5% level of significance. When testing for differences in the distribution of individual SNPs mutants, mutants and mixed wildtype/mutant infections were pooled and tested against wildtypes.

Results

Patient characteristics

Between 176 and 200 patients below the age of 5 years were enrolled in the TES studies from the six sites (table 1). The density of parasites/ μl ranged from median values of 25,304 parasites/ μl (Mikalayi) to 30,343 parasites/ μl (Kimpese). A total of 1,115 patients were diagnosed with malaria by microscopy, from which 1,065 DBS samples were available for analysis of molecular markers (table 1).

Prevalence of SNPs in the genes *PfK13*, *Pfdhfr*, *Pfdhps* and *Pfmdr1* in *P. falciparum* infections of patients from six sites across DRC

For SNPs in the *PfK13* gene, a total of 59 different SNPs were found in the propeller region of the gene, whereof 34 were non-synonymous (Supplemental Table 1). Two of the non-synonymous mutations occurred more than once; Q613E and V637I. Moreover, one SNP, the candidate P441L mutant found in one sample from Rushuru and the validated R561H, found in one sample from Kabondo are associated with artemisinin partial resistance by delayed parasite clearance [10].

Full data on the prevalence of SNPs in the genes *Pfdhfr*, *Pfdhps* and *Pfmdr1* can be observed in supplementary table 2. For *Pfdhfr* and *Pfdhps*; Mikalayi was an outlier (low prevalence on all resistance SNPs) and was set as the reference site and compared to the other sites. For *Pfdhfr*, SNPs in codons 51, 59 and 108 were observed and in all sites and except in Mikalayi the prevalence of the 51I and 59R mutants were over 94% and 80%, respectively. In Mikalayi the prevalence was significantly lower, at 81.0% for 51I and 58.3% for 59R ($\chi^2=15.52$, $P\leq 0.001$ and $\chi^2=38.35$, $P\leq 0.001$; wildtypes set against mutant types including mixed wildtypes/mutant types).

Regarding the *Pfdhps* gene, SNPs in codons 431, 436, 437, 540, 581, and 613 was observed with large differences between the sites. The prevalence of the 437G mutant varied from 69.3% (Mikalayi) to 96,0% (Kimpese) ($\chi^2=16.25$, $P\leq 0.001$), while the 540E mutant varied from 14.5% (Mikalayi) to 89.6% (Rutshuru) ($\chi^2=153.26$, $P\leq 0.001$) and the 581G as well varied significantly between sites from 0% (Mikalayi) to 41.8% (Rutshuru). Lastly, the *Pfdhps*-431V and the 613S was identified in Boende and Kimpese only, at below 3%. To the best of our knowledge, the 431V SNP has not been recorded in the DRC until now.

For *Pfmdr1*, SNPs in codons 86, 184, 1042, 1034 and 1246 were observed. The 86Y mutant was identified at low prevalence (<10%) in all sites except in Rutshuru at 17.3% while the 184F varied significantly between sites from 20.6% in Kapolowe to 44.1% in Kabondo ($\chi^2=6.85$, $P=0.009$; Y184 wildtype set against 184F mutant type including mixed wildtypes/mutant types). The prevalence of the 1246Y mutant was high only in Rutsuru (20.9%) and very low elsewhere ($\leq 2\%$). The 1034C and the 1042D mutants were found in two samples, from Mikalayi and Rutsuru, respectively.

Frequency of constructed haplotypes in the genes *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfprt* at six sites across DRC

Figures 1-3 (and supplemental figure 1) present the frequencies and distribution of the constructed haplotypes in the four genes (for full frequency data, see supplementary table 3). The constructed haplotypes of *Pfdhfr* (codons 51-59-108-164; figure 1) show almost absence of the wildtype NCSI in all settings (frequency $\leq 2\%$) while the frequency of the triple mutant **IRNI** haplotype were above 85% in all sites except Mikalayi where prevalence of **IRNI** was significantly lower (57.9%; $\chi^2=85.98$, $P\leq 0.0001$, Mikalayi set against all the other sites). Large differences in the frequencies of various constructed *Pfdhps* haplotypes (codons 431-436-437-540-581-613) were observed and are shown in Figure 2. In Rutshuru, the frequency

of the single ISGKAA mutant haplotype was low (4.8%). Compared to Rutshuru, frequency of ISGKAA was higher in Kabondo and Kapolowe (both at 30.8%; $\chi^2=47.18$, $P\leq 0.001$) while much higher in central and western DRC (Mikalayi, Kimpese and Boende, where this haplotype accounted for approximately 60%; $\chi^2=0.38$, $P=0.829$). Conversely, the double (ISGEAA) and triple mutant (ISGEGA) haplotypes were observed at the highest frequency in Rutshuru (at 44.6% and 42.2%, respectively), while in central and western sites (Kimpese, Boende and Mikalayi) frequency of the double ISGEAA mutant haplotype was low (11.0%, 12.7% and 18.6%, respectively). The triple mutant varied between the remaining sites between 0.8% (Mikalayi) and 20.8% (Kapolowe). Notably, the *Pfdhps*-431V containing haplotypes were identified in Eastern sites of Boende (VAGKGS) and Kimpese (VAGKGS and VAGEGA) only, albeit in very few samples (n=1 and 4, respectively).

The distribution of constructed haplotypes in *Pfjndr1* (codons 86-184-1034-1042-1246) are shown in figure 3, displaying large heterogeneity between the six sites. The haplotype, NYSND was highly prevalent in most sites (range: 53.0%-70.9%) except for Rutshuru (30.5%). The NFSND haplotype was the most prevalent in Rutshuru (39.0%), which also showed a prevalence of 11.0% of the YYSNY haplotype (n=10).

Finally, the haplotypes in codon 72-76 of the *Pfprt* gene identified consisted of only the wildtype CVMNK and the mutant type CVIET haplotype. The frequency of the CVMNK haplotype ranged from 72.9% in Kimpese to 100% in Kapolowe, except for Rutshuru where only 15.2% were of the CVMNK wildtype (Supplemental Figure 1).

Discussion

It is evidently clear from the history of antimalarial drug use in the malaria endemic world that once a new antimalarial drug has been introduced large-scale, resistance in the *P. falciparum* malaria parasite populations will always emerge [18]. Consequently, continued surveillance of molecular markers of drug resistance in *P. falciparum* populations is key to monitor the distribution of drug resistance to inform efforts to mitigate the inevitable reduction in effectiveness of these drugs. Strategies of routine sampling of *P. falciparum* populations across sentinel sites and *timely* analyses and interpretation of data is essential to provide inputs for models guiding antimalarial drug policy in malaria endemic countries. The recent discovery of emerging *Pfk13* SNPs in Eastern Africa associated with partial artemisinin resistance underline the necessity.

In this study, we explored such molecular markers in *P. falciparum* populations obtained from patients enrolled in TES performed recently (in 2020-21) in six sites across DRC.

Using high-throughput NGS, we identified 34 non-synonymous mutations in the *PfK13* gene, whereof one validated (R561H) and one candidate (P441L) mutation associated with delayed clearance by artemisinin [10] were observed in Kabondo and Rutshuru, respectively. The R561H mutation has been reported in neighbouring countries of Uganda [14] and Rwanda [11] (see figure 4) and as well in the Coastal region of Tanzania [11] while only detected once in a sample from Kinshasa by Ménard *et al.* (2016) [9].

The P441L mutation has only been reported on the African continent in Uganda and Rwanda – bordering the DRC near Rutshuru and seems to be increasing in prevalence as well [14, 19, 20] (Figure 4). Thus Rwanda and Uganda seem to constitute a hotspot for emerging *PfK13* mutations associated with delayed clearance by artemisinin as the prevalence of these SNPs is increasing in both countries [14,19, 21]. The observed dispersal to bordering countries, such as the DRC shown in the present study is an alarming trend.

This study also explored the genes associated with SP resistance; *Pfdhfr* codons 51, 59, 108 and 164 and *Pfdhps* codons 431, 436, 437, 540, 581 and 613 [22]. The spatial distribution of different *Pfdhfr* haplotypes was variable with the triple **IRNI** mutant haplotype dominating the population in all locations, as expected. Generally, an increase in the triple mutant haplotype has been recorded historically since the use of SP was introduced in a large scale [7]. Southern Mikalayi was the only site with somewhat lower frequency of the triple mutant (57.9%, due to lower prevalence of the 59R genotype).

The distribution of *Pfdhps* haplotypes varied greatly between sites. The double ISGEAA and in particular the triple ISGEGA mutant haplotypes have been associated with loss of protective effectiveness of SP when used for IPT in pregnant women [22] or infants [23]. In Rutshuru, both mutant haplotypes were highly prevalent compared to other sites, especially central and western sites. Analysis of data from two demographic and health surveys conducted in the DRC in 2007, and 2013-2014 noted an increase in the prevalence of the double and triple mutant from 14.6% to 27.2% [24]. Our study shows a further increase in some sites since then. Interestingly, a 2018 study reported a prevalence of just 9.6% (N=137/1435) of the double mutant and 4% (N=58/1435) of the triple mutant haplotype in western DRC [25]. The 2018 study by Aydemir *et al.* (2018) suggests that the triple *Pfdhps* mutant spread from Tanzania in 2006 to Rwanda, Uganda and the DRC (amongst other bordering countries) [24]. Similarly, our study seems to suggest that the *Pfdhps* haplotypes in northeastern DRC more closely resemble parasites in Rwanda and Uganda than the parasite populations in Western and Southern DRC.

Furthermore, the 431V mutation was identified in five individual samples in the two most Western sampling sites of Kimpese and Boende. This mutation has emerged recently and has previously been identified exclusively in West or Central African countries such, Nigeria and

Benin amongst others, where seasonal malaria chemoprevention (SMC) using SP+amodiaquine is being implemented [21, 26]. It has often been seen to co-occur with the 581G/613S mutations (as the *Pfdhps*-VAGKGS haplotype) [26] - also only observed in this study in Kimpese and Boende, albeit at a low prevalence. This SNP/haplotype has previously been hypothesized to compromise the protective efficacy of preventive strategies using SP similarly to the East African *Pfdhps* SGEGA haplotype [21].

The SNPs/combined haplotypes in *Pfmdr1* codons 86, 184, 1034, 1042 and 1246 have been associated with various levels of resistance/tolerance to various antimalarial drugs [5, 8, 20]. This study found significantly different haplotype profiles between the six DRC sites. Particularly, the 86-184-1246 haplotype, NFD is associated with reduced susceptibility to lumefantrine [6] which is detected at a relatively high frequency throughout most sites (ranging from 24,1% in Mikalayi to 47,2% in Kabondo). In the same codons, the **YYY** haplotype was only observed in Rutshuru (11,0%) and Kapolowe (0.8%). Since the introduction of artemether-lumefantrine as first-line treatment in most of sub-Saharan African countries, several countries like Tanzania have witnessed to have an increase in the NFD haplotype [5, 27]. This study could be indicative of a similar pattern in the DRC as a 2018 study suggested that 29% of samples collected between 2012 and 2014 near Kimpese harboured the NFD haplotype [28] while in this study, 37.9% of the Kimpese site samples were of the NFD haplotype, suggestive of an increase. In areas where AL is widely used, selection of *Pfmdr1* NFD haplotype was shown to occur at a faster rate while ASAQ seems to antagonistically select for *Pfmdr1* **YYY** haplotype [5]. This trend could have implications for the use of AL and ASAQ as first-line treatment.

The *Pfcr1* CVIET mutant haplotype is highly associated with chloroquine (CQ) resistance, and in the present study the frequencies varied from 0% in Kapolowe to 84,8% in Rutshuru.

CQ was used as first-line treatment in many African countries, but was widely abandoned around the turn of the millennium due to widespread reports of resistance. However, most sites in this study, with the exception of Rutshuru, seem to be CQ susceptible once again, based on *Pfprt* and *Pfmdr1* data.

The emergence of partial artemisinin resistance and partner drugs is a public health threat that requires urgent and coordinated response beyond national borders to delay the spread of resistant strains in the region. In regions affected by military conflicts such as the border between Rwanda and DRC, malaria disproportionately affects displaced populations, increasing their vulnerability and may enhance the spread of resistant strains. Strong surveillance through TES and genetic surveillance leveraging recent technological advances is critical to timely inform case management and resistance mitigation strategies. As malaria burden is still extremely high in DRC, enhancing malaria control interventions through increasing coverage and use of interventions including vector control interventions (ITNs, larviciding, IRS), improved patient access and adherence to quality-assured antimalarial medicine is essential. Moreover, integrated community case management of malaria (CCM) [29], use of single low dose primaquine with transmission blocking potential may reduce malaria transmission and hence reduce drug pressure on the existing ACTs, delay the spread of partial artemisinin resistance and prolong the ACTs use in the region. Other innovative approaches in the pipeline such as new therapeutics such triple ACTs and non-artemisinin-based treatments, deployment of sequential or multiple first-line therapies [30] may provide options to limit the spread of resistance and prevent treatment failures in endemic regions of Africa.

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Ethical approval

The DRC Ethics Committee of the School of Public Health of the University of Kinshasa provided ethical clearance for the study in DRC. The study protocol was registered in an approved public register (www.ClinicalTrials.gov, registration number NCT04618523). Informed consent was available in French and translated into local languages (Lingala, Kikongo, Swahili, and Tshiluba). All patient information was kept confidential and was known only to the research team. The analyzed dataset was anonymized.

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Contributions

GMK, SWT, LWT, VB, DTRM and MA conceptualized the study. HMM, PMM, PNM, DMM, DMN, EMS and JJMT assisted in the recruitment of patients and collection of

samples and reporting the patient characteristics. SWT, LWT, EFH and HH performed the molecular analyses and validated the results. SWT, LWT, EFH, HH, MA, CR and AM performed the statistical and bioinformatics analysis of data and SWT, LWT, GMK and MA drafted the manuscript. All authors read and approved the final draft of the manuscript.

Competing interests: All authors declare that they do not have any competing interests.

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References

- [1] WHO: **World Malaria Report**. In. <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022>; 2022.[accessed 4 June 2023]
- [2] Kayiba NK, Yobi DM, Devleeschauwer B, Mvumbi DM, Kabututu PZ, Likwela JL, Kalindula LA, DeMol P, Hayette MP, Mvumbi GL *et al*: **Care-seeking behaviour and socio-economic burden associated with uncomplicated malaria in the Democratic Republic of Congo**. *Malar J* 2021, **20**(1):260. <https://doi.org/10.1186/s12936-021-03789-w>
- [3] Akilimali A, Bisimwa C, Aborode AT, Biamba C, Sironge L, Balume A, Sayadi R, Ajibade SB, Akintayo AA, Oluwadairo TO *et al*: **Self-medication and Anti-malarial Drug Resistance in the Democratic Republic of the Congo (DRC): A silent threat**. *Trop Med Health* 2022, **50**(1):73. <https://doi.org/10.1186/s41182-022-00466-9>
- [4] Arieu F, Witkowski B, Amaratunga C, Beghain J, Langlois A-C, Khim N, Kim S, Duru V, Bouchier C, Ma L *et al*: **A molecular marker of artemisinin-resistant Plasmodium falciparum malaria**. *Nature* 2014, **505**(7481):50-55. Doi: [10.1038/nature12876](https://doi.org/10.1038/nature12876)
- [5]. Okell LC, Reiter LM, Ebbe LS, Baraka V, Bisanzio D, Watson OJ, Bennett A, Verity R, Gething P, Roper C *et al*: **Emerging implications of policies on malaria treatment: genetic changes in the Pfmdr-1 gene affecting susceptibility to artemether-lumefantrine and artesunate-amodiaquine in Africa**. *BMJ Glob Health* 2018, **3**(5):e000999. <http://dx.doi.org/10.1136/bmjgh-2018-000999>
- [6] Sisowath C, Petersen I, Veiga MI, Mårtensson A, Premji Z, Björkman A, Fidock DA, Gil JP: **In Vivo Selection of Plasmodium falciparum Parasites Carrying the Chloroquine-Susceptible pfcr1 K76 Allele after Treatment with Artemether-Lumefantrine in Africa**. *J Infect Dis* 2009, **199**(5):750-757. doi: [10.1086/596738](https://doi.org/10.1086/596738)

- [7] Okell LC, Griffin JT, Roper C: **Mapping sulphadoxine-pyrimethamine-resistant Plasmodium falciparum malaria in infected humans and in parasite populations in Africa.** *Sci Rep* 2017, **7**. DOI: [10.1038/s41598-017-06708-9](https://doi.org/10.1038/s41598-017-06708-9)
- [8] Plowe CV: **Malaria chemoprevention and drug resistance: a review of the literature and policy implications.** *Malar J* 2022, **21**(1):104. <https://doi.org/10.1186/s12936-022-04115-8>
- [9] Ménard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, Rahim-Awab G, Barnadas C, Berry A, Boum Y *et al*: **A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms.** *N Engl J Med* 2016, **374**(25):2453-2464. DOI: [10.1056/NEJMoa1513137](https://doi.org/10.1056/NEJMoa1513137)
- [10] WHO: **Malaria: Artemisinin partial resistance.** In. <https://www.who.int/news-room/questions-and-answers/item/artemisinin-resistance>: World Health Organization [accessed 14 July 2023].
- [11] Uwimana A, Umulisa N, Venkatesan M, Szigel SS, Zhou Z, Munyaneza T, Habimana RM, Rucogoza A, Moriarty LF, Sandford R *et al*: **Association of Plasmodium falciparum kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study.** *Lancet Infect Dis* 2021, **21**(8):1120-1128. [https://doi.org/10.1016/S1473-3099\(21\)00142-0](https://doi.org/10.1016/S1473-3099(21)00142-0)
- [12] Bwire GM, Ngasala B, Mikomangwa WP, Kilonzi M, Kamuhabwa AAR: **Detection of mutations associated with artemisinin resistance at k13-propeller gene and a near complete return of chloroquine susceptible falciparum malaria in Southeast of Tanzania.** *Sci Rep* 2020, **10**(1):3500. <https://doi.org/10.1038/s41598-020-60549-7>

- [13] Balikagala B, Fukuda N, Ikeda M, Katuro OT, Tachibana SI, Yamauchi M, Opió W, Emoto S, Anywar DA, Kimura E *et al*: **Evidence of Artemisinin-Resistant Malaria in Africa.** *N Engl J Med* 2021, **385**(13):1163-1171. DOI: 10.1056/NEJMoa2101746
- [14] Conrad MD, Asua V, Garg S, Giesbrecht D, Niaré K, Smith S, Namuganga JF, Katairo T, Legac J, Crudale RM *et al*: **Evolution of Partial Resistance to Artemisinins in Malaria Parasites in Uganda.** *N Engl J Med* 2023, **389**(8):722-732.
- [15] Owoloye A, Olufemi M, Idowu ET, Oyebola KM: **Prevalence of potential mediators of artemisinin resistance in African isolates of Plasmodium falciparum.** *Malar J* 2021, **20**(1):451. DOI: 10.1056/NEJMoa2211803
- [16] Moriarty LF, Nkoli PM, Likwela JL, Mulopo PM, Sompwe EM, Rika JM, Mavoko HM, Svigel SS, Jones S, Ntamabyaliro NY *et al*: **Therapeutic Efficacy of Artemisinin-Based Combination Therapies in Democratic Republic of the Congo and Investigation of Molecular Markers of Antimalarial Resistance.** *Am J Trop Med Hyg* 2021, **105**(4):1067-1075. DOI: [10.4269/ajtmh.21-0214](https://doi.org/10.4269/ajtmh.21-0214)
- [17] WHO: **Tools for monitoring antimalarial drug efficacy.** In., vol. 2023. <https://www.who.int/teams/global-malaria-programme/case-management/drug-efficacy-and-resistance/tools-for-monitoring-antimalarial-drug-efficacy>. [accessed 10 August 2023].
- [18] Nag S, Ursing J, Rodrigues A, Crespo M, Krogsgaard C, Lund O, Aarestrup FM, Alifrangis M, Kofoed PE: **Proof of concept: used malaria rapid diagnostic tests applied for parallel sequencing for surveillance of molecular markers of anti-malarial resistance in Bissau, Guinea-Bissau during 2014-2017.** *Malar J* 2019, **18**(1):252. <https://doi.org/10.1186/s12936-019-2894-8>

- [18] Haldar K, Bhattacharjee S, Safeukui I: **Drug resistance in Plasmodium**. *Nat Rev Microbiol* 2018, **16**(3):156-170. doi: 10.1038/nrmicro.2017.161
- [19] Kirby R, Giesbrecht D, Karema C, Watson O, Lewis S, Munyaneza T, Butera JDD, Juliano JJ, Bailey JA, Mazarati JB: **Examining the Early Distribution of the Artemisinin-Resistant Plasmodium falciparum kelch13 R561H Mutation in Areas of Higher Transmission in Rwanda**. *Open Forum Infect Dis* 2023, **10**(4). DOI: [10.1093/ofid/ofad149](https://doi.org/10.1093/ofid/ofad149)
- [20] Asua V, Conrad MD, Aydemir O, Duvalsaint M, Legac J, Duarte E, Tumwebaze P, Chin DM, Cooper RA, Yeka A *et al*: **Changing Prevalence of Potential Mediators of Aminoquinoline, Antifolate, and Artemisinin Resistance Across Uganda**. *J Infect Dis* 2021, **223**(6):985-994. DOI: 10.1093/infdis/jiaa687
- [21] Oguike MC, Falade CO, Shu E, Enato IG, Watila I, Baba ES, Bruce J, Webster J, Hamade P, Meek S *et al*: **Molecular determinants of sulfadoxine-pyrimethamine resistance in Plasmodium falciparum in Nigeria and the regional emergence of dhps 431V**. *Int J Parasitol Drugs Drug Resist* 2016, **6**(3):220-229. DOI: 10.1016/j.ijpddr.2016.08.004
- [22] Hansson H, Minja DTR, Moeller SL, Lusingu JPA, Bygbjerg IC, Yde AM, Jensen RW, Nag S, Msemu OA, Theander TG *et al*: **Reduced Birth Weight Caused by Sextuple Drug-Resistant Plasmodium falciparum Infection in Early Second Trimester**. *J Infect Dis* 2021, **224**(9):1605-1613. DOI: 10.1093/infdis/jiab117

- [23] Gosling RD, Gesase S, Mosha JF, Carneiro I, Hashim R, Lemnge M, Mosha FW, Greenwood B, Chandramohan D: **Protective efficacy and safety of three antimalarial regimens for intermittent preventive treatment for malaria in infants: a randomised, double-blind, placebo-controlled trial.** *Lancet* 2009, **374**(9700):1521-1532. [https://doi.org/10.1016/S0140-6736\(09\)60997-1](https://doi.org/10.1016/S0140-6736(09)60997-1)
- [24] Aydemir O, Janko M, Hathaway NJ, Verity R, Mwandagalirwa MK, Tshefu AK, Tessema SK, Marsh PW, Tran A, Reimonn T *et al*: **Drug-Resistance and Population Structure of Plasmodium falciparum Across the Democratic Republic of Congo Using High-Throughput Molecular Inversion Probes.** *J Infect Dis* 2018, **218**(6):946-955. DOI: 10.1093/infdis/jiy223
- [25] Nkoli Mandoko P, Rouvier F, Matendo Kakina L, Moke Mbongi D, Latour C, Losimba Likwela J, Ngoyi Mumba D, Bi Shamamba SK, Tamfum Muyembe JJ, Muepu Tshilolo L *et al*: **Prevalence of Plasmodium falciparum parasites resistant to sulfadoxine/pyrimethamine in the Democratic Republic of the Congo: emergence of highly resistant pfdhfr/pfdhps alleles.** *J Antimicrob Chemother* 2018, **73**(10):2704-2715. DOI: 10.1093/jac/dky258
- [26] Adegbola AJ, Ijarotimi OA, Ubom AE, Adesoji BA, Babalola OE, Hocke EF, Hansson H, Mousa A, Bolaji OO, Alifrangis M *et al*: **A snapshot of the prevalence of dihydropteroate synthase-431V mutation and other sulfadoxine-pyrimethamine resistance markers in Plasmodium falciparum isolates in Nigeria.** *Malar J* 2023, **22**. DOI: 10.1186/s12936-023-04487-5

- [27] Thomsen TT, Ishengoma DS, Mmbando BP, Lusingu JP, Vestergaard LS, Theander TG, Lemnge MM, Bygbjerg IC, Alifrangis M: **Prevalence of single nucleotide polymorphisms in the Plasmodium falciparum multidrug resistance gene (Pfmdr-1) in Korogwe District in Tanzania before and after introduction of artemisinin-based combination therapy.** *Am J Trop Med Hyg* 2011, **85**(6):979-983.
DOI: 10.4269/ajtmh.2011.11-0071
- [28] Baraka V, Delgado-Ratto C, Nag S, Ishengoma DS, Madebe RA, Mavoko HM, Nabasumba C, Lutumba P, Alifrangis M, Van Geertruyden JP: **Different origin and dispersal of sulfadoxine-resistant Plasmodium falciparum haplotypes between Eastern Africa and Democratic Republic of Congo.** *Int J Antimicrob Agents* 2017, **49**(4):456-464. DOI: 10.1016/j.ijantimicag.2016.12.007
- [29] Oliphant NP, Manda S, Daniels K, Odendaal WA, Besada D, Kinney M, White Johansson E, Doherty T: **Integrated community case management of childhood illness in low- and middle-income countries.** *Cochrane Database Syst Rev* 2021, **2**(2):Cd012882. doi: 10.1002/14651858.CD012882.pub2.
- [30] Boni MF, White NJ, Baird JK: **The Community As the Patient in Malaria-Endemic Areas: Preempting Drug Resistance with Multiple First-Line Therapies.** *PLoS Med* 2016, **13**(3):e1001984. doi: 10.1371/journal.pmed.1001984

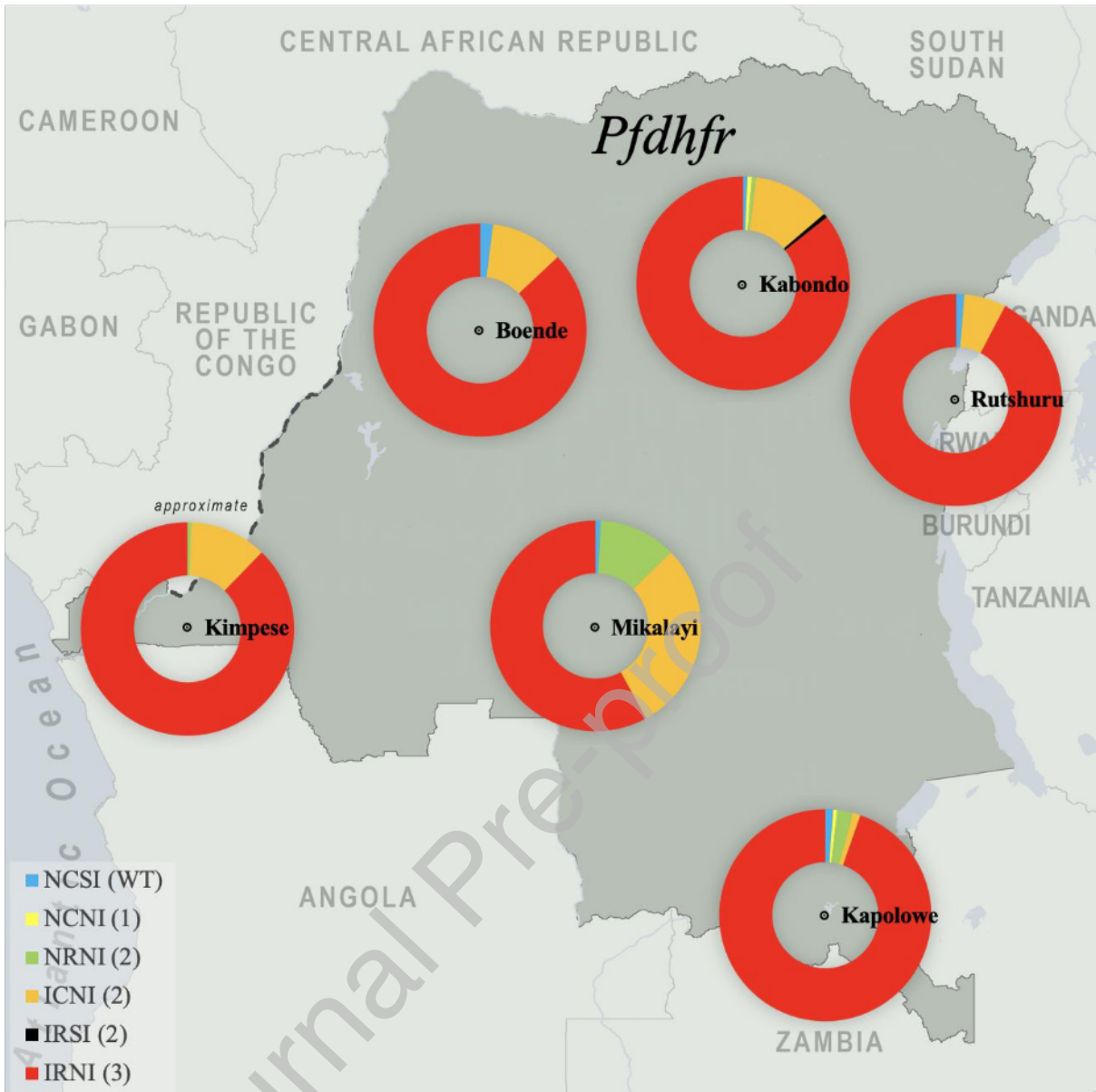


Figure 1. *Pfdhfr* haplotype frequency in 6 sites across the DRC. Haplotype frequency of *Pfdhfr* (codons 51-59-108-164) (mutant in **fat**: NCSI, NCNI, NRNI, ICNI, IRSI and IRNI. The numbers next to the haplotypes indicate the amount of mutations while WT is indicative of the wild-type haplotype. Mixed infections were not included. Haplotype diagrams depicted on the DRC map (CDC 2023) (<https://wwwnc.cdc.gov/travel/destinations/traveler/none/democratic-republic-of-congo>).

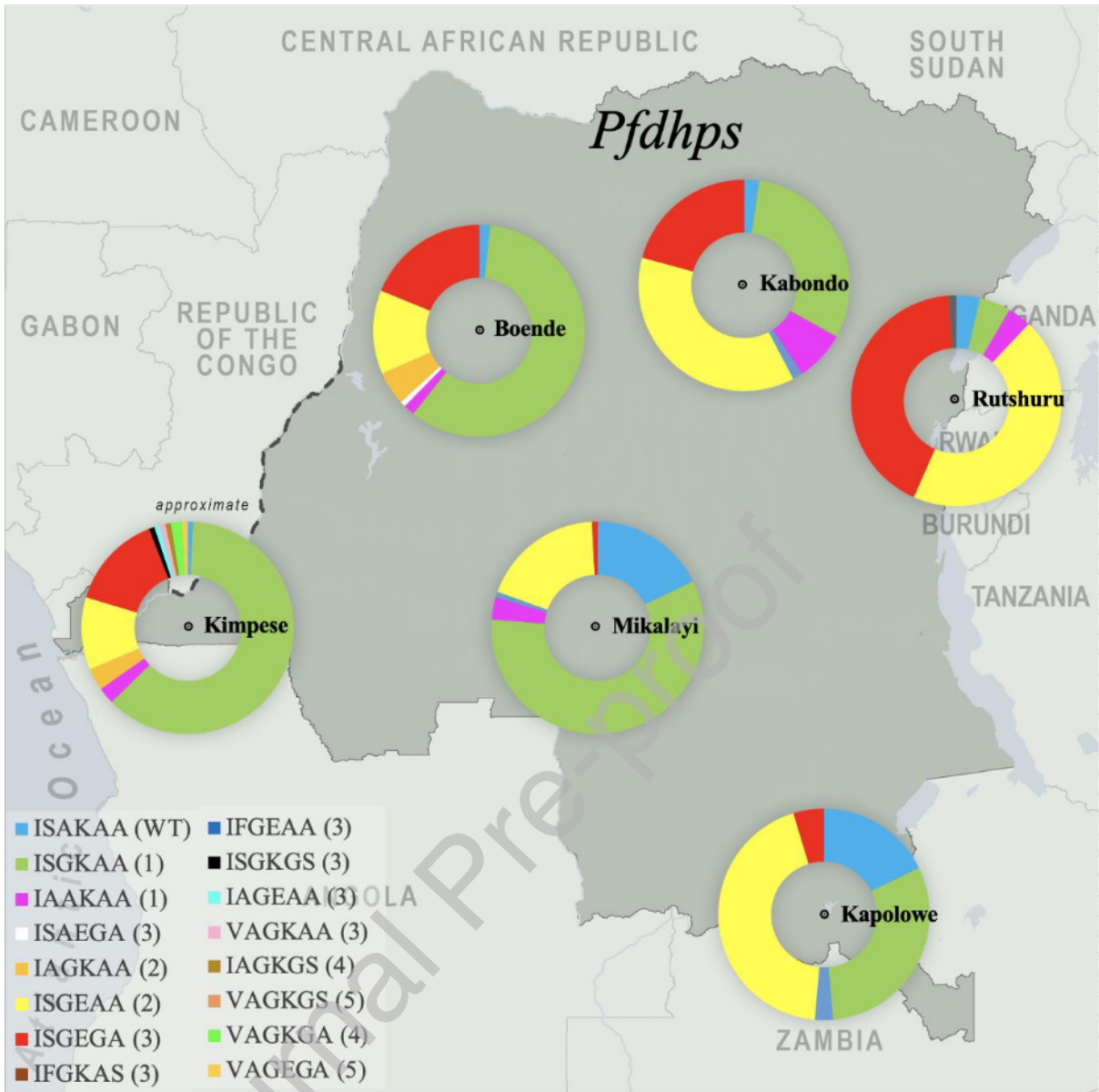


Figure 2. *Pfdhps* haplotype frequency in 6 sites across the DRC. Haplotype frequency in *Pfdhps* (codons 431-436-437-540-581-613); mutants in **fat**: ISAKAA, IAAKAA, ISGKAA, IAGKAA, ISAEGA, IAGEAA, ISGEAA, ISGEGA, IFGEAA, ISGKGS, VAGKAA, IAGKGS, VAGKGA, VAGKGS and VAGEGA. The numbers next to the haplotypes indicate the amount of mutations while WT is indicative of the wild-type haplotype. Haplotype diagrams depicted on the DRC map (CDC 2023) (<https://wwwnc.cdc.gov/travel/destinations/traveler/none/democratic-republic-of-congo>)

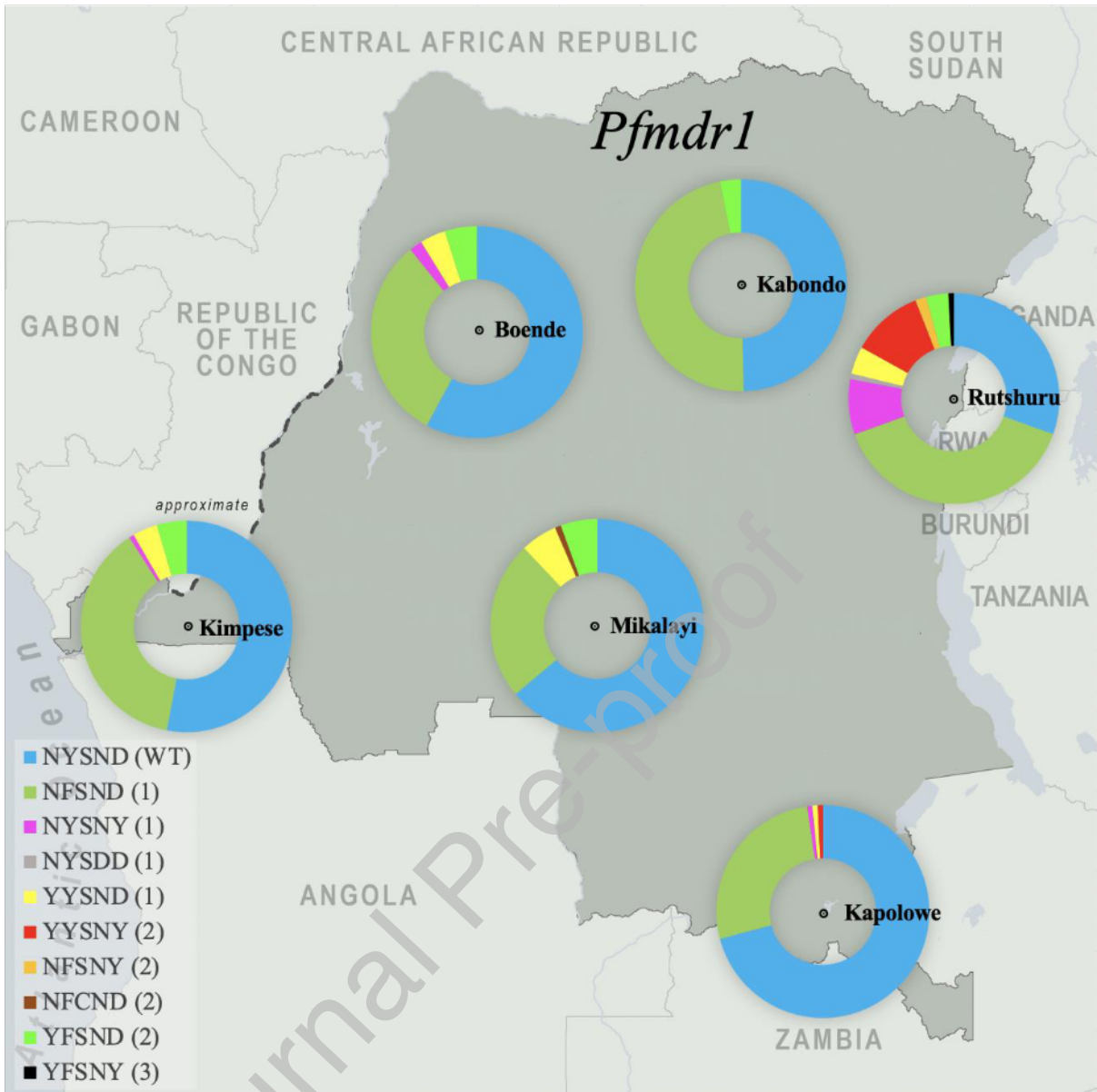


Figure 3. *Pfmdr1* haplotype frequency in 6 sites across the DRC. Haplotype frequency of *Pfmdr1* (codons 86-184-1034-1042-1246); mutants in **fat**: NYSND, NFSND, NYSNY, NYSDD, YYSND, YYSNY, NFSNY, NFCND, YFSND, YFSNY. The numbers next to the haplotypes indicate the amount of mutations while WT is indicative of the wild-type haplotype. Haplotype diagrams depicted on the DRC map (CDC 2023) (<https://wwwnc.cdc.gov/travel/destinations/traveler/none/democratic-republic-of-congo>)

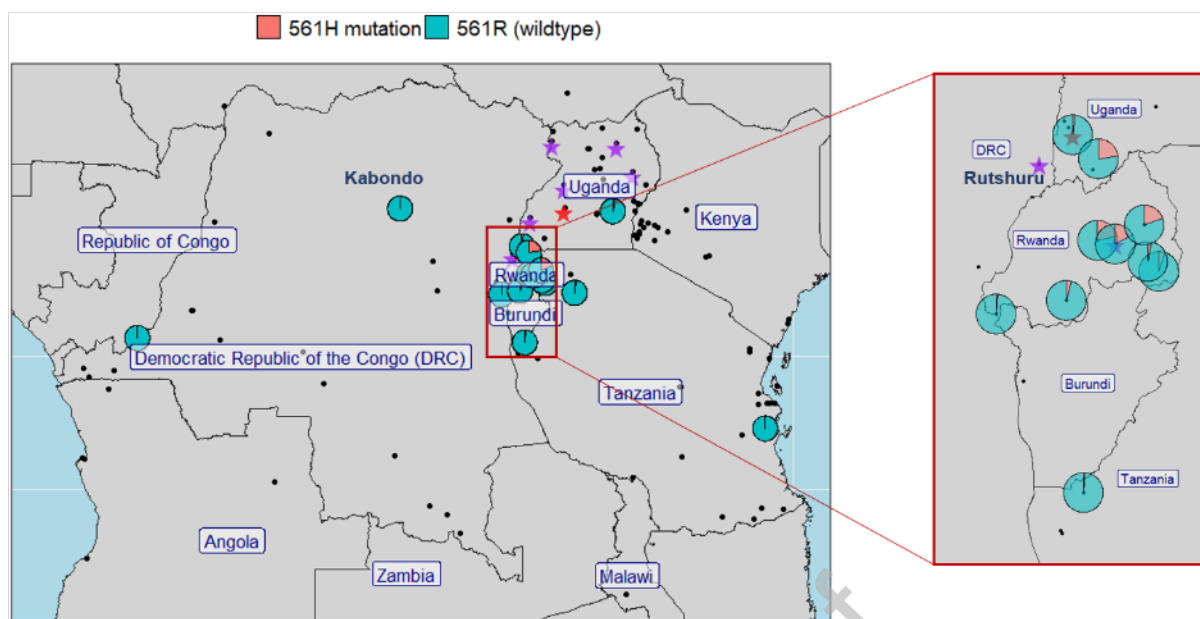


Figure 4. Prevalence of *Pfk13* R561H and of P441L mutations in DRC and neighbouring countries. The data are based on the WorldWide Antimalarial Resistance Network (WWARN) database (<http://www.wwarn.org/molecular/surveyor/k13/index.html?t=201608031200#0>), data from the current study (Kabondo and Rutshuru) and data from a site in Rwanda [19] and a multi-centre study in Uganda [14]. The pie charts show the prevalence of the R561H mutations, and purple stars denote the presence of P441L. For all studies where the P441L mutation was identified, it was only found in a single sample, with the exception of two sites in Uganda where prevalence of P441L was 23.1% (15/65) in Kanungu and 11.5% (6/52) in Mubende marked as red stars [14]. Black dots show the sites where prevalence of other *Pfk13* mutations is reported, based on the WWARN database.

Table 1. General demographic data of enrolled patients in the therapeutic efficacy studies (TES).

Sites	Boende	Kabondo	Kapolowe	Kimpese	Mikalayi	Rutshuru
Year of sampling	2021	2021	2021	2020	2021	2020
Enrolled (n)	179	176	180	200	180	200
Median age, months (25-75% percentiles)	32.0 (18.0-47.0)	36.0 (23.0-48.0)	30.5 (19.0-47.8)	41.0 (24.0-55.0)	24.0 (13.3-36.0)	30.0 (16.3-48.0)
Gender (M:F) %	47.5:52.5	43.8:56.2	41.1:58.9	46.5:53.5	53.9:46.1	51.5:48.5
Median temperature C, (25-75% percentiles)	38.8 (38.0-39.5)	38.4 (37.8-39.2)	38.0 (37.8-38.8)	37.9 (37.5-38.7)	38.6 (37.7-39.3)	38.1 (37.7-39.0)
Median parasite density (parasites/ul) (25%-75% percentiles)	27,764 (11,612-46,917)	26,526 (13,954-49,862)	27,850 (12,652-53,281)	30,343 (10,687-63,719)	25,304 (11,889-52,837)	29,871 (12,506-63,662)