Author's response to reviews

Title: Inhaled Nitric Oxide for the Adjunctive Therapy of Severe Malaria: Protocol for a Randomized Controlled Trial

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Author's response to reviews: see over
Dear Editor,

Thank you for the review of our manuscript. We appreciate the interest expressed by the reviewer and the opportunity to revise the manuscript.

We have addressed each of the reviewer’s comments and suggestions for discretionary revisions in a point-by-point manner below.

#1

**Page 9 "Positive malaria rapid diagnostic test."**

These tests significantly vary in performance parameters. Please address test selection, staff proficiency training in test use, and concomitant or retrospective requirement to confirm presence of asexual parasites in peripheral blood.

In selecting a rapid diagnostic test (RDT) for the purpose of inclusion in the trial, our primary consideration is test specificity (in order to minimize false positive results) such that all patients enrolled are truly parasitemic. "Three band" immunochromatographic tests are available that utilize multiple *Plasmodium* antigens, including histidine-rich protein 2 (HRP-2) and parasite lactate dehydrogenase (pLDH). We have elected to use the commercially available First Response Malaria Ag Combo (pLDH/HRP2) (Premier Medical Corporation Ltd., India). This RDT includes both HRP-2 and pLDH detection bands. For trial inclusion, we will require that both bands be positive. The requirement for the detection of two separate *P. falciparum* antigens reduces the likelihood of a false positive result, and circumvents the well recognized challenge of persistence of HRP-2 in serum or plasma up to 4 weeks following eradication of *P. falciparum* infection (i.e., enrolling patients with recent past infection who are not truly parasitemic at the time of admission). The WHO has evaluated commercially available RDTs in a standardized fashion against cultured parasites at fixed densities, as well as against a panel of wild type *P. falciparum* isolates and parasite-negative control samples. First Response Malaria Ag Combo (pLDH/HRP2) was among the top-ranked RDTs in this head to head comparison, with a detection rate of 95% and a false positive rate of 0% [1]. Medical officers performing the RDT will be thoroughly trained in the performance and interpretation of the RDT. Of note, standardized training tools exist and health care workers can be trained to safely and accurately perform RDTs in less than one day [2].

Secondly, to the extent possible, we will independently verify parasitemia using on site microscopy at the time of enrolment, and exclude patients from the study if the peripheral smear is negative by microscopy. Logistical challenges at the peripheral study site may limit the 24 hour availability of microscopy, and therefore the result will only be used if available in a timely fashion prior to randomization.

Finally, peripheral smears will be sent out for quantification of parasite density at multiple points throughout the trial: admission, 12 hours, days 1, 2, 3 of hospitalization and day 14 follow-up. This will be performed at an external, quality assured laboratory. This result will only be available retrospectively, after the point of randomization, and therefore can be used only to exclude patients from a *per protocol* analysis if the
admission peripheral smear is negative. We expect that this will occur very rarely given our stringent inclusion criteria (requirement for HRP-2 and pLDH positivity on RDT and positive peripheral smear at the trial site, if available).

We have added a paragraph to the manuscript describing our strategy for parasitologic confirmation prior to enrolment (pages 9 and 10).

#2
Page 9 "Features of severe malaria."
Please be explicit if one or more features are required for inclusion.

Any one or more of the features of severe malaria will qualify a patient for inclusion in the study. This is consistent with the WHO definition of severe malaria [3].

We have clarified this in the manuscript (page 9).

#3
Page 10 "Severe malnutrition"
Please provide objective criteria for making this determination.

The World Health Organization (WHO) definition of severe malnutrition will be used: weight-for length/height below -3 SD based on WHO reference charts (termed “severely wasted”), or who have symmetrical oedema involving at least the feet (termed “oedematous malnutrition”) [4].

We have added this objective definition to the protocol (page 10).

#4
Page 10 and 11.
"Treatment groups."
It is highly desirable to standardize treatment (Ugandan standard of care) within the trial (quinine versus artesunate), since their efficacy is known to be different, and the study might not be adequately powered to determine the incremental benefit of iNO in two versus one treatment groups.

We agree that standardization of the anti-parasitic agent is important for this trial. In light of recent data (AQUAMAT trial) demonstrating the superiority of intravenous artesunate over quinine for the treatment of severe malaria in African children [5], we have elected to use intravenous artesunate for all children enrolled in the trial.

We have added a sentence to this effect in the protocol (page 11).

#5
Page 12
"Outcome measures."

The method for determination of the primary study endpoint, Ang-2 concentration, should be stipulated and referenced in this section.

Angiopoietin-2 can be measured by ELISA from plasma or serum samples, and is readily detectable in samples frozen for storage and later thawed [6-9]. Commercially available ELISA kits will be used (DuoSets, R&D Systems, Minneapolis, MN).

We have added a statement to the manuscript (page 13) to address this.

#6
Page 14
"Duration of Study Participation."
It would be helpful to stipulate that interim medical history would be collected, with special attention to severe intercurrent illness including cerebral malaria that might affect neurocognitive testing.

We thank the reviewer for this comment. We have made this addition to the manuscript (page 15).

#7
Page 20
Interim analysis.
Agree with approach, but perhaps useful to clarify "no plan to stop the trial prematurely for efficacy or futility based on the primary or secondary trial endpoints....." by noting the exception of the planned use of the control chart for detecting excess mortality.

We thank the reviewer for this remark. We have clarified the trial halting rules in the manuscript (page 21). The trial will be halted for safety review in the case of excess mortality as determined by control charts. However, we do not plan to halt the trial for overwhelming efficacy or futility based on biochemical or parasitological endpoints, or differences in recovery times.

#8
Page 22
Secondary outcomes.
The protocol as presented makes reference to, but does not list, the cognitive and neurologic endpoints used for the secondary outcome analyses. Inclusion of such a list or named indices of performance/cognition would make the protocol complete.

As in previous studies [10], the instruments that will be used for the neurocognitive testing are standardized and include the Kaufman Assessment Battery for Children, the visual form of the computerized Test of Variables of Attention, and the Tactual Performance Test. The cognitive domains evaluated with these instruments are working
memory, executive attention and tactile-based learning, respectively. Summary variables from each test will be used to measure each of these cognitive areas, after conversion of raw outcomes into an age-specific standardized (z) score: sequential processing of the Kaufman Assessment Battery for Children (working memory), signal detection D prime test of the Test of Variables of Attention(executive attention), and total time per block of the Tactual Performance Test (tactile-based learning). Details of these tests are described elsewhere [11].

We have added a description of the neurocognitive testing and outcome measures to the manuscript (page 23).

Again, we appreciate the opportunity to revise and improve our manuscript to address the concerns and criticisms raised by the reviewers.

Sincerely,

Michael Hawkes, MD and Kevin C. Kain, MD (for the co-authors)

References


