Effectiveness of pyronaridine-artesunate against *Plasmodium malariae, Plasmodium ovale* spp, and mixed-*Plasmodium* infections: a post-hoc analysis of the CANTAM-Pyramax trial

Mirjam Groger, Gaston Tona Lutete, Ghyslain Mombo-Ngoma, Nsengi Y Ntamabyaliro, Gauthier Kahunu Mesia, Trésor Bodjick Mujobu, Lia Betty Dimessa Mbadinga, Rella Zoleko Manego, Diane Egger-Adam, Isabelle Borghini-Führer, Jangsik Shin, Robert Miller, Sarah Arbe-Barnes, Stephan Duparc, Michael Ramharter

Summary

**Background** High-quality evidence for the therapeutic efficacy and effectiveness of antimalarials for infections caused by *Plasmodium malariae, Plasmodium ovale* spp, and mixed-*Plasmodium* infections is scarce. In this study, we aimed to analyse the efficacy of pyronaridine–artesunate for the treatment of non-falciparum and mixed-species *Plasmodium* infections from a large phase 3b/4 clinical trial in central Africa.

**Methods** This post-hoc analysis was done in a random subset of samples from two sites (in the Democratic Republic of the Congo and in Gabon) of the CANTAM-Pyramax trial assessing pyronaridine-artesunate therapy. We randomly selected paired dried blood spot samples from day 0 and day 28 (or unforeseen visit) and assessed them by quantitative PCR for mixed *Plasmodium* infections or non-falciparum mono-infections. Day 28 (or unforeseen visit) samples positive for non-falciparum malaria were re-assessed by microscopy to identify microscopic versus submicroscopic infections. Analyses were done on two sample sets: a per-protocol set and an intention-to-treat set.

**Findings** Among 1502 randomly selected samples, 192 (12.8%) showed mixed-*Plasmodium* infections or non-falciparum mono-infections. We did not detect *P. vivax* in the samples. For both the per-protocol and intention-to-treat sets, the overall day 28 cure rates for *P. malariae, P. ovale curtisi*, and *P. ovale wallikeri* were 96–3% or higher (95% CIs from 81–0–99·9 to 95·7–100). Cure rates were consistently high in *P. malariae* (99·2%, 95·7–100) and *P. ovale* spp (97·9%, 88·7–99·9, for *P. ovale curtisi* and 96·3%, 81·0–99·9, for *P. ovale wallikeri*) infections.

**Interpretation** This post-hoc analysis provides important evidence supporting the high efficacy of pyronaridine–artesunate against mono-infections with *P. malariae, P. ovale curtisi*, or *P. ovale wallikeri* and mixed-*Plasmodium* infections in a real-world setting.

**Funding** Medicines for Malaria Venture.

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Infections have the potential to challenge progress of malaria control programmes. Although asymptomatic malaria frequently remains untreated in high-transmission settings, treatment of all Plasmodium infections is important to reduce morbidity and, especially in malaria-elimination settings, to interrupt transmission.

A fixed dose combination of pyronaridine and artesunate (Pyramax, Seoul, South Korea) is the only artemisinin-based combination therapy with positive assessments from stringent regulatory authorities for both the treatment of P falciparum and blood-stage P vivax malaria. For malaria control and malaria elimination programmes, efficacious antimalarial treatments for all Plasmodium species and mixed infection are indispensable. Generating more evidence for the treatment of infections with P malariae and P ovale spp is thus a much-needed undertaking.

For this post-hoc analysis, we analysed blood samples from the CANTAM-Pyramax real-world study, in which pyronaridine–artesunate was administered to patients with acute uncomplicated malaria, to provide a patient pool of considerable size from endemic countries with a known prevalence of P malariae and P ovale spp.

We aimed to assess the proportions of non-falciparum, non-vivax mono-infections and mixed Plasmodium infections and corresponding efficacy of pyronaridine–artesunate for infections with Plasmodium species and mixed infections to provide urgently needed evidence based on systematically collected data.

**Methods**

**Participants**

We did a post-hoc analysis assessing samples collected during the CANTAM-Pyramax trial (NCT03201770), which has been published in more detail elsewhere. In short, the CANTAM-Pyramax trial was an open label, non-randomised, phase 3b/4, cohort event monitoring study designed to evaluate, in a real-life setting, the safety, tolerability, and efficacy of pyronaridine–artesunate therapy administered daily over 3 consecutive days in patients with uncomplicated malaria. Patients were included on the basis of a positive rapid diagnostic test (RDT) result or malaria microscopy. The first dose was administered as directly observed treatment, and the subsequent two doses were taken unobserved by the participants. On day 7 (±1) and day 28 (±2), a trained community health worker visited the participants at home to monitor safety, assess compliance by collecting empty blisters and any leftover pills or sachets, and collect blood for malaria microscopy and molecular analyses. Participants were further encouraged to contact the community health worker in case of health concerns outside the scheduled visits. Participants could be retreated in the CANTAM-Pyramax trial for subsequent malaria episodes. Therefore, blood spots from multiple malaria events might be available for a participant.

**Procedures**

We did a retrospective real-time quantitative PCR (qPCR) analysis using dried blood spot (DBS) samples from the
two CANTAM-Pyramax African sites: the Centre Hospitalier du Mont-Amba in Kinshasa (Democratic Republic of the Congo [DR Congo]), representing an urban setting in a large metropolitan region of central Africa, and the Centre de Recherches Médicales de Lambaréné (CERMEL) in Lambaréné (Gabon),

representing a rural central African setting. These sites were selected as they had the highest recruitment rates in the CANTAM-Pyramax trial, represent different endemic settings (urban vs rural), and are both known regions of relevant prevalence of non-falciparum malaria. The CANTAM-Pyramax trial and the ancillary protocol covering this post-hoc analysis were approved by the responsible regulatory and ethics review bodies in DR Congo (reference CEUPC0048) and Gabon (number 041/2018/PR/SG/CNE), as applicable.

For this post-hoc analysis, a random subset of malaria episodes for whom paired samples from day 0 and day 28 (or day 0 and unforeseen visit between day 4 and day 28) were available was identified by generation of a random list by an independent statistician, with the aim of including 750 cases per study site.

Sample analyses
We analysed DBS samples by qPCR at the Department of Biomedical Sciences, Institute of Tropical Medicine (Antwerp, Belgium), to detect and differentiate *P falciparum*, *P malariae*, *P ovale curtisi*, *P ovale wallikeri*, and *P vivax* with previously published methods. Parasite counts by qPCR were species specific, and singleplex assays were used to individually identify *P falciparum* (varATS, limit of detection 0-05 parasites per μL), *P vivax* (Po-mtCOX1 qPCR, 1 parasite per μL), *P malariae* (Mal qPCR, 5 parasites per μL), and *P ovale* spp (Ova, 5 parasites per μL). Samples positive for *P ovale* spp were further analysed for *P ovale curtisi* and *P ovale wallikeri*. A patient was considered cured for the species of interest if the species was detected in the day 0 DBS and not detected in the day 28 DBS, irrespective of any other species being present in the day 28 DBS. Patients were considered not cured if the same species of interest was detected in a DBS taken on day 0, as well as in DBSs taken between day 4 and day 28.

When a mixed infection was present at day 0, the patient was considered for inclusion in the analysis of each species that was present in the day 0 sample, meaning that a patient could be allocated multiple times. *P falciparum* cure rates were not determined because no further qPCR analyses of pure *P falciparum* samples at day 28 were done.

Each cure rate is presented as the number and percentage of patients with exact Clopper Pearson 95% CIs overall, by species, and separately for each site. An additional summary of cure rates for the post-hoc per-protocol set is also provided.

Role of the funding source
The funder was involved in the conceptualisation, funding acquisition, methods, project administration, resources, supervision, validation, and review and editing of the manuscript.

Results
We included day 0 samples of 1502 malaria episodes in 1413 patients in this analysis; 750 malaria episodes in 715 patients from the DR Congo site and 752 malaria episodes in 698 patients from the Gabon site. Of these, 64 (9-0%) patients at the DR Congo site and 96 (13-8%) at the Gabon site (160 [11-3%] overall) had multiple episodes of malaria. On day 28, three samples were qPCR-positive for non-falciparum malaria at the DR Cong
Congo site and none at the Gabon site. There were no non-falciparum-positive samples from an unscheduled visit between days 0 and 28 on either site.

Overall, slightly more randomly assigned malaria events occurred in male participants than in female participants (table 1), which was similar between the two sites (405 [54·0%] events in male participants and 345 [46·0%] events in female participants in DR Congo, and 399 [53·1%] in male participants and 353 [46·9%] in female participants in Gabon). The overall median age in this randomly selected population was 10·0 years (SD 14·8) and was also similar at the DR Congo and Gabon sites (9·0 years [15·6] at the DR Congo site and 11·0 years [13·9] at the Gabon site). The majority of infections overall and at each of the two sites occurred in participants aged 5–12 years (634 [42·2%] overall, 314 [41·9%] at the DR Congo site, and 399 [53·0%] at the Gabon site). The overall median age in this randomly selected population was 10·0 years (SD 14·8) and was also similar at the DR Congo and Gabon sites (9·0 years [15·6] at the DR Congo site and 11·0 years [13·9] at the Gabon site). The majority of infections overall and at each of the two sites occurred in participants aged 5–12 years (634 [42·2%] overall, 314 [41·9%] at the DR Congo site, and 399 [53·0%] at the Gabon site). The overall median age in this randomly selected population was 10·0 years (SD 14·8) and was also similar at the DR Congo and Gabon sites (9·0 years [15·6] at the DR Congo site and 11·0 years [13·9] at the Gabon site).

Of the 1502 malaria episodes on day 0, 1237 [82·4%] were \textit{P} falciparum mono-infections, six [0·4%] were \textit{P} malariae mono-infections, three [0·2%] were \textit{P} ovale spp mono-infections, and 73 [4·9%] were negative (table 2). The overall mean parasite index was similar overall and at each of the two sites at the DR Congo and Gabon sites (17·4 kg/m², 4·1, at the DR Congo site; and 18·0 kg/m², 4·7, at the Gabon site).

Patients with a \textit{P} falciparum, \textit{P} malariae, \textit{P} ovale curtisi, or \textit{P} ovale wallikeri mono-infection all had headache and rigours or chills as the two most commonly reported
malaria symptoms on day 0. By contrast with patients with a *P falciparum* mono-infection, patients with a *P malariae*, *P ovale curtisi*, or *P ovale wallikeri* mono-infection did not show any events of sweating, jaundice, and haemoptoemegaly. Patients with mixed *Plasmodium* infections commonly reported symptoms of headache (124 [67·8·%] of 183 episodes), rigours or chills (92 [50·3·%]), cough (67 [36·6·%]), and loss of appetite or anorexia (61 [33·3·%]). Mean body temperatures at day 0 were similar overall and for patients infected with a *P falciparum* mono-infection, *P malariae* mono-infection, *P ovale curtisi* mono-infection, *P ovale wallikeri* mono-infection, or mixed-*Plasmodium* infection, ranging from 36·7°C to 37·2°C (SD 0·35 to 0·98). Detailed listings of signs and symptoms are shown in the appendix.

Overall, two samples on day 28 (both from DR Congo) had the same non-falciparum infection by qPCR as on day 0 and were further assessed by microscopy. In the microscopic reassessment, one slide was positive for *P falciparum* (qPCR result was *P falciparum* 9591 per μL, *P malariae* 6 per μL, *P falciparum* 9591 per μL, *P malariae* 6 per μL, *P falciparum* 9591 per μL, *P malariae* 6 per μL, with reappearance of *P malariae* with a *P falciparum* mono-infection, *P malariae* mono-infection, *P ovale curtisi* mono-infection, *P ovale wallikeri* mono-infection, or mixed-*Plasmodium* infection, ranging from 36·7°C to 37·2°C (SD 0·35 to 0·98). Detailed listings of signs and symptoms are shown in the appendix.

The cure rate at day 28 was defined on the basis of the post-hoc qPCR analysis separately for each *Plasmodium* species other than *P falciparum*. The day 28 cure rates in the post-hoc intention-to-treat analysis set for *P malariae*, *P ovale curtisi*, and *P ovale wallikeri* were 96·3·% or higher (95% CIs from 81·0·–99·9 to 95·7–100). We observed slightly higher point estimates of cure rates in patients with *P malariae* infection than in those infected with *P ovale curtisi* or *P ovale wallikeri*, but the 95% CIs crossed in all subanalyses (table 4). In the post-hoc per-protocol set, we observed similar cure rates at day 28, with a likewise overall cure rate of 96·3·% or higher (95% CIs from 81·0·–99·9 to 95·7–100; table 5).

**Discussion**

With this post-hoc analysis of the CANTAM-Pyramax real-world, safety, tolerability, and efficacy trial, we aimed to provide high-quality data to fill the current gap of high-grade evidence for treatment recommendations for blood-stage clearance of non-falciparum and non-vivax malaria. The results of this study provide important evidence supporting the use of pyronaridine–artesunate for the treatment of blood stages of mixed *Plasmodium* malaria and malaria caused by mono-infections of *P malariae*, *P ovale curtisi*, and *P ovale wallikeri* in a real-world setting. To our knowledge, to date, this is the largest cohort in a study systematically assessing the treatment outcome of patients with such infections.

The distributions of baseline characteristics in this post-hoc subset were similar between both study sites, which suggests an unbiased randomisation process for the selection of samples. The underlying expectation that approximately 10% of the 1502 randomly selected malaria episodes would be non-falciparum mono-infections or mixed *Plasmodium* infections was exceeded by the observed 192 (12·8·%) such infections in this analysis, which were equally distributed between the urban site in DR Congo and the rural site in Gabon. 183 (12·2·%) of all episodes were mixed infections. This underlines the epidemiological importance of non-falciparum and mixed *Plasmodium* malaria in central Africa and the surrounding countries.
Table 5: Data are n or n (%; 95% CI) for the post-hoc per-protocol analysis set. Percentages are based on the available observations.

|                      | Democratic Republic of the Congo | Gabon | Total       |
|----------------------|----------------------------------|-------|-------------|
| **Plasmodium malariae** |                                  |       |             |
| Available observations | 68                               | 59    | 127         |
| Number of patients with cure on day 28 | 67 (98.5%; 92.1–100) | 59 (100%; 93.9–100) | 126 (99.2%; 95.7–100) |
| **Plasmodium ovale curtisi** |                                  |       |             |
| Available observations | 20                               | 26    | 46          |
| Number of patients with cure on day 28 | 20 (100%; 83.2–100) | 26 (100%; 86.8–100) | 46 (100%; 92.3–100) |
| **P ovale wallikeri** |                                  |       |             |
| Available observations | 16                               | 11    | 27          |
| Number of patients with cure on day 28 | 15 (93.8%; 69.8–99.8) | 11 (100%; 71.5–100) | 26 (96.3%; 81.0–99.9) |

Data are n or n (%) (Clopper-Pearson CI) for the post-hoc per-protocol analysis set. Percentages are based on the number of available observations. No Plasmodium vivax infections were detected.

Articles

Determining antimalarial treatment efficacy for non-falciparum and mixed Plasmodium infections can be challenging. Information about the epidemiology of P. malariae and P. ovale spp is scarce, which makes it difficult to identify suitable geographical settings where sufficiently large sample sizes can be obtained for clinical trials in this field. Additionally, the diagnosis of non-falciparum malaria requires microscopists to be well trained in identifying potential study participants. In case of reappearance of parasites during the observational period, no established methods are available to reliably distinguish recrudescence from reinfection and relapse, if applicable, which might lead to overestimation or underestimation of failure rates. In this post-hoc analysis, we chose the more conservative approach to consider reappearance of the same Plasmodium species within the follow-up period as failure.

To reflect a real-world setting, patients with malaria were recruited in the CANTAM-Pyramax trial on the basis of either a positive RDT result (using only tests with WHO prequalification detecting HRP-2 antigens alone or HRP-2 and pLDH or aldolase antigens) or malaria microscopy—depending on what was routine procedure at the respective sites. On the basis of the fact that some RDTs were targeting P. falciparum infection alone and that microscopic identification of low-level parasitaemia of non-falciparum malaria is challenging, the observed prevalence in this study might underestimate somewhat the true prevalence of non-falciparum malaria in this setting.

Fewer than 5% of samples of patients recruited on the basis of RDT tests had a negative result in qPCR testing. This discrepancy of results might be explained by the test characteristics of RDTs. Positive predictive values (PPVs) and negative predictive values of RDTs for clinical malaria have been modelled previously, and PPVs differed considerably between countries when used for screening, suggesting that there are substantial error rates in RDT-based test-and-treat algorithms in low-PPV settings (ie, ≤10% of false positives). Positive RDT test results might, for example, be explained by successfully treated malaria episodes within up to 4–6 weeks before RDT testing due to persistence of circulating antigens. This suggests that the presence of a positive RDT result for a proportion of patients, particularly in low-PPV settings, might not always reflect presence of infection at the time of testing. This finding in this subset is thus in line with the test characteristics of RDTs. Additionally, we cannot rule out that degradation of DNA due to handling, storage, and shipment of samples might also have played a role in subsequent testing of individuals with low-level parasitaemia. In summary, the low proportion of discrepant test results using different diagnostic assays seems in line with the performance characteristics of the tests.

The microscopic reassessment of qPCR-positive day 28 samples showed one slide being microscopically positive for P. falciparum and one microscopically negative slide, suggesting there were submicroscopic non-falciparum parasitaemia in both samples. The effect of antimalarial treatment regimens with and without 8-aminoquinolines on P. falciparum gametocytes have been described previously, and a 2022 publication has detailed the specific clinical effects of pyronaridine–artesunate on the sexual stages of this species. However, the effects of antimalarials on the sexual stages of Plasmodium malariae and Plasmodium ovale spp, and the characteristics of those stages, have not been described conclusively so far. Therefore, we cannot rule out that these submicroscopic parasitaemias were due to sexual stages of said non-falciparum parasite species that, to date, cannot be reliably distinguished from asexual stages with the available molecular methods.

Overall cure rates on day 28 in this post-hoc analysis were very good for all non-falciparum species and similar to the effectiveness of pyronaridine–artesunate in P. falciparum and P. vivax (>95%). Overall cure rates were similar in mixed infections. The only outlier was the noticeably decreased cure rate (93.8%) for P. ovale wallikeri at the DR Congo site. However, this is based on one observation alone, which had such a big effect because of the low sample size in this group (one of 16 observations). Given that the clinical trial was an effectiveness trial with unsupervised treatment intake on days 2 and 3, these high cure rates further support the use of pyronaridine–artesunate in clinical routine. Therefore, our results give confidence that pyronaridine–artesunate can be used to effectively treat blood-stage infections of all African Plasmodium spp reported in this study. This finding can simplify the future management of patients with malaria, given the challenges of routine...
diagnostics of non-falciparum and mixed-species malaria by RDT and microscopy. In this sample subset, *P. malariae* was more prevalent than *P. ovale* spp, which reflects what is reported from other endemic regions. SD, IB-F, SA-B, JS, RM, GTL, GM-N, NYN, GKM, TBMM, LBDM, and RZM were responsible for resources. MR, SD, and IB-F supervised the study. SD, IB-F, MR, MG, SA-B, JS, RM, and DE-A did oversight of data analysis and validation of study results. MG wrote the original draft. MR, SD, IB-F, SA-B, JS, RM, DE-A, GTL, GM-N, NYN, GKM, TBMM, LBDM, and RZM reviewed and edited the manuscript. SD, IB, SA-B, MR and MG verified the underlying data. All authors contributed to the development of the paper, provided critical review, and approved the final version for submission. All authors had access to the data in the study and accept responsibility to submit for publication.

**Declaration of interests**

IB-F and SD are full-time employees of Medicines for Malaria Venture (MMV). JS is a full-time employee of Shin-Poong Pharmaceutical. RM and SA-B are consultants paid by Shin-Poong Pharmaceutical. All other authors declare no competing interests.

**Data sharing**

All relevant data are presented in this Article and the appendix. The data underlying the results presented in the study are available from Medicines for Malaria Venture (https://www.mmv.org) on reasonable request.

**Acknowledgments**

This work was funded in whole by MMV. MMV is funded by a number of donors including USAID, the Bill & Melinda Gates Foundation, the UK Department for International Development, the Norwegian Agency for Development Cooperation, Irish Aid, Newcrest Mining, Australian Aid, the Swiss Agency for Development and Co-operation, and the Wellcome Trust. The Bill & Melinda Gates Foundation contributed to the cost of open access publication: this work was supported, in part, by the Bill & Melinda Gates Foundation (INV-007155). Data from this study were presented as a poster at the American Society of Tropical Medicine and Hygiene 2021 Virtual Congress (Nov 19, 2021, Abstract LB-5200).

We recognise the contributions of Sherry Armstrong-Wilkinson, who was a consultant for MMV Switzerland, and Helen Demarest, who is a clinical trial manager at MMV.

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