Author’s response to reviews

**Title:** Association of non-synonymous mutations in the Plasmodium falciparum kelch13 gene (Pf3D7_1343700) with parasite clearance rates after artemisinin-based treatments - a WWARN individual patient data meta-analysis

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**Author’s response to reviews:**

Reviewer 1

I would suggest that the authors include all the wild type amino acids when mentioning mutations so that the readers can immediately know which types of mutations they are (for example C580Y instead of 580Y) - like in Table 7. Also in Figures 3, 4.

This has been completed in the text and tables and added to figures 3 and 4.

That request could also apply to supplementary figures S3- S8. For the latter figures, the designation of the wild type amino acid to the codon number and the mutant amino acid, e.g. C 580Y, have been added to the legend of each supplementary figure S3- S8.

Be consistent with either US or UK spelling: for example in most places the authors used "parasitaemia, haemoglobin, haematocrit", whereas they also use "analyzed, neighboring" throughout the manuscript.

This has been completed; spelling has been changed to the US conventional spelling.

Page 3: Southern Yunnan Province, China - remove "Southern". The study sites are actually in western Yunnan.

This has been completed
Page 4, line 4-5: the statement that "these mutations are common in GMS" is true for three of the mutations, but not for M597I - since this mutation was not detected in the GMS (or at least not common in GMS). So reference 43 needs to be removed from here.

Thanks for the correction, that reference has been removed.

That sentence now reads

Exceptions include recent molecular reports of independent emergence of parasites that carry pfk13 propeller mutant alleles observed previously in the GMS in Guyana (C580Y) [39] and Rwanda (P574L and A675V) [40]. On p. 3, lines 22-25

Page 11, line 1: "Although a mutation"

On p. 11 beginning on line 1, this paragraph has been re-written to add that information. It now reads

Many of the parasites from the Thai Western Border (Site 20) shared an allele with a codon change at position 252 from glutamic acid to glutamine (E252Q). Parasites with this allele were observed in Study 1 (n=1/7, 14%), Study 4 (n=68/950, 7%) and study 16 (n=14/116, 12%). Parasites with this genotype were also observed in Shwe Kyin in 2011-2013 (study 16, site 16), where they comprised 10/74 (14%) of the isolates tested (Additional File 2: Table S2A) and this allele has been observed near the Myanmar-China border, as well [14]. In the relatively conserved stem domain (350-440), mutants were observed only transiently in parasites from Shwe Kyin and the Thai Western Border.

Changed to “Although mutations in the 5’ region of the pfk13 gene have been observed in the GMS {Wang, 2015 #10155}, only one, E252Q, has been associated with slow parasite clearance {Phyo, 2016 #8697}”.

Table 3, 4, 5: Specify the mutations: e.g., F446I.

This information has now been added in tables 3, 4 and 5.

Page 13: first line: > 5.5 hours (also make sure you either abbreviate hours as h or use hours throughout).
This has been assured.

Table 7: Is xPC1/2 stands for fold increase? If yes, revise the footnote (to be consistent with that in Table 3).

In both Table 3 and Table 7, this has been done.

In addition, in response to Reviewer 2, Page 6, line 1 query, the data sets for I543T with and without study 8 and P574L with and without Site ID 15 (Study ID 13) removed have been analyzed. The conclusion that these codons are strongly associated with slow clearance was verified without study 8 (I543T) or without study 15 (P574L)

Both outcomes are now entered in Table 3 and in Table 7 and additional explanation has been added to Supp figure S2 where the discrepancy between the WT half life/half-life of parasites in each of these sites is so obvious.

Page 15: Since P1/2 in different places seem to vary, what about the ratio of mutant K13 vs WT K13 in the two studies mentioned in Myanmar and Vietnam (given most analysis compared their ratios - e.g. Table 3)?

The description of how these anomalies were tested to determine whether their inclusion biased the conclusion on their effect on parasite clearance is on page 6, beginning on line 23 for both Tra Lang, Vietnam (site ID 23; study ID 8) and Pyin Oo Lwin, Myanmar (Site ID 15; Study ID 13). These outcomes and the explanations are included in the legends to tables 3 and 7 as described above.

The above information has now been added to the manuscript on page 15, beginning on line 3.

“At both sites, no significant differences were observed in PC1/2 between K13 wild type and K13 mutant isolates. The corresponding factor for change in PC1/2 in mutant isolates compared with wild type was estimated as 0.91 (0.74-1.12) p=0.367 for Vietnam, Tra Lang and 0.90 (0.73 – 1.10) p=0.274 for Myanmar, Pyin Oo Lwin. “ This has been added to the text on p.

Page 22: "northwestern" as one word.

Corrected
Page 23: The description of increased prevalence of certain mutations in Africa needs to include a follow-up sentence to indicate that these are from small sample sizes (e.g., 31, 26, 29), and thus may not represent the true scenarios.

Text added on p. 23, beginning on line 16:

The low prevalence of pfk13 mutant parasites in the African studies in our data set depends on relatively small numbers of parasites (e.g. 31, 26, 29). However, the generality of this observation is supported by extensive studies in many sites in Africa including some assessments of parasite clearance in vivo [37, 38, 62-64]....

Reviewer 2

Page 6, Line 1: The authors assumed a wild type if no mutation is reported. Please mention if the whole pfk13 was genotyped by sequencing and the whole DNA sequence was analysed rather than reported candidate point mutations. In Supplementary material 1, there was one study (No. 8) reported to have moderate risk of bias due to genotyping method. Was the analysis (Supp Fig S2) also done by excluding this study?

Yes, in Study 8, the authors assayed by TaqMan allelic discrimination and sequencing all 4 mutant positions known at that time to be associated with delayed clearance (Y493H, R539T, I543T, and C580Y). Numerous I543T carrying parasites were found, plus one C580Y. One possible explanation of the high median half-life of the ostensibly wild type parasites is that parasites with other propeller mutants were inadvertently included in the wild type group. However, there are no longer samples available to test this idea. This explanation is included in a box added to figure S2

These data are presented in supplementary figure S2 (labelled Tra Lang) and but we have not provided a figure excluding these data. In that study, only parasites carrying I543T were present in large enough numbers to be assessed.

On p. 21, beginning on line 6, in the risk of bias section, we have added.

ewe have now repeated a calculation for change in PC1/2 associated with mutation I543T after excluding study 8 (xPC1/2 = 2.8 (2.3-3.4) p<0.001 in univariable analysis and xPC1/2 = 2.8 (2.3-3.5) p<0.001 in multivariable analysis) and have been added to Tables 3 and 7 and further discussed in the Risk of Bias section.
Page 6, Line 4: The authors excluded mixed genotypes and the authors did not provide rational for exclusion and possibly provide evidence that mixed genotypes does not correlate with clearance rate.

The rationale for this exclusion was discussed in the supplementary material in the TRAC paper from which many of these data sets came,


“Because of the complex effect on phenotype, samples with heterozygosity at one or more positions were also excluded from genotype-phenotype analyses, as were samples with missingness at any of the kelch13 SNP positions”

In our meta-analysis all excluded isolates, except for 3 were from TRAC study, so the same rationale underpins the exclusion in this study.

Page 6, Line 20-25: Are the cut-off values random or based on previous findings? Please clarify.

The cutoff values refer either to biologically or statistically plausible results and have been used previously in


The process is described in the methods, p. 6, beginning on line 15

“In order to determine a PC1/2 threshold value that defined slow parasite clearance,.....”

This has now been added to the text.

In addition, in this paper, the process of selecting a cut-off for slow clearance was described briefly under the section “Defining the groups of slow and fast clearing parasites” on page 12 beginning on line 9

Page 17, Line 25: When PC1/2 was compared to microscopic positive on day 3 false positive (when parasitaemia is very high) and false negative (when the initial parasitaemia is low) was
observed. Was there qPCR data in any of the studies? It would be interesting to see whether qPCR, either Parasite Reduction Ratio at 48 hours (PRR48) after treatment or days 3 qPCR positivity, can reduce the false positive and the false negative respectively.

This is an interesting idea, but we did not have any qPCR data for these isolates, so could not have made the comparison.

Page 23, Line 56: remove }

Done

Page 24, Line 7: remove gap between multidrug- and resistant

Done