

Rising Prevalence of *Plasmodium falciparum* Artemisinin Partial Resistance Mutations in Ethiopia

Corresponding Author: Mr Bokretsion Brhane

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Review of Manuscript

Rising prevalence of *Plasmodium falciparum* artemisinin resistance mutations in Ethiopia

Summary and Importance

This manuscript examines drug resistance markers in *Plasmodium falciparum* across 12 sentinel sites in Ethiopia from 2019 to 2022. The study analyzes 855 isolates using two molecular inversion probe (MIP) panels, one previously published targeting drug resistance genes and another newly developed panel targeting common SNPs. The identification of increased prevalence of the K13 R622I mutation (15% of all infections), associated with partial artemisinin resistance, is a significant finding for Ethiopia and the broader region. This prevalence is notably higher compared to prior reports, indicating a potential emerging threat to malaria treatment efficacy. Furthermore, the first-time detection in Ethiopia of three WHO-validated K13 mutations (A675V, P441L, and P574L) underscores the need for robust surveillance to monitor resistance patterns. Additionally, the study evaluates markers associated with ACT partner drug resistance (e.g., *mdr1*, *crt*, *dhfr*, and *dhps*) and provides insights into genetic relatedness patterns.

The findings will be of particular interest to malaria policy makers in Ethiopia and the region, as well as researchers engaged in drug resistance monitoring. However, the broader implications for treatment policy require careful framing, as association of the investigated markers with clinical resistance to lumefantrine is not validated.

Additionally, the spatial relatedness analyses contribute valuable insight into the distribution of resistant strains. Although the study lacks some depth in genomic population structure analysis and statistical validations, it still provides a foundation for future work to explore these patterns further.

While the data offer important contributions to malaria treatment strategies in Ethiopia, several methodological and analytical issues need to be addressed to strengthen the manuscript's conclusions and impact.

Major Comments

1. Clarity and Presentation

The manuscript requires substantial revision for grammar and sentence structure. Several sentences lack clarity due to missing verbs, incorrect phrasing, or ambiguous meanings.

2. Analysis of Genetic Relatedness, population structure and Diversity

- The focus on IBD networks with high cut-offs (e.g., 0.99) limits the scope of conclusions about genetic diversity and relatedness in the whole population. Including analyses at lower IBD thresholds or using different population genetic metrics could reveal whether mutant parasites cluster separately or emerge within the local population.
- Pairwise IBD comparisons and PCA results suggest that there is no clearly differentiated cluster of parasites with the R622I mutation. This observation contrasts with previous findings, where a single cluster with R622I mutations was reported. A direct comparison to earlier datasets could clarify whether the mutation is spreading more broadly in the population.
- The manuscript claims to provide insights into genetic population structure but lacks standard population genetics metrics (e.g., genetic diversity, differentiation). These analyses could strengthen the study's conclusions.
- Using a high IBD threshold limits the analysis to a subset of samples, overlooking broader population diversity and relatedness between clusters with and without 622I. Figures 2B and 5B show only a median IBD of 0.25, and PCA indicates no distinct clustering of 622I parasites within the population. This could be investigated and reported in more detail.
- Line 290: "Tracking the emergence and spread etc." is contradicting the limitations described in the discussion.

3. Spatial and Statistical Analysis

- While the manuscript highlights associations between K13 622I mutation and the *mdr1* NFD haplotype, given the high prevalence of this NFD haplotype (~95%), statistical analyses such as Chi-square tests or multilocus linkage disequilibrium are needed to confirm whether these associations are significant beyond chance.
- Figure 2B: Pairwise IBD sharing within kelch13 R622I mutant versus wild parasites, show significant differences. Did you include in this analysis also comparisons where there was no pairwise IBD (i.e. where IBD-sharing =0. Or only included those where IBD>0)? Did you correct/stratify this analysis for health centre? Cause you report later on that you see differences in pairwise- IBD by health centre, while you also see different levels of the mutations in the different health centres.
- For ACT partner drug resistance markers, the manuscript focuses on individual proportions but does not provide statistical summaries or confidence intervals for haplotypes. This is critical since combinations of alleles often determine resistance phenotypes especially for the partner drugs. Summary estimates of haplotype prevalences should complement the upset plots.
- Spatial heterogeneity analyses (e.g., 5B) require statistical validation to support claims of differences in IBD-sharing across health centers.

4. Partner Drug Resistance Markers

- The discussion overemphasizes the implications for ACT failure without sufficient supporting evidence for lumefantrine resistance. While ongoing monitoring is essential, the absence of clinical or in vitro data limits the strength of the conclusions. Consider discussing also the lack of "strong" or validated markers for lumefantrine resistance and potential alternative explanations for the selection of the *mdr1* haplotype, such as selection due to chloroquine pressure from treatment of co-endemic *P. vivax*.
- the important measure to report on for SP resistance is the prevalence of samples that have the quintuple mutation taking both genes into account.

5. Data and Methods Transparency

- The flow of samples from collection to analysis is unclear. A flowchart detailing recruitment, processing, and inclusion/exclusion criteria should be included in supplementary materials. Explain how 12 out of 25 sentinel sites were selected for this study.
- The new MIP panel is insufficiently described. Details on its design, targeted SNPs, and validation are necessary for evaluating its robustness. If publication or preprint of the panel is pending, a more detailed description should be provided in this manuscript. This is important for the interpretation of the population genetics and relatedness data.
- The analysis of genome-wide SNPs is mentioned but not clearly linked to specific results or methods. Provide clarity when and how these SNPs were used in the analysis. Add how many SNPs were in the analyzed dataset.

Minor Comments

1. Grammar and Wording

- Line 96: Replace "frank partner drug resistance mutations" with "validated partner drug resistance mutations." Or correct otherwise if this does not capture the correct meaning
- Line 99: what does it means to "partially affect susceptibility diametrically"
- Line 104-105: check this sentence
- The methods come after the results, so it would be better to explain a better what you did in the results section. For example in line 136, first sentence of the results, you start with "after filtering,". But it is not clear at all what you are filtering (what type of data, how etc.)
- Line 157: "a Somali region Eastern part of" correct to "a Somali region in the Eastern part of".
- Line 178: check sentence, "clustering clustered"
- Line 197: Clarify "significant frequencies" with specific thresholds or statistical definitions.
- Line 217: the reference to the figure breaks up the sentence, move to the end of that sentence.
- Line 219: Instead of saying 'low spatial heterogeneity', say 'with equal distribution at regional level across the country'
- Line 227: "infections" instead of "infection"
- Line 230: add a word linking "Gambela in the southwest" and "7.6% ([95% CI 5.2-8.4%])"
- Line 251: Add verb: "The lowest average IBD sharing was observed at Abol health center, Gambella Region"
- Line 252-255: check this sentence
- Line 260: some words seem missing in this sentence
- Line 300: correct to "The K13 441L mutation was found"
- Line 310: The haplotype is or the haplotypes are
- Line 388: remove bracket
- Line 411-413: check the grammar of this sentence
- Line 426: correct "infectious" to "infection"
- Line 434: Correct to "visualized using ggplot2"
- Line 445: correct to "or geographic origin (health facilities) were created "
- Line 452-454: check sentence

2. Figures and Tables

- Figure 2C: Indicate the number of samples included in the network analysis. Consider showing the other validated K13 mutations in this network.
- Figure 3: Please report somewhere how many/what proportion of samples have calls at all positions.
- Figure 5C: Consider using color coding by region or district rather than health center to enhance clarity.
- Table S3: Verify the reported genotype L in crt position 73 resulting in haplotype (CLIET at positions 72-76) and correct if necessary.
- Figure S1: does this figure show only included samples? Cause there seem to still be samples with low depth across many MiPs probes. It might help to show also how this looks for the excluded samples, and/or to quantify 'low' and 'high'. (as low

might still be sufficient for analysis of course).

3. Additional References and Data Access

- Ensure that all programs and packages (e.g., The Real McCOIL, SNPRelate) used in analyses are appropriately cited.
- Include individual sample accession numbers in Table S2 to support data sharing.
- Add details on sequencing performance (e.g., target and achieved depth) and the variability in probe success rates.
- Line 218: the confidence interval for the triple mutant (61.6%) is missing
- Line 283: Include references to prior studies cited in the discussion (e.g., comparisons of R622I prevalence).
- Line 283-285: "Here we see a prevalence of 622I up to 53.7% in Andansa Health Centre within the Amhara region, as compared to 10% in 20185 and 2.4% in 201434."
- o Are these proportions from the same site? It does not appear to me that this health centre was sampled in those 2 studies.
- Line 337: You do not necessarily need whole genome sequencing to do this. For example in SEA this was initially done with MS markers, as at that time WGS was not easily implemented on field samples.
- Line 339: Clinical resistance and in vitro efficacy are two different things. One is related to treatment failure. The other to parasite survival in the presence of drugs. You indeed have information on neither in this population at this time.
- Line 382-384: add a reference for the drug resistance MIPs panel.
- Line 389: "the same MIP panel": please clarify, The drug resistant one, or both?
- Line 406-408: How many SNPs did you retain before and after this step? How many samples (%) were removed with less than 50% of SNPs?
- Line 411: before you mentioned a threshold UMI of 10, but here you mention 3; please clarify the different thresholds used and which data and analysis this impacted.
- Line 431: what settings did you use for the COI analysis?
- Line 446: Please add more details how you did this: "We then assessed IBD sharing at the regional and local (health..."
- The github contains the code for data visualization, not (yet) data analysis as claimed in line 456.

Conclusion

This study provides valuable data on drug resistance markers in *P. falciparum* in Ethiopia and highlights the potential for genomic surveillance to guide malaria control strategies. However, the manuscript would benefit significantly from improvements in clarity, additional statistical analyses, and more robust methodological descriptions to enhance its impact and reproducibility.

Reviewer #2

(Remarks to the Author)

The manuscript by Brhane et al. provides results from molecular surveillance of Plasmodium parasites in Ethiopia with a focus on mutations causing partial resistance to antimalarials of the artemisinin class.

The paper is well written and the conclusion that there is an increase in prevalence of relevant mutations in the Kelch13 gene is valid. It is important work and great to see that this setup is being established in Ethiopia. There is a lot of debate on the consequences of emerging drug resistance in Eastern Africa and this manuscript is providing important information. The main limitation that I see, is a lack of efficacy data or references to efficacy studies. The clinical impact of the described mutations in Ethiopia is not clear. I would recommend to include data or references from recent TES studies performed in Ethiopia.

About a quarter of the samples were already collected in 2019, which is quite some time ago. It would be good to discuss, if this might have any impact on the result analysis and the conclusions of the manuscript.

Some further suggestions for the authors:

In line 127 it is mentioned that the sentinel site collect data on efficacy, however there is no reference to such data available in the entire manuscript. Even if patient level correlation of mutated parasites and treatment outcome is not available, the overall efficacy of ACT should be mentioned.

The start of the result section, line 136, is a bit odd. The paragraph should provide more details on patient population. The type of "filtering" performed on the samples needs to be clarified.

Minor comments of editorial nature:

Number of dried blood spots is 1999 in abstract, but 1199 in result section

sampling was done 2019 to 2023 and not as mentioned in the abstract 2019 to 2022

Sentence starting in line 104 is not clear

Sentence starting in row 324 is not clear

use potentially when mentioning lumefantrine resistance as there is no clear evidence at the moment (it is not done in discussion section)

Reviewer #3

(Remarks to the Author)

The manuscript by Brhane et al describes surveillance efforts to track artemisinin resistance markers at sentinel sites across Ethiopia using a combination of dried blood spots and molecular inversion probe sequencing. The authors focus on mutations within a series of known drug resistance genes including k13, mdr1, crt, dhfr and dhps and supplement this with a

selection of genome wide SNPs.

The results are observational but of importance as they provide a crucial update on drug resistance within Ethiopia and identify the presence of WHO validated K13 mutants that had not previously been seen within the country. This is supported by a large number of samples from multiple sites and years with haplotypes constructed from SNPs spread across the genome. While WGS could provide additional resolution of the observed haplotypes the number of SNPs used are sufficient for the purpose of the study and appropriate given the use of dried blood spots for sample collection.

The figures are largely clear and informative, with a substantial amount of supporting material that provides additional depth to the results. The methods are clearly written, use appropriate techniques and provide enough detail that they could be replicated. The included statistics are straightforward but appropriate for the results that are described. The authors identify a number of key limitations of the study in the discussion including that it does not address whether mutations are emerging locally or imported from neighbouring countries. Have these haplotypes been explored in the substantial malariaGen Pf7 release (or prior)? While the exact same SNP sets may not be available there is likely to be overlap within K13. International reporting, especially between countries sharing borders and substantial flow of people are key to understanding the spread of drug antimalarial drug resistance so this manuscript will be of most value when considered alongside comparable results from the region, Africa as a whole and the rest of the world.

Specific comments:

How many samples were there? In the abstract it says 1999 (line 42) but in the results it states there were 1199 (line 136). This is likely a typo but should be corrected and subsequent numbers rechecked.

The description of sample numbers in the opening results paragraph is confusing in the way it alternates between the filtered samples and total sample numbers. The breakdown by region is for the filtered numbers, while the breakdown by year is for the total. It would be better to highlight the total number of samples and their breakdown first and then present the breakdown of the filtered sample set. This is presented better in Table S1 and the authors may want to consider making this a main table and add in the successfully genotyped breakdown (for example for each cell presenting data as x / y (%) to concisely state the number of sequenced samples (x), total number (y) and the percentage sequenced (%)).

For the P574L (Figure S4B) what was the p-value for the significant difference between wildtype and mutant? Please either add to the main text or the supplementary figure.

Were clustered R622L parasites in figure 2C found within the same regions or were related haplotypes observed between difference regions of the country? Given the generally low transmission levels (as shown by the COI estimates later in the manuscript) it would be expected that related parasites would arise from clonal transmission cycles within a local region.

The majority of the MIP panel is newly designed but references an in preparation manuscript (Niare et al) by a lead author who is not named as an author on this study. It concerns me that the paper describing and validating these new MIPs will probably not be published until after work using them. At the very least Niare (and possibly co-authors) should be credited as an author on this work as this will represent the first publication of the MIP sequences.

Line 388 - Extra) that should be removed

I was unable to access the study data via the SRA or BioProject accession numbers. I assume they are embargoed prior to release but it should be checked that they have been released prior to the manuscript being published.

The typed signature for the editorial checklist differs from the name of the lead author. This appears to be a typo (Berhan vs Brhane) but should be confirmed and corrected given the document relates to ethics and reproducibility.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study provides valuable data on drug resistance markers in *P. falciparum* in Ethiopia and highlights the potential for genomic surveillance to guide malaria control strategies. The authors have addressed the issues raised in the previous review with much care and the manuscript has much improved in detail and clarity.

A few minor comments: (line numbers refer to the version with tracked changes)

Line 275 – the word genome is duplicated

Line 414 and Supplementary Fig. 11. – CVIET is now corrected from CLIET in the text and table S2, but in the text and supplementary figure there is now mention of CLMNK and CLMNT, should this be CVMNK and CVMNT? CVMNK is the wildtype haplotype (CQ sensitive).

Line 401 – z?

Check abbreviations, sometimes use ArtR, sometimes ART-R

Reviewer #2

(Remarks to the Author)

Thank you for providing the opportunity to review the revised manuscript.

The manuscript looks good, please consider addressing the editorial comments below prior finalization.

1. The sentence starting in line 123 appears to be a duplication of content of the prior sentence
2. In line 323 provide full name for IBD as it is mentioned here for the first time in result section
3. In line 365 consider to list the 3 residues by sequence order, so that it is aligned with NFD haplotype referred to in the following sentences

Reviewer #3

(Remarks to the Author)

Following the first round of review by myself and others Brhane et al have made substantial revisions to the points raised, including a detailed response and updates to the manuscript itself which is now much stronger. I am happy to recommend the amended manuscript for publication, it provides a detailed and valuable description of the rising prevalence of artemisinin resistance in Ethiopia. Surveillance work such as this is essential for understanding the spread of drug resistance and collected with studies from across Africa and the rest of the world provide a valuable resource in fighting malaria.

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Response to the Reviewers

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Review of Manuscript

Rising prevalence of *Plasmodium falciparum* artemisinin resistance mutations in Ethiopia

Summary and Importance

This manuscript examines drug resistance markers in *Plasmodium falciparum* across 12 sentinel sites in Ethiopia from 2019 to 2022. The study analyzes 855 isolates using two molecular inversion probe (MIP) panels, one previously published targeting drug resistance genes and another newly developed panel targeting common SNPs. The identification of increased prevalence of the K13 R622I mutation (15% of all infections), associated with partial artemisinin resistance, is a significant finding for Ethiopia and the broader region. This prevalence is notably higher compared to prior reports, indicating a potential emerging threat to malaria treatment efficacy. Furthermore, the first-time detection in Ethiopia of three WHO-validated K13 mutations (A675V, P441L, and P574L) underscores the need for robust surveillance to monitor resistance patterns. Additionally, the study evaluates markers associated with ACT partner drug resistance (e.g., *mdr1*, *crt*, *dhfr*, and *dhps*) and provides insights into genetic relatedness patterns. The findings will be of particular interest to malaria policy makers in Ethiopia and the region, as well as researchers engaged in drug resistance monitoring. However, the broader implications for treatment policy require careful framing, as association of the investigated markers with clinical resistance to lumefantrine is not validated.

Additionally, the spatial relatedness analyses contribute valuable insight into the distribution of resistant strains. Although the study lacks some depth in genomic population structure analysis and statistical validations, it still provides a foundation for future work to explore these patterns further.

While the data offer important contributions to malaria treatment strategies in Ethiopia, several methodological and analytical issues need to be addressed to strengthen the manuscript's conclusions and impact.

Response: Thank you for your thoughtful comments and suggestions, which have strengthened our manuscript. We have carefully addressed each point raised, making revisions to clarify our findings and improve the overall quality of the study.

Major Comments

1. Clarity and Presentation

The manuscript requires substantial revision for grammar and sentence structure. Several sentences lack clarity due to missing verbs, incorrect phrasing, or ambiguous meanings.

Response: Thank you for your feedback on the manuscript's clarity and presentation. We have revised it to address issues with grammar, sentence structure, and phrasing, ensuring clearer and more concise language throughout.

2. Analysis of Genetic Relatedness, population structure and Diversity

- The focus on IBD networks with high cut-offs (e.g., 0.99) limits the scope of conclusions about genetic diversity and relatedness in the whole population. Including analyses at lower IBD thresholds or using

different population genetic metrics could reveal whether mutant parasites cluster separately or emerge within the local population.

Response: We conducted additional IBD analysis (in line 341-343) with a lower threshold and observed that the mutant parasites began to group with the wildtype parasites. This finding indicates that mutant parasites not only share similarities with each other but also have a broader genetic background in common with the wild type, extending beyond the k13 gene. We have included this result in the revised manuscript and added the results as a **supplementary Figure. 7.**

- Pairwise IBD comparisons and PCA results suggest that there is no clearly differentiated cluster of parasites with the R622I mutation. This observation contrasts with previous findings, where a single cluster with R622I mutations was reported. A direct comparison to earlier datasets could clarify whether the mutation is spreading more broadly in the population.

Response: Thank you for your suggestion. We respectfully disagree with the reviewer's statement that "Pairwise IBD and PCA findings contrast with the IBD network of clonal parasites." The IBD network in **Figure 1C** focuses on highly related, clonal parasite pairs, whereas the pairwise IBD plot and PCA analysis include all parasite populations in the dataset. Our PCA results (see lines 431-437 and **Figure S12**) support our new analysis at lower IBD thresholds, where mutant parasites group with wild-type parasites.

Additionally, it's important to note that IBD and PCA are distinct genomic metrics. IBD is less affected by allele frequency and is more robust in capturing genetic relatedness, whereas PCA is influenced by allele frequency variations in the population. These differences in methodology may explain the observed variation in clustering patterns.

Regarding the Fola et al. (2023) previous study, where a single cluster with R622I mutations was reported, this study focused on a geographically restricted region (the North), where the parasite populations are more closely related. In contrast, our study covers five regions (12 sites) across the country, encompassing diverse malaria transmission areas and significant geographic distances. These factors, particularly variations in transmission dynamics and human migration (including refugees), likely contribute to the differences observed in parasite population structures. Specifically, the Gambella region, which shares borders with Sudan and South Sudan, may be particularly impacted by these factors, further distinguishing its parasite populations from those in other regions. Moreover, the key difference here is that more time has passed since the earlier study, allowing for more intermixing of parasite populations. The parasites from the North, particularly those from earlier times, may have been among the first to exhibit the emergence of the R622I mutation, with subsequent spread and intermingling across different regions contributing to the broader distribution observed in our study. Nevertheless, our current findings do support previous observations when considering a subset of parasites from the North (Amhara Region). In this region, we observe a cluster of parasites based on the K13 622I status (**Figure 4B**), and closely cluster within health facilities compared to wild types consistent with previous reports.

- The manuscript claims to provide insights into genetic population structure but lacks standard population genetics metrics (e.g., genetic diversity, differentiation). These analyses could strengthen the study's conclusions.

Response: Thank you for your suggestion. In response, we have conducted additional population genetic analyses, including **SNP π** as measures of genetic diversity at the regional level. We also calculated F_{ST} as a measure of genetic differentiation. **We have added this to that line 435-438 in the revised manuscript.** Our new findings support the conclusion that the Gambella population exhibits greater genetic diversity and higher F_{ST} (indicating higher genetic differentiation) compared to the other populations. This finding is consistent with expectation given the presence of multiple refugee camps and

displaced persons in the region, who could be a source of parasite importation. These new results are now included as a main figure (**Figure 4C and D**) in the manuscript.

- Using a high IBD threshold limits the analysis to a subset of samples, overlooking broader population diversity and relatedness between clusters with and without 622I. Figures 2B and 5B show only a median IBD of 0.25, and PCA indicates no distinct clustering of 622I parasites within the population. This could be investigated and reported in more detail.

Response: Thank you for this valuable feedback. We have extended our analysis to include the full distribution of IBD values (ranging from 0 to 1) for both mutant and wildtype parasites. This additional analysis has been included in **Figure 2B (new)**, which demonstrates that IBD values are broadly distributed across the population. Notably, we observe that 622I parasites tend to show higher IBD values compared to wildtype parasites.

Additionally, we have updated the PCA analysis in **Figure 4B** to show clustering based on both parasite mutation status (represented by different shapes) and geography (indicated by color). This update provides a clearer view of how the parasite population is structured according to both mutation status and geographic origin, which helps to better interpret the observed clustering patterns. We apologize for any lack of clarity in the original submission and appreciate the opportunity to improve the presentation of our findings.

In addition, we have conducted further **population genetic analyses**, including genetic differentiation, and SNP π assessments. These results have been incorporated into the manuscript (**line 247-250**) to offer a more comprehensive understanding of the population dynamics, as detailed in the previous response.

- Line 290: "Tracking the emergence and spread etc." is contradicting the limitations described in the discussion.

Response: We have restructured the manuscript to focus on the spread of mutations within Ethiopia, as we currently do not have data from other neighboring countries. We have also changed this text in **revised manuscript line 537-539** "tracking the emergence and spread of drug-resistant strains in different parts of Ethiopia ". As correctly noted, one of the main limitations of the study is the inability to determine whether the mutations, such as the K13 622I, are emerging locally within Ethiopia or being imported from nearby regions. Unfortunately, we do not have publicly available data on K13 622I data to compare to from neighboring countries, which limits our ability to track the origin and broader spread of the mutations. We have clarified this limitation in the revised manuscript.

3. Spatial and Statistical Analysis

- While the manuscript highlights associations between K13 622I mutation and the *mdr1* NFD haplotype, given the high prevalence of this NFD haplotype (~95%), statistical analyses such as Chi-square tests or multilocus linkage disequilibrium are needed to confirm whether these associations are significant beyond chance.

Response: We have added a statistical analysis to assess the association between the K13 622I mutation and the *mdr1* NFD haplotype. A Chi-squared test was performed, yielding a Chi-squared value of 5.5404, degrees of freedom = 1, and a p-value of 0.0186, which indicates a statistically significant association between these two genetic factors. Added this finding in the result section **in line 381-382 in the revised manuscript**.

- Figure 2B: Pairwise IBD sharing within kelch13 R622I mutant versus wild parasites, show significant differences. Did you include in this analysis also comparisons where there was no pairwise IBD (i.e. where IBD-sharing = 0. Or only included those where IBD > 0)? Did you correct/stratify this analysis for health centre? Cause you report later on that you see differences in pairwise- IBD by health centre, while you also see different levels of the mutations in the different health centres.

Response: I We now included comparisons where IBD-sharing = 0, we focused our analysis on pairwise comparisons where IBD > 0, as we aimed to examine relatedness between isolates with non-zero IBD sharing. We also added a new analysis to adjust for health centre variability by including health centre as a random effect in our linear mixed-effects model. The linear mixed-effects model, fitted using the REML (Restricted Maximum Likelihood) method, showed that K13 R622I mutants consistently had significantly higher IBD estimates compared to the wild-type isolates. Specifically, there was a decrease of 0.24 in IBD for wildtype-mutant isolates and a decrease of 0.14 for wildtype-wildtype isolates, relative to the baseline group. This model accounted for health centre as a random effect, allowing for variability across different health centre groups. **The Results (added in a revised manuscript in 332-334)** suggest that K13 R622I mutants exhibit consistently sharing across health centres.

- For ACT partner drug resistance markers, the manuscript focuses on individual proportions but does not provide statistical summaries or confidence intervals for haplotypes. This is critical since combinations of alleles often determine resistance phenotypes especially for the partner drugs. Summary estimates of haplotype prevalences should complement the upset plots.

Response: Thank you for this suggestion. We did additional haplotype analysis for key drug resistance genes as the reviewer suggested. Specifically, **supplementary Figure 10** now includes an overall haplotype distribution pie chart (**Panel A**) and a regional stacked bar plot (**Panel B**), showing haplotype prevalence. Additionally, we have provided haplotype prevalence at regional level, in **Supplementary Table 5**. These additions complement the upset plots and offer a more comprehensive understanding of haplotype distribution and prevalence. The new analysis and results have been incorporated in the revised manuscript.

- Spatial heterogeneity analyses (e.g., 5B) require statistical validation to support claims of differences in IBD-sharing across health centers.

Response: To address the concern regarding statistical validation of the observed spatial heterogeneity in IBD-sharing, we have added a new analysis in the revised manuscript (**in line 473-476**). Specific to **Figure 5B** - We conducted a one-way ANOVA to formally assess whether the variation in IBD-sharing differs significantly across health centers. This approach was chosen to compare the means of IBD-sharing among the multiple health centers. The results indicate highly significant differences across the sites ($F = 483.2$, $p < 2e-16$), providing statistical support for the claim of spatial heterogeneity in IBD-sharing across the health centers.

4. Partner Drug Resistance Markers

- The discussion overemphasizes the implications for ACT failure without sufficient supporting evidence for lumefantrine resistance. While ongoing monitoring is essential, the absence of clinical or in vitro data limits the strength of the conclusions. Consider discussing also the lack of "strong" or validated markers for lumefantrine resistance and potential alternative explanations for the selection of the *mdr1* haplotype, such as selection due to chloroquine pressure from treatment of co-endemic *P. vivax*.

Response: We have revised the discussion to clarify that while ongoing monitoring of lumefantrine susceptibility is essential, the absence of clinical or in vitro data limits our ability to draw definitive conclusions. We have also adjusted the terminology to refer to "reduced susceptibility" rather than "lumefantrine resistance," given the lack of validated markers for clinical failure. Additionally, we address

the gap in validated markers for lumefantrine resistance and explore alternative explanations for the selection of the *mdr1* haplotype. This includes considering potential chloroquine pressure from co-endemic *P. vivax* and these in discussion revised manuscript in line 588-596. There is strong evidence supporting its role in reducing sensitivity and contributing to more rapid reinfection, although this does not constitute clinical resistance at therapeutic drug levels.

- the important measure to report on for SP resistance is the prevalence of samples that have the quintuple mutation taking both genes into account.

Response: Thank you for the valuable comments. In light of the importance of combined mutations in both DHPS and DHFR for SP resistance, we focused on determining the prevalence of the IRNGE quintuple mutation. Our analysis revealed notable regional variation in the prevalence of this quintuple mutation across Ethiopia. Specifically, the highest prevalence was observed in the Southern and Oromia regions, while the lowest prevalence was found in the Amhara and Gambela regions. Added this result as supplementary Table 5 in the revised manuscript.

5. Data and Methods Transparency

- The flow of samples from collection to analysis is unclear. A flowchart detailing recruitment, processing, and inclusion/exclusion criteria should be included in supplementary materials. Explain how 12 out of 25 sentinel sites were selected for this study.

Response: We appreciate your suggestion to clarify the flow of samples from collection to analysis. In response, we have included a flowchart in the supplementary materials that outlines the recruitment process, as well as the inclusion and exclusion criteria.

Regarding the selection of sentinel sites, 12 out of the 25 national sentinel sites were strategically chosen to ensure comprehensive representation of the five major regions, spanning from the northwestern to the southern parts of the country. This deliberate selection was based on geographical diversity and accessibility, ensuring that all key areas are covered for robust data collection and monitoring efforts.

These sites were selected for their accessibility (i.e., ease of access via available transportation routes) and safety, as they were located in areas free from conflict, but chosen to include as many regions as possible. This ensured that researchers and medical personnel could access the sites without significant delays, thus facilitating timely data collection and monitoring.

Additionally, we have clarified the study's inclusion criteria, which included all cases of microscopically confirmed positive *P. falciparum* infections. Excluded cases involved mixed infections (*P. falciparum* + *P. vivax*), severe malaria, or individuals unable to provide consent or assent. Present this flow chart as figure S1.

- The new MIP panel is insufficiently described. Details on its design, targeted SNPs, and validation are necessary for evaluating its robustness. If publication or preprint of the panel is pending, a more detailed description should be provided in this manuscript. This is important for the interpretation of the population genetics and relatedness data.

Response: We appreciate the importance of providing a clear description of the new MIP panel. To address this, we have added more detailed information regarding its design, targeted SNPs, and validation in the revised manuscript. Additionally, we have included a reference to a preprint describing the development of the MIP panel in detail: [doi: https://doi.org/10.1101/2025.03.07.25323597](https://doi.org/10.1101/2025.03.07.25323597), which provides further details for those interested in evaluating its robustness. This will aid in the interpretation of the population genetics and relatedness data presented in the manuscript.

- The analysis of genome-wide SNPs is mentioned but not clearly linked to specific results or methods. Provide clarity when and how these SNPs were used in the analysis. Add how many SNPs were in the analyzed dataset.

Response: In the revised manuscript (lines 216-219), we provide additional details regarding the analysis. A total of **855 retained samples** and **508 high-quality, biallelic SNPs** were included, spanning all 14 chromosomes of *Plasmodium falciparum*. These SNPs were filtered based on quality and missingness criteria to ensure robust data for subsequent analyses. To better illustrate their distribution, we have included a supplementary figure (**Supplementary Figure 2B**) showing the genome-wide distribution of the retained SNPs. This plot displays SNP density across chromosomes in 25 kb windows, with the horizontal axis representing chromosome length (Mb) and the green color indicating SNP density. The SNPs were specifically incorporated in the pairwise IBD analysis and PCA, as well as used to calculate F_{ST} to assess population differentiation.

Minor Comments

1. Grammar and Wording

- Line 96: Replace “frank partner drug resistance mutations” with “validated partner drug resistance mutations.” Or correct otherwise if this does not capture the correct meaning

Response: We have replaced “frank partner drug resistance mutations” with “validated partner drug resistance mutations” to accurately reflect the intended meaning and ensure clarity.

- Line 99: what does it means to “partially affect susceptibility diametrically”

Response: We have restructured the sentence for better clarity and readability. The revised sentence now reads: “Amino acid polymorphisms in the multidrug resistance protein 1 (MDR1) and chloroquine resistance transporter (CRT) are well-documented and may influence susceptibility to both amodiaquine and, potentially, lumefantrine in opposing ways.”

- Line 104-105: check this sentence

Response: We have revised the sentence to improve clarity by adding a linking word.

- The methods come after the results, so it would be better to explain a better what you did in the results section. For example in line 136, first sentence of the results, you start with “after filtering,”. But it is not clear at all what you are filtering (what type of data, how etc.)

Response: We have revised the results section to provide more clarity on the methods used, particularly in regard to the filtering process. The sentence now includes specific details on what data was filtered, how it was done, and the criteria used for filtering. This revision aims to ensure that the methodology is better understood in the context of the results. Additionally, we have made similar improvements in other parts of the results section to enhance clarity and readability.

- Line 157: “a Somali region Eastern part of” correct to “a Somali region in the Eastern part of”.

Response: We have corrected the text from “a Somali region Eastern part of” to “a Somali region in the Eastern part of”

- Line 178: check sentence, “clustering Clustered”

Response: We have revised the sentence for clarity and consistency. The issue with “clustering ... Clustered” has been corrected.

- Line 197: Clarify “significant frequencies” with specific thresholds or statistical definitions.

Response: We have modified the term “significant frequencies” to “high frequencies” for clarity and precision in the manuscript.

- Line 217: the reference to the figure breaks up the sentence, move to the end of that sentence.

Response: We have moved the reference to the figure to the end of the sentence to improve sentence flow and readability.

- Line 219: Instead of saying 'low spatial heterogeneity', say 'with equal distribution at regional level across the country'

Response: We have replaced "low spatial heterogeneity" with "equal distribution at the regional level across the country" for greater clarity.

- Line 227: "infections" instead of "infection"

Response: We have corrected "infection" to "infections" as suggested.

- Line 230: add a word linking "Gambela in the southwest" and "7.6% ([95% CI 5.2-8.4%])"

Response: Thank you for your suggestion. We have revised the sentence to improve clarity by adding a linking word. The revised sentence now reads: All regions had high prevalence, with the exception of Gambela in the southwest, which had a prevalence of 7.6% ([95% CI 5.2-8.4%]).

- Line 251: Add verb: "The lowest average IBD sharing was observed at Abol health center, Gambella Region".

Response: Thank you for your suggestion. We have revised the sentence as follows: The lowest average IBD sharing was observed at Abol Health Center, Gambella Region.

- Line 252-255: check this sentence

Response: We have revised the sentence as follows: We then conducted relatedness network analysis to assess where haplotypes sharing IBD above the specified threshold clustered by geographic region or sample origin. We found clear clustering of highly related parasite pairs ($IBD \geq 0.90$) within health centers, suggesting inbreeding and clonal transmission at a local scale (**Fig. 5C**).

- Line 260: some words seem missing in this sentence

Response: We have revised the sentence to address the missing words and improve clarity.

- Line 300: correct to "The K13 441L mutation was found"

Response: We have corrected the sentence to "The K13 441L mutation was found" as requested.

- Line 310: The haplotype is or the haplotypes are

Response: We have corrected the sentence to use "haplotypes" in plural form, as the context refers to multiple haplotypes.

- Line 388: remove bracket

Response: We have removed the bracket as requested.

- Line 411-413: check the grammar of this sentence

Response: Thank you for your feedback. We have corrected the grammar of the sentence as follows: Variants with a unique UMI count of 3 or greater were used to estimate the prevalence of key and known drug resistance mutations associated with different forms of antimalarial resistance, using the mipicorn package in R 4.2.1 (<https://github.com/bailey-lab/mipicorn>) and a custom R script.

- Line 426: correct "infectious" to "infection"

Response: We have corrected "infectious" to "infection" as requested.

- Line 434: Correct to "visualized using ggplot2"

Response: We have corrected the sentence to "visualized using ggplot2" as requested.

- Line 445: correct to "or geographic origin (health facilities) were created "

Response: We have corrected the sentence to "or geographic origin (health facilities) were created" as requested.

- Line 452-454: check sentence

Response: Thank you for your feedback. We have revised the sentence as follows: The dataset includes de-identified clinical information, a data dictionary, and drug resistance genotypes, all of which are available in the supplementary tables."

2. Figures and Tables

- Figure 2C: Indicate the number of samples included in the network analysis.

Response: Thank you for your suggestion; the figure legend in **Figure 2C** and other figures has been updated to specify the number of samples included in each network analysis, providing clarity on the sample sizes for each analysis.

- Figure 3: Please report somewhere how many/what proportion of samples have calls at all positions.

Response: We have now included the information regarding how many and what proportion of samples have calls at all positions for the haplotype upset plotting. This information is reported in the figure legend for clarity.

- Figure 5C: Consider using color coding by region or district rather than health center to enhance clarity.

Response: We have elected to retain the original color coding because the main aim of this analysis is to better understand the fine-scale spatial heterogeneity of parasite relatedness and connectivity, and focusing on the health facility level makes more sense for this purpose. Additionally, in Ethiopia, there is significant variation in malaria transmission dynamics within regions, so regional data may not accurately reflect local transmission patterns. Since we have precise latitude and longitude coordinates at the health facility level, we decided that the health facility network would provide the most meaningful analysis.

- Table S3: Verify the reported genotype L in crt position 73 resulting in haplotype (CLIET at positions 72-76) and correct if necessary.

Response: Thank you for catching this typo. The reported genotype was incorrectly listed as L. Thus the predominant haplotype is (CVIET at positions 72-76). The revised version features both the corrected haplotype and a detailed spatial distribution analysis (**supplementary Figure 11C**), providing further support for our findings.

- Figure S1: does this figure show only included samples? Cause there seem to still be samples with low depth across many MiPs probes. It might help to show also how this looks for the excluded samples, and/or to quantify 'low' and 'high'. (as low might still be sufficient for analysis of course).

Response: Yes, Figure S1 (**Supplementary Figure 2A**) displays only the samples that were included after the filtering process. We acknowledge that there are still samples with low depth across many MiPs probes, and we understand that the clarity regarding "low" and "high" depth is important for the interpretation of our results. We also added a detailed description of the filtering criteria and thresholds used for the inclusion of samples to the Methods section in the manuscript, to help clarify the rationale behind which samples were included or excluded. **Supplementary Figure 2B** has been added, which

shows the SNP density plot. This plot illustrates the distribution of retained SNPs, giving a clearer view of the depth distribution across the samples.

3. Additional References and Data Access

- Ensure that all programs and packages (e.g., The Real McCOIL, SNPRelate) used in analyses are appropriately cited.

Response: Thank you for your helpful suggestion. We have carefully reviewed the manuscript and ensured that all programs and packages used in our analyses, including *The Real McCOIL* and *SNPRelate*, are now properly cited in the text. These citations have been added to the relevant sections to ensure full transparency and proper attribution.

- Include individual sample accession numbers in Table S2 to support data sharing.

Response: We have addressed this by ensuring that all accession numbers for individual samples will be made available alongside the raw sequence data upon publication.

- Add details on sequencing performance (e.g., target and achieved depth) and the variability in probe success rates.

Response: We calculated the mean coverage, standard deviation (SD), and coefficient of variation (CV) for each probe targeting five key drug resistance genes (*crt*, *dhfr*, *dhps*, *k13*, and *mdr1*). The minimum target coverage was set at 10x, with only five probes failing to meet this threshold, while the majority of probes achieved a coverage of 100x. The mean coverage reflects the achieved sequencing depth, while SD and CV provide insights into the variability and consistency in probe coverage across samples. Probes were categorized into coverage ranges (<10x, 10-50x, 50-100x, >100x) to evaluate their performance, and high SD and CV values were used to identify probes with variable success rates, which may indicate differences in probe efficiency or sequencing bias. These metrics offer a comprehensive view of sequencing performance and probe variability, and the results are presented in **supplementary Tables 1** and **Supplementary Figure 3** and included revised manuscript in **line 194-198**.

- Line 218: the confidence interval for the triple mutant (61.6%) is missing

Response: We have added the missing confidence interval for the triple mutant, which is now presented as [95% CI 58.6-65.4%].

- Line 283: Include references to prior studies cited in the discussion (e.g., comparisons of R622I prevalence).

Response: We have now included references to prior studies in the discussion, particularly regarding the comparisons of R622I prevalence, to provide proper context and support for our findings.

- Line 283-285: "Here we see a prevalence of 622I up to 53.7% in Andansa Health Centre within the Amhara region, as compared to 10% in 20185 and 2.4% in 201434."

o Are these proportions from the same site? It does not appear to me that this health centre was sampled in those 2 studies.

Response: You are correct that the prior studies (2018 and 2014) did not include data from Andansa Health Centre specifically. The proportions mentioned (10% in 2018 and 2.4% in 2014) were reported from studies conducted within the same region, Amhara, where the 622I mutation was first identified. Although these studies did not sample Andansa Health Centre, we included these figures for comparison to highlight regional trends in the prevalence of 622I. The prevalence we report in Andansa Health Centre (53.7%) could reflect the influence of more recent malaria control interventions in this particular health centre, which may differ from those in previous studies.

- Line 337: You do not necessarily need whole genome sequencing to do this. For example in SEA this was initially done with MS “microsatellite) markers, as at that time WGS was not easily implemented on field samples.

Response: You are correct that whole genome sequencing (WGS) is not the only approach for investigating the origin of mutations. Because our study did not utilize microsatellite data, and since our analysis relies on MIP genotype data, comparing it directly with microsatellite-based studies would be challenging. To clarify, we cannot confirm whether the haplotype carrying the K13 675V mutation is imported or locally emerged due to the lack of publicly available WGS data specifically for this mutation from East Africa. We have revised the manuscript (in line 561-564) to reflect that while microsatellite markers could provide useful insights, our current data and methodology do not allow for such comparisons.

- Line 339: Clinical resistance and in vitro efficacy are two different things. One is related to treatment failure. The other to parasite survival in the presence of drugs. You indeed have information on neither in this population at this time.

Response: Thank you for raising this important point. You are correct in pointing out that clinical resistance and in vitro efficacy are distinct concepts, with clinical resistance related to treatment failure and in vitro efficacy concerning parasite survival in the presence of drugs. We appreciate the clarification and have revised the manuscript (in lines 572-574) to accurately reflect this distinction. Additionally, we acknowledge that we do not have data on either clinical resistance or in vitro efficacy in this population.

- Line 382-384: add a reference for the drug resistance MIPs panel.

Response: We have now added the appropriate reference for the drug resistance MIPs panel in lines 382-384, as requested.

- Line 389: “the same MIP panel”: please clarify, The drug resistant one, or both?

Response: To clarify, when we refer to “the same MIP panel,” we are specifically referring to the drug resistance MIP panel. We have revised the text accordingly to avoid any confusion.

- Line 406-408: How many SNPs did you retain before and after this step? How many samples (%) were removed with less than 50% of SNPs?

Response: We have now included the number of SNPs retained before and after the filtering step, as well as the number and percentage of samples that were removed due to having less than 50% of SNPs. Additionally, we have added a SNP density plot (**Supplementary Figure 2B**) to visually represent the distribution of the retained SNPs.

- Line 411: before you mentioned a threshold UMI of 10, but here you mention 3; please clarify the different thresholds used and which data and analysis this impacted.

Response: You are correct that there is a need for clarity regarding the UMI thresholds. The threshold of 10 UMIs across the entire population was used to filter for high-confidence variants to reduce false positives. However, for variants related to drug resistance, we allowed a lower threshold of 3 unique UMIs to ensure that we captured relevant mutations even when the allele was present at lower frequencies. We have revised the manuscript (see line 222-225) to clearly distinguish between these two thresholds and their impact on the data and analysis.

- Line 431: what settings did you use for the COI analysis?

Response: For the COI analysis, we used filtered VCF files to generate genotypes and used genotypes as the input data. The analysis was performed using the default settings of the categorical method in *The Real McCOIL*. We have added these details to the Methods section for clarity.

- Line 446: Please add more details how you did this: “We then assessed IBD sharing at the regional and local (health...”

Response: We have added more details on how IBD sharing was assessed at both the regional and local levels. Specifically, the data visualization was performed using the ggplot2 and sf libraries in R, along with additional packages such as rnaturalearth, ggspatial, and scatterpie for spatial and map-based visualizations. The map was generated using administrative, river, and lake shapefiles from the Natural Earth database. Pairwise IBD values were calculated for each sample and visualized using geom_sf and geom_curve. The IBD values for the individual sites were represented with color gradients, and the mean IBD values for each unique site were also generated. We focused on pairs with an IBD ≥ 0.90 for visualizing the genetic relatedness. These details have been added to the Methods section **in line 263-274** for clarity.

- The github contains the code for data visualization, not (yet) data analysis as claimed in line 456.

Response: The GitHub repository contains the code for both data analysis and data visualization, along with the respective input files used to generate the figures and tables in this paper. The repository is publicly accessible and can be found at https://github.com/Abefola/EPHI_SentinelSites_DR_project.git. We have checked for access issues and confirmed that it is available to anyone with this link.

Conclusion

This study provides valuable data on drug resistance markers in *P. falciparum* in Ethiopia and highlights the potential for genomic surveillance to guide malaria control strategies. However, the manuscript would benefit significantly from improvements in clarity, additional statistical analyses, and more robust methodological descriptions to enhance its impact and reproducibility.

Thank you for your constructive feedback. We appreciate your comments about the value of our study and have made improvements to enhance the manuscript's clarity, robustness, and reproducibility:

Reviewer #2 (Remarks to the Author):

The manuscript by Brhane et al. provides results from molecular surveillance of Plasmodium parasites in Ethiopia with a focus on mutations causing partial resistance to antimalarials of the artemisin class. The paper is well written and the conclusion that there is an increase in prevalence of relevant mutations in the Kelch13 gene is valid. It is important work and great to see that this setup is being established in Ethiopia. There is a lot of debate on the consequences of emerging drug resistance in Eastern Africa and this manuscript is providing important information.

The main limitation that I see, is a lack of efficacy data or references to efficacy studies. The clinical impact of the described mutations in Ethiopia is not clear. I would recommend to include data or references from recent TES studies performed in Ethiopia.

About a quarter of the samples were already collected in 2019, which is quite some time ago. It would be good to discuss, if this might have any impact on the result analysis and the conclusions of the manuscript.

Response to Reviewer Comment:

Response: Thank you for your thoughtful and encouraging comments on our manuscript. We greatly appreciate your comments about the importance of molecular surveillance of *Plasmodium* parasites in Ethiopia and the contribution it makes to understanding emerging resistance in the region.

Regarding the clinical impact of the mutations:

We agree with the reviewer and acknowledge the importance of efficacy data to understand the clinical impact of the mutations described in the manuscript. We reviewed recent therapeutic efficacy studies (TES) conducted in Ethiopia and have referenced relevant data that can provide context for the clinical significance of the mutations and **added in line 123-235 in revised manuscript**. These references have been added to the manuscript to better frame the implications of the mutations identified in our study. Furthermore, there is ongoing work (from our group and others) to better understand the clinical impact of different mutations, including the R622I mutation, in Ethiopia. While this is an important avenue of research, it is not part of the current manuscript.

On the inclusion of samples from 2019:

We have addressed this in the revised manuscript, noting that while the study includes data from a few years ago, the mutation frequencies observed remain relevant in the broader context of malaria control and resistance surveillance efforts in Ethiopia. We have clarified this point in the discussion, emphasizing the continuing importance of this data, even if it was collected several years ago. We acknowledge this as a limitation of the study, as newer data could provide a more up-to-date understanding of the current trends in resistance.

Some further suggestions for the authors:

-In line 127 it is mentioned that the sentinel site collect data on efficacy, however there is no reference to such data available in the entire manuscript. Even if patient level correlation of mutated parasites and treatment outcome is not available, the overall efficacy of ACT should be mentioned.

Response: Thank you for your insightful comments. We completely agree that the patient-level correlation between mutated parasites and treatment outcomes is important. However, while we utilized samples collected from the sentinel site, our study was not a Therapeutic Efficacy Study (TES). We have now clarified in the methods section how the sites were selected and how the survey was conducted. In response to your suggestion, we have included the overall efficacy of ACT in the country, referencing data from previous studies conducted at various sentinel sites. These additions and references have been incorporated into the revised manuscript.

-The start of the result section, line 136, is a bit odd. The paragraph should provide more details on patient population. The type of "filtering" performed on the samples needs to be clarified.

Response: We received similar feedback from Reviewer 1, and we have addressed the concern as follows: We have revised the results section to provide more clarity on the methods used, particularly regarding the filtering process. We have now included specific details about what data was filtered, how the filtering was performed, and the criteria used. This revision aims to improve the reader's understanding of the methodology and how it relates to the results. Additionally, we have made similar enhancements in other parts of the results section to improve clarity and readability.

Minor comments of editorial nature:

-Number of dried blood spots is 1999 in abstract, but 1199 in result section

Response: Thank you for catching this typo. You are correct that there was an inconsistency in the number of dried blood spots reported. The correct number is 1199. We have updated the abstract to reflect the correct number of samples as mentioned in the results section.

-sampling was done 2019 to 2023 and not as mentioned in the abstract 2019 to 2022

Response: We originally intended to highlight the years when the majority of samples were collected (2019-2022), but agree that the entire sampling period should be listed in the abstract. We have corrected the abstract to note that samples were collected from 2019 to 2023.

-Sentence starting in line 104 is not clear

Response: We have revised the sentence starting in line 104 for better clarity. The updated version reads: Despite Ethiopia's progress in malaria control over the past two decades, the country is experiencing an increase in malaria cases across various regions, due to several challenges.

-Sentence starting in row 324 is not clear

Response: The revised sentence now reads: "The majority of genotyped samples carried the MDR1 NFD haplotype. Additionally, most samples that harbored WHO-validated ArtR markers also carried the MDR1 NFD haplotype as a background mutation, which could potentially contribute to reduced susceptibility to lumefantrine, a key component of ACT." We also modified the term "ACT treatment failure" to "reduced susceptibility to lumefantrine" to more precisely describe the potential implications of the MDR1 NFD haplotype in the context of ACT efficacy.

-use potentially when mentioning lumefantrine resistance as there is no clear evidence at the moment (it is not done in discussion section)

Response: Thank you for your valuable feedback. We appreciate your suggestion to use "potentially" when discussing lumefantrine resistance, as there is currently no definitive evidence linking the MDR1 NFD haplotype to lumefantrine resistance. In response to your comment, we have revised the sentence in line 324 to better reflect this uncertainty. Additionally, we have clarified in the discussion section that, while the MDR1 NFD haplotype may be associated with reduced susceptibility to lumefantrine, further research is needed to identify definitive lumefantrine resistance markers, as this falls outside the scope of our current study.

Reviewer #3 (Remarks to the Author):

The manuscript by Brhane et al describes surveillance efforts to track artemisinin resistance markers at sentinel sites across Ethiopia using a combination of dried blood spots and molecular inversion probe sequencing. The authors focus on mutations within a series of known drug resistance genes including k13, mdr1, crt, dhfr and dhps and supplement this with a selection of genome wide SNPs.

The results are observational but of importance as they provide a crucial update on drug resistance within Ethiopia and identify the presence of WHO validated K13 mutants that had not previously been seen within the country. This is supported by a large number of samples from multiple sites and years with haplotypes constructed from SNPs spread across the genome. While WGS could provide additional resolution of the observed haplotypes the number of SNPs used are sufficient for the purpose of the study and appropriate given the use of dried blood spots for sample collection.

The figures are largely clear and informative, with a substantial amount of supporting material that provides additional depth to the results. The methods are clearly written, use appropriate techniques and provide enough detail that they could be replicated. The included statistics are straightforward but appropriate for the results that are described. The authors identify a number of key limitations of the study in the discussion including that it does not address whether mutations are emerging locally or imported from neighbouring countries. Have these haplotypes been explored in the substantial malariaGen Pf7 release (or prior)? While the exact same SNP sets may not be available there is likely to be overlap within

K13. International reporting, especially between countries sharing borders and substantial flow of people are key to understanding the spread of drug antimalarial drug resistance so this manuscript will be of most value when considered alongside comparable results from the region, Africa as a whole and the rest of the world.

Response: Thank you for your thoughtful feedback. We agree that whole-genome sequencing (WGS) and haplotype comparison would provide valuable additional resolution. This is an important aspect to consider in the broader context of drug resistance surveillance. However, we would like to clarify that WGS/ haplotypes, including those involving the 622I mutation, from locations outside of Ethiopia are not available in public databases, which limits our ability to compare these haplotypes directly with those from other regions.

Specific comments:

-How many samples were there? In the abstract it says 1999 (line 42) but in the results it states there were 1199 (line 136). This is likely a typo but should be corrected and subsequent numbers rechecked.

Response: Thank you for catching this error. We corrected this typo as noted in our response to Reviewer #2 above.

-The description of sample numbers in the opening results paragraph is confusing in the way it alternates between the filtered samples and total sample numbers. The breakdown by region is for the filtered numbers, while the breakdown by year is for the total. It would be better to highlight the total number of samples and their breakdown first and then present the breakdown of the filtered sample set. This is presented better in Table S1 and the authors may want to consider making this a main table and add in the successfully genotyped breakdown (for example for each cell presenting data as x / y (%) to concisely state the number of sequenced samples (x), total number (y) and the percentage sequenced (%)).

Response: Thank you for your valuable suggestion. We have revised the manuscript to ensure clarity by first presenting the total number of samples and their breakdown, followed by the breakdown for the filtered sample set as suggested. We agree Table S1 (now **Supplementary Table 2**) that provides a clearer presentation, and we have kept it as a supplementary table to maintain the flow of the main manuscript. Additionally, we have addressed the breakdown of successfully genotyped samples by presenting the data in the x/y (%) format, as you recommended (see **Supplementary Table 2**).

-For the P574L (**Figure S4B**) what was the p-value for the significant difference between wildtype and mutant? Please either add to the main text or the supplementary figure.

Response: We have added the p-value for this comparison to the main text of the manuscript.

-Were clustered R622L parasites in figure 2C found within the same regions or were related haplotypes observed between difference regions of the country? Given the generally low transmission levels (as shown by the COI estimates later in the manuscript) it would be expected that related parasites would arise from clonal transmission cycles within a local region.

Response: We have conducted additional analysis to address your question about the clustering of mutant parasites. Our results indicate that the majority of mutant clonal parasites were indeed clustered at the regional level, particularly within the Amhara region. Furthermore, we observed even more localized clustering at the district level, which suggests a rapid spread of mutant parasites within that region. We have included this new analysis and its corresponding results (see **Supplementary Figure 6A and B**) in the revised manuscript, providing additional context to the findings and addressing your concern regarding the potential local spread of mutant parasites.

-The majority of the MIP panel is newly designed but references an in preparation manuscript (Niare et al) by a lead author who is not named as an author on this study. It concerns me that the paper describing

and validating these new MIPs will probably not be published until after work using them. At the very least Niare (and possibly co-authors) should be credited as an author on this work as this will represent the first publication of the MIP sequences.

Response: Pre-print describing this work is now available ([doi: https://doi.org/10.1101/2025.03.07.25323597](https://doi.org/10.1101/2025.03.07.25323597)) and has been properly referenced

-Line 388 - Extra) that should be removed

Response: This was a typo, now corrected.

-I was unable to access the study data via the SRA or BioProject accession numbers. I assume they are embargoed prior to release but it should be checked that they have been released prior to the manuscript being published.

Response: Data was initially embargoed in SRA but has now been publicly released.

-The typed signature for the editorial checklist differs from the name of the lead author. This appears to be a typo (Berhan vs Brhane) but should be confirmed and corrected given the document relates to ethics and reproducibility.

Response: The discrepancy between the typed signature on the editorial checklist and the lead author's name was a typographical error. The correct name is Bokretsion G. Brhane, and we have updated the document to reflect this correction. This ensures accuracy and consistency, particularly concerning ethics and reproducibility. We appreciate your attention to detail and have made the necessary revision.

Response to Reviewers' Comments

Manuscript Title: *Rising Prevalence of Plasmodium falciparum Artemisinin Partial Resistance Mutations in Ethiopia*

We thank the reviewers for their valuable feedback, which has helped improve the clarity, accuracy, and presentation of our manuscript. Below, we provide detailed point-by-point responses to each comment.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

This study provides valuable data on drug resistance markers in *P. falciparum* in Ethiopia and highlights the potential for genomic surveillance to guide malaria control strategies. The authors have addressed the issues raised in the previous review with much care and the manuscript has much improved in detail and clarity.

A few minor comments: (line numbers refer to the version with tracked changes)

Line 275 – the word genome is duplicated

Response: Thank you for spotting this. The duplication has been removed and the sentence revised for clarity (see Line 275).

Line 414 and Supplementary Fig. 11. – CVIET is now corrected from CLIET in the text and table S2, but in the text and supplementary figure there is now mention of CLMNK and CLMNT, should this be CVMNK and CVMNT? CVMNK is the wildtype haplotype (CQ sensitive).

Response: We appreciate this observation. The incorrect haplotypes "CLMNK" and "CLMNT" were typographical errors and have been corrected to **CVMNK** and **CVMNT**, respectively, in both the main text and Supplementary Figure 11 (see Line 414 and Supplementary Materials)

Line 401 – z?

Response: This appears to have been a stray character and has been removed (see Line 401)

Check abbreviations, sometimes use ArtR, sometimes ART-R

Response: We have standardized the abbreviation to **ART-R** (Artemisinin Resistance) throughout the manuscript and supplementary files for consistency and clarity.

Reviewer #2 (Remarks to the Author):

Thank you for providing the opportunity to review the revised manuscript. The manuscript looks good, please consider addressing the editorial comments below prior finalization.

1. The sentence starting in line 123 appears to be a duplication of content of the prior sentence

Response: Thank you for pointing this out. The duplicated sentence has been removed to eliminate redundancy.

2. In line 323 provide full name for IBD as it is mentioned here for the first time in result section

Response: We have added the full term “Identity-By-Descent (IBD)” at its first mention in the Results section.

3. In line 365 consider to list the 3 residues by sequence order, so that it is aligned with NFD haplotype referred to in the following sentences

Response: We agree and have reordered the amino acid residues accordingly to reflect the correct sequence consistent with the NFD haplotype.

Reviewer #3 (Remarks to the Author):

Following the first round of review by myself and others Brhane et al have made substantial revisions to the points raised, including a detailed response and updates to the manuscript itself which is now much stronger. I am happy to recommend the amended manuscript for publication, it provides a detailed and valuable description of the rising prevalence of artemisinin resistance in Ethiopia. Surveillance work such as this is essential for understanding the spread of drug resistance and collected with studies from across Africa and the rest of the world provide a valuable resource in fighting malaria.

Response: We sincerely thank Reviewer #3 for the positive and encouraging feedback. We are glad the revisions were effective in strengthening the manuscript and that the study was found to provide valuable insights into artemisinin resistance trends in Ethiopia.

We have also implemented all editorial suggestions and formatting requirements as outlined in the attached checklist and editorial request file.

Sincerely,

Mr. Brhane and co-authors