# PCR correction strategies for malaria drug trials: updates and clarifications

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# PCR correction strategies for malaria drug trials: updates and clarifications

*Ingrid Felger, Georges Snounou, Ian Hastings, Joerg J Moehrle, Hans-Peter Beck*

**Malaria drug trials conducted in endemic areas face a major challenge in their analysis because it is difficult to establish whether parasitaemia in blood samples collected after treatment indicate drug failure or a new infection acquired after treatment [A: Please confirm that the original meaning of this sentence has been retained]. It is therefore vital to reliably distinguish drug failures from new infections in order to obtain accurate estimates of drug failure rates. This distinction can be achieved for *Plasmodium falciparum* by comparing parasite genotypes obtained at the time of treatment (the baseline) and on the day of recurring parasitaemia. Such PCR correction is required to obtain accurate failure rates, even for new effective drugs. Despite the routine use of PCR correction in surveillance of drug resistance and in clinical drug trials, limitations inherent to the molecular genotyping methods have led some commentators [A: please clarify whether this refers to researchers/clinicians/health-care professionals etc.) to question the validity of current PCR correction strategies. Here we describe and discuss recent developments in these genotyping strategies, with a particular focus on method validation and limitations [A: please clarify whether this is referring to limitations of the genotyping strategies or to method validation limitations here]. Our aim is to update scientists from public and private bodies who are working on the development, deployment, and surveillance of new malaria drugs. We aim to promote discussion around these issues and argue for the adoption of improved standardised PCR correction methodologies.**

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## Introduction

Cure rates in clinical trials of antimalarial drugs are based on clinical assessments and microscopy analyses done during several weeks of post-treatment follow-up. These rates can be corrected by genotyping parasites in patient blood samples so that recrudescent infections (ie, those samples containing parasites that survived drug treatment) can be distinguished from new infections acquired after treatment. ~~In previous years, recrudescence typing and molecular correction methods, such as PCR correction, have become integral parts~~ of malaria drug efficacy trials [A: Please confirm that this sentence is correct]. The PCR-corrected efficacy excludes all new infections from treatment failures and is essential when trials are conducted in areas of high malaria transmission with frequent new infections. Without using PCR correction methods in high-transmission areas, even a perfectly efficacious drug could have a high apparent failure rate, as new infections would be mistaken for drug failures.

The European Medicines Agency and WHO consider [A: Please clarify whether 'require' would be more appropriate] PCR-corrected cure rates and adequate clinical and parasitological responses as primary endpoints. These primary endpoints are now routinely reported in regulatory trials of new drugs [A: Please include appropriate references for these trials if possible]. However, the US Food and Drug Administration (FDA) only requires uncorrected cure rates as primary endpoints, and the FDA assessment of PCR-corrected rates is still pending. Nevertheless, there is a wide consensus that the use of the best available genotyping methodology, which follows critical and systematic validation [A: Please clarify whether this refers to the critical and systematic validation of the genotyping methodology. In which case, would '...the best available genotyping methodology, which has been critically and systematically validated' be more suitable?], would be highly relevant for regulatory trials of antimalarial drugs, as it would enable more precise estimation of drug efficacy.

PCR-corrected outcomes are also accepted endpoints for monitoring drug resistance. WHO widely implements PCR correction in the surveillance of drug efficacy in malaria-endemic areas and recommends changing first-line antimalarial therapy if the PCR-corrected failure rate exceeds 10%. Without PCR correction, this threshold would be reached in high-transmission areas even with highly efficient [A: would 'effective' be more appropriate here?] drugs because new infections would be mistaken as drug failures [A: is there a different failure rate threshold for non-PCR-corrected studies/surveillance strategies?]. Genotyping is performed on archived blood samples at a later point in time and does not inform or influence treatment of recurrent parasitaemia. Treatment is always given for all episodes of parasitaemia, either per protocol in clinical trials or according to local treatment guidelines in trials monitoring drug efficacy. Many laboratories in malaria-endemic countries already have the necessary equipment for genotyping. Therefore, molecular analyses can be performed routinely within these countries to monitor the effectiveness of first-line antimalarial drugs.

## The need for molecular correction

Molecular correction should [A: please confirm the change from 'will' to 'should' or clarify whether molecular correction is currently not used to guide the physician's decision] not be used to guide the decision of a physician on the treatment of a patient presenting with recurrent

malaria. Genotyping is done because it permits estimation of the therapeutic effectiveness of a particular drug in clearing existing infections, which is distinct from the prophylactic effectiveness of the drug in preventing new infections. Malaria control and elimination programmes require estimates of prophylaxis to predict their impact. The most likely end-users of drug efficacy trials are national programme managers who (assuming they follow WHO guidelines) are mandated to change their first-line antimalarial once failure rates exceed 10%. As uncorrected failure rates include new infections, these managers could find themselves in a position in which all available drugs are considered as failing using this metric, and no rational basis for drug deployment would remain. In addition, we argue that there is an ethical obligation to maximise the value of recruited participants in clinical trials of new antimalarial drugs. Molecular correction uses blood samples that have already been provided by participants at the time of recurrence and treatment, therefore, no further action is required from participants for molecular correction to occur. Although we acknowledge the differing opinions on how best to utilise molecular correction, our strongly held personal view is that to wilfully ignore molecular correction is, at best, a missed opportunity to understand malaria treatment, prophylaxis, and resistance and, at worst, a liability for effective public provision. **[A: Please ensure that suitable references have been cited in the 'Introduction' and 'The need for molecular correction' subsections, particularly those that support the agency requirements mentioned].**

## Principles of PCR correction for *Plasmodium falciparum* infection [A: Please confirm this addition, as only *P falciparum* has been discussed in this article]

Multiclonal infections are characterised by the concurrent persistence of genetically distinct parasite clones over long periods of time.[1] Cases of multiclonal *P falciparum* infections are frequent **[A: do you mean that a high proportion of individuals infected with *P falciparum* present with multiclonal disease?]** and ~~multiclonal disease is~~ a hallmark of *P falciparum* epidemiology. The number of concurrent infections in a host (multiplicity of infection) depends on transmission intensity, acquired immunity, as well as other host factors. Individuals in regions of high malaria transmission harbour an average of approximately five parasite clones, whereas patients in regions of intermediate or low malaria transmission harbour one or two parasite clones.[2,3] High-length, polymorphic *P falciparum* genes **[A: please confirm this change from 'high length-polymorphic']** can be used to differentiate these coinfecting clones.

Three molecular markers are routinely genotyped to distinguish new infections from recrudescent infections in clinical trials of antimalarial drugs: the merozoite surface proteins (*msp*) 1 and 2 and glutamate-rich protein (*glurp*). In 2007 a group of experts convened by WHO and the Medicines for Malaria Venture (MMV), released recommendations for genotyping protocols and presented a consensus for the analysis of genotyping data and for classifying outcomes.[4] The recommendations stated that any recurrent microscopy-positive parasitaemia apparent 7 or more days after treatment (day X) should be genotyped and compared with the baseline sample (day 0). Recrudescence was **[A: please confirm that this is still referring to the released recommendations for classifying outcomes]** defined as a genotype that had already been detected in the blood sample taken before treatment (ie, the same alleles are present **[A: please confirm this change from 'alleles are shared']** at day 0 and day X at all three loci). A new infection was defined as the absence of a shared allele between day 0 and day X at any of the three loci. Molecular correction strategies enable statistical estimation of drug efficacy by survival analysis or when using the WHO per-protocol method to censor new infections.[5]

Inherent limitations of currently used genotyping techniques, together with a reluctance of laboratories **[A: Please use an alternative word instead of 'laboratories' here, eg, 'clinicians' or 'researchers']** to adopt more precise methods, have prompted caution in adopting PCR correction as an endpoint.[6,7] There are two major technical drawbacks of PCR correction: first a difficulty in detecting all parasite clones present in a blood sample, particularly the so-called minority clones; and second the limited discriminatory power of gel electrophoresis in distinguishing PCR fragments of similar sizes.[6]

## Biological and epidemiological limitations of the WHO-MMV-recommended method of PCR correction

### Biological constraints resulting in undetected *P falciparum* clones [A: Please confirm this addition]

Parasite clones can occasionally remain undetected by PCR despite being present in the host; an observation known as imperfect detectability. Imperfect detectability is caused by sequestration of the *P falciparum* parasite in its late developmental stages **[A: Please confirm that the meaning of this statement has been retained]** (a biological characteristic of this species) and due to naturally acquired immunity **[A: of the host to the parasite?]**, which leads to fluctuations in parasite concentrations that can be below the PCR detection limit **[A: please confirm that the original meaning has been retained]**. For example, clone detectability in a host has been estimated at 79% on the basis of samples collected 24 h apart.[8] In addition, daily samples collected from infected children over 14 days showed a 48-h periodicity for some clones.[9] Thus, the near-complete sequestration of late-stage parasites can prevent the detection of some clones. One way to improve detectability is by taking blood samples on consecutive days. The effect of analysing consecutive samples (day 0 + day 1 and day X + [day X + 1][**A: OK to add parentheses?**]) was evaluated[10] and shown to significantly

increase detection of recrudescence [A: Please confirm the addition of 'detection']. Nevertheless, following a lengthy discussion of the practicalities of genotyping two blood samples taken 24 h apart, the WHO-MMV consultation of experts[4] did not recommend including these extra samples. From an operational aspect, genotyping two blood samples was considered impracticable and not feasible by sponsors, as it would require detaining patients for 24 h after treatment or asking that they return the following day when they might still be symptomatic. Moreover, there is invariably an ethical obligation to provide immediate treatment with fast-acting artemisinin combination therapies (ACTs), where the parasites disappear [A: Perhaps consider: 'so that parasites are eradicated'] 8 h after ACT treatment. Therefore, the likelihood of detecting minority clones [A: Please confirm change to 'minority' from 'minor'?] at day 1 and beyond becomes very small. In addition, the likelihood of detecting any clones that are sequestered when the infected individual is admitted would be reduced, and detection would be even more unlikely for samples collected on day 2 [A: please confirm that the meaning of this sentence has been retained]. For single-exposure cures that are currently under development [A: If possible, please consider including references to relevant previously published studies about single-exposure cures], there would be great difficulties in obtaining PCR samples beyond day 0. [A: Do you mean that detection of clones would be difficult beyond day 0 for single-exposure cures, or that obtaining samples from individuals given single-exposure cures would be difficult?]

### Effect of long follow-up periods and local transmission intensities: the need to genotype several markers

Generally, the longer the follow-up period, the more likely patients infected with malaria are to acquire new infections [A: Please confirm that this sentence retains its original meaning]. Therefore, the need for PCR correction to distinguish new infections from recrudescence [A: please confirm the addition of this statement] becomes increasingly important. The follow-up period for patients treated with drugs that have a long half-life can be up to 63 days. New infections accumulating during that period can contain, purely by chance, an allele identical to that present at baseline and could be falsely classified as recrudescence [A: please confirm this change from 'This mimics recrudescence']. To prevent this outcome, genotyping of more markers is crucial.

Even when three markers are analysed, extremes [A: Please clarify what is meant by 'extremes' in this context] in both low and high malaria transmission regions can cause problems for PCR correction. In areas of intermediate or high malaria transmission, a genotype can reach an allelic frequency of 16%.[3] However, in regions of low malaria transmission, the parasite population is genetically less diverse and the frequency of the most abundant allele can reach 25%. Consequently, independent infections can share the same genotype by chance. In the context of a drug trial, new infections carrying the same genotype by chance might therefore be misclassified as recrudescence, which can lead to overestimation of drug failure.[11,12] To prevent this error caused by limited genetic diversity in molecular markers, WHO recommendations suggest genotyping three markers to confirm recrudescence [A: Please clarify whether this could be changed to 'a minimum of three markers'?].[4] In areas of known low endemicity, or situations in which PCR-corrected failure rates exceed 10%, baseline frequencies should be determined from a representative set of admission samples to statistically calculate the likelihood of misclassifications. However, despite a multimarker strategy, genotyping methods will be limited by lack of discriminatory power in areas with an almost clonal parasite population structure. In the past [A: Please consider including a defined time-period and a suitable reference(s)], conducting trials in regions of diverse transmission intensities was considered important; however, from a genotyping perspective, conducting trials in regions with intermediate transmission [A: changed from 'moderate' for consistency.] regions would be ideal.

### The dangers of low-level genetic signals after treatment: gametocytes, and dying and dead asexual parasites

Early gametocyte stages are susceptible to certain antimalarial drugs, but only 8-aminoquinolines (ie, primaquine and tafenoquine)[13] are effective against circulating stage 5 gametocytes. Most currently available drugs (ACTs) are given without 8-aminoquinolines (although this may change [A: Please consider expanding on this statement and explain why this may change]). Therefore, mature gametocytes can persist for a few weeks after asexual forms have been cleared.[14,15] Gametocytes are detectable by PCR, which can lead to incorrect classification as treatment failures. The WHO-MMV consultation recognises this limitation and recommends that genotyping should only be performed if asexual parasites are observed by microscopy.[4] [A: Please confirm movement of reference 4 to here] The persistence of gametocytes alone is not considered a criterion for treatment failure. Results from simulations[16] support this recommendation by suggesting that genetic signals from gametocytes will have a negligible impact on PCR correction, provided that only those patients who are positive for asexual parasites by microscopy at day X are genotyped. Similarly, ring-specific transcripts have been observed up to 14 days after ACT treatment.[15] In addition, DNA from parasites persisting at very low levels, or potentially from dead parasites, can sometimes be detected by PCR weeks after clearance.[17] Similar to gametocytes, DNA from low levels of persistent parasites produce minor genetic signals [A: Please confirm change from 'these are low-level genetic signals'] that should be

negligible (see below) in patients whose asexual parasitaemia is patent by microscopy. These observations highlight the importance of performing genotyping analysis only in patients with microscopy-positive infections.

## Technical genotyping limitations of the WHO-MMV-recommended method of PCR correction

### Allelic suppression and the detection limit of minority clones

Systematic investigation of technical limitations has revealed that PCR template competition during amplification (known as allelic suppression) contributes to the imperfect detectability of individual clones.[9,18–21] The detection probability of a genotype depends on its fragment length and the ratio of dominant-to-minority clones, with shorter fragments more efficiently amplified during PCR.[21] Amplification bias, and thus the limited detection of minority clones, has been observed in previous studies.[19,22] However the extent and consequences amplification bias were largely ignored, as quantification of such effects requires systematic analysis of mixed culture strains. For *msp1* and *msp2,* a reciprocal dilution series of two precisely quantified strains in increasingly discrepant proportions indicated that, when a minority clone fragment was longer than the dominant clone fragment, it was detectable up to a dilution **[A: Correct to add here?]** ratio of 1:5, but not at increasingly discrepant ratios. If the minority clone carried a shorter allele (in the reciprocal experiment) it was still detectable at ratios of 1:500 to 1:1000.[21] Importantly, if two genotypes of the same marker belonged to different allelic families and the families were amplified in different reactions, template competition was removed and the detection of minority clones was possible to the lowest tested ratio of 1:5000.[21] Such excellent sensitivity in detecting minority clones has not been achieved using methods other than allele-specific PCR.

### Sequential genotyping of three markers and the questionable suitability of the glurp marker

The sequential genotyping strategy recommended by WHO-MMV[4] has some drawbacks **[A: Do you mean 'problems', 'disadvantages' or 'limitations' here?]**. The strategy recommends the genotyping of no additional markers if the results for one marker indicates the presence of a new infection on day X. Consequently, if genotyping of the first marker leads to the erroneous identification of a new infection due to a technical fault **[A: please clarify how this could occur due to a technical fault]**, no additional genotyping result would question that outcome. Previously, many laboratories adopted *glurp* as the first marker in sequential genotyping because of its technical simplicity—ie, only one nPCR reaction **[A: please clarify whether this is referring to 'nested PCR'. Please also include a suitable reference(s) to support this statement]** was necessary. However, using *glurp* as the first genotyping marker could be problematic because *glurp* has the greatest propensity for amplification bias among all of the possible markers **[A: Please confirm that this sentence retains its original meaning following this addition (in red)]**.[21] The large size differences between *glurp* fragments lead to preferential amplification of shorter fragments and a loss of larger fragments. A previous study found that a two-fold overrepresentation of the clone with the shortest allele was sufficient to suppress detection of three larger fragments.[21] The lack of detection of genotypes **[A: please confirm this change]** at baseline can lead to an overestimation of new infections, which consequently results in an underestimation of treatment failure rates. Despite its high genetic polymorphism, *glurp* might not be a useful genotyping marker for *P falciparum* infections **[A: please confirm that this sentence retains its original meaning]**. However, the majority of individuals in trials conducted in regions with a very low multiplicity of infection will have single-clone infections, therefore, clone competition would be largely absent. In cases such as these, the large genetic diversity of *glurp* could make it a useful third marker to increase discriminative power.

## Improving on the current WHO-MMV method: corrective actions to minimise the impact of genotyping limitations

### Maximising the precision of fragment sizing

Substantial efforts have been made to overcome the aforementioned limitations and improve genotyping methodologies. One key strategy has been to measure the precise size of length-polymorphic markers by capillary electrophoresis (CE), which has become standard practice in many laboratories.[11,23,24] CE is vastly superior to fragment sizing by gel electrophoresis, which has a limited resolution to discriminate between similar-sized alleles, for which the unequal loading of PCR products can strongly impair correct sizing. CE has excellent resolution and can be considered highly robust if carefully determined cutoffs eliminate stutter peaks. However, some laboratories still use gels, as this technique is technically simpler and does not require access to an automated sequencer.

### New algorithms for the analysis of genotyping data

An increased appreciation of the technical limitations of PCR correction has led to suggested changes to the methods used to analyse genotyping data **[A: please confirm that this sentence retains its original meaning]**. One such revision (herein referred to as the 2/3 approach), demands concordant results from at least two markers for classification of a new infection or recrudescence **[A: please provide a reference(s) to this approach if possible]**. If discordant, a third marker, which could either be an established microsatellite marker[25–27] or *glurp,* should be genotyped. A previous study compared two new

approaches for interpreting genotyping data from PCR correction with the standard WHO-MMV recommendations in a small set of 44 paired samples.[21] The results revealed substantial differences in outcomes **[A: Please include more information about the observed differences]**. Reanalysis of the results from a larger clinical trial conducted in Rwanda using simulation models of different analysis approaches, indicated a two-fold difference in how frequently these methods identify cases of recrudescence.[16]

Modelling provides a new approach to validate potential algorithms for interpreting molecular data. Pharmacological models can simulate a population of patients in a drug efficacy trial, their therapeutic outcomes, and the genotyping results that could occur at day 0 and day X.[16,28] A previous study compared the PCR-corrected failure rates using simulation models of several molecular correction approaches.[16] The 2/3 approach, for which *glurp* was used only if *msp1* and *msp2* results were discordant, provided the best fit with the simulated treatment failure rates when compared with the WHO-MMV and non-PCR-corrected strategies **[A: Please note that figure 1 has been removed from this article, as it is similar to figure 2 from Jones et al (2019)[16] and more information about how this figure was derived is available in the published article]**. Adopting the 2/3 approach instead of the current WHO-recommended sequential typing method gave higher failure rate estimates that were closer to the theoretical true failure rate.[16]

The current WHO-MMV method and new algorithms under investigation,[16] seek to define recurrent infections as either a new infection or drug failure. In reality, the results are often uncertain, and a method that incorporates this uncertainty could lead to improved efficacy estimates. A Bayesian algorithm has been developed to adjust **[A: would 'correct' be more suitable instead of 'adjust' here?]** drug efficacy results for length of microsatellite PCR products and the population frequency of each genotype detected in paired samples,[29] thus permitting an estimation of the misclassification and allelic suppression probabilities **[A: Please confirm that this sentence retains its original meaning]**. This highly promising new approach considers the uncertainty around the classification of new infection and recrudescence.

## Possible use of alternative genotyping techniques

There are several developments that show great potential for improving the genotyping of multiclonal *P falciparum* infections. One suggested strategy is to identify regions less than 500 base pairs in length that are rich in single-nucleotide polymorphisms (SNPs), which can be genotyped by next generation sequencing (NGS).[30–34] SNP-based genotyping has improved abilities to detect minority clones **[A: Please clarify, improved compared with which method/technique?]**. Using this method, low-abundance *P falciparum* clones can be detected at a ~~dilution~~ ratio of 1:1000 in mixed infections.[33] In addition, NGS can quantify the relative abundance of concurrent clones in a host.[30,32,33] Molecular inversion probes can be useful for highly multiplexed targeted sequencing.[34] However, NGS-based genotyping has not yet been validated for molecular correction in clinical trials. Nevertheless, its greatly improved sensitivity **[A: please clarify: improved sensitivity compared with what method/technique? Would 'high sensitivity' be appropriate?]** to detect minor clones indicates an urgent need to do so.

A second technique involves genotyping a large number of SNPs distributed over the entire genome to generate a molecular barcode.[35] Using allele-specific probes and high-resolution melting curve analyses, individual quantitative PCRs are performed for all SNPs. Multiclonal infections yield mostly mixed signals, and haplotypes for concurrent clones cannot be established for samples of high multiplicity. Molecular barcodes could be suitable for clonal infections in low malaria transmission areas; however, their use in high malaria transmission areas still needs to be validated.

Some of these new genotyping methods have the potential to improve the detection of minority clones and overcome allelic suppression. Validation of these methods in clinical trials should therefore have high priority. Regulatory trials in particular would benefit from state-of-the-art techniques that overcome the aforementioned technical limitations. However, the same biological constraints would remain, such as sequestration or the detection limits of minority clones. For surveillance trials, optimised protocols that use established genotyping techniques could be implemented more easily than NGS-based methods in laboratories located in malaria-endemic countries.

## Steps forward and conclusions

The community of technical experts, trial investigators, regulators, and policy makers should consider the rapid adoption of new consensual protocols for genotyping in regulatory malaria drug trials, with harmonised laboratory procedures and data analyses. In particular, obtaining FDA validation of PCR correction in drug efficacy trials requires a demonstrably robust genotyping strategy that can generate reproducible results. Although the intrinsic biological constraints of malaria parasite biology on PCR correction strategies cannot be resolved **[A: Has the meaning of this statement been retained? Consider changing 'cannot be resolved' to 'remain unresolved'?]**, all technical issues should be addressed together by laboratories involved in recrudescence typing. We propose the adoption of four technical procedures. First, PCR reactions for each allelic family (*msp1* and *msp2*) should be separate rather than multiplexed, which will improve the detection of minority clones **[A: Please confirm that this sentence retains its original meaning]**. Second, the use of CE by

automated sequencing should be combined with fluorescently-labelled primers for better accuracy of fragment sizing. Third, *msp1* and *msp2* markers should be used to provide the minimal essential data for PCR correction. If both markers give congruent results (either recrudescence or a new infection), no further marker needs to be typed. However, one additional marker should be analysed (either *glurp* or new marker) when there are discrepant results. If the 2/3 approach is not possible, the result should be classified as a recrudescence—a stringent interpretation that avoids underestimation of treatment failures. During the transition phase, both the 2/3 approach and previous WHO-MMV algorithm should be used for comparing compatibility with previous studies and to build an evidence base that documents the differences between algorithms ~~as~~ estimated failure rates. Finally, *glurp* could still be a valuable marker in regions of low malaria endemicity, where it can be expected that only one allele is present per sample and that no allelic suppression occurs.

PCR correction is necessary to obtain accurate measures of outcomes in malaria clinical trials. Although it is subject to biological and technical limitations, the basic methodology has consistently provided better estimates of drug efficacy in clinical trials than non-PCR-corrected outcomes. Considering technical developments and improvements in our understanding since the publication of the WHO-MMV consensus protocols in 2008, it is important that these protocol recommendations are updated. Another technical meeting should be convened, particularly to address the methodological requirements for drug registration trials, which are more demanding than studies monitoring drug resistance. Regulatory trials require precise methodologies and should be implemented as state-of-the-art **[A: The meaning of this statement is unclear. Do you mean that the genotyping protocols used for regulatory trials should use the most advanced methods, as they are required to be precise?]**. This article will serve as a basis for discussion towards a revised consensus.

In addition to adopting the 2/3 approach, Bayesian methodologies should also be applied to incorporate the inherent uncertainty of genotyping. A consensus to implement strictly standardised PCR correction **[A: please confirm addition]** protocols should be reached. PCR correction should also become an acceptable and mandatory endpoint in both surveillance and regulatory trials of new antimalarial drugs.

#### Contributors

HPB and IF conceptualised the article and designed the typing methodology. IF wrote the original draft. IH validated genotyping approaches by modelling and edited the article. GS contributed to methodology, article writing, and editing. JJM critically revised the manuscript and contributed expertise in regulatory trials of antimalarials. All authors agreed with the submitted version.

#### Declaration of interests

We declare no competing interests.

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