

# Inhaled Nitric Oxide as an Adjunctive Treatment for Cerebral Malaria in Children: A Phase II Randomized Open-Label Clinical Trial

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**Background.** Children with cerebral malaria (CM) have high rates of mortality and neurologic sequelae. Nitric oxide (NO) metabolite levels in plasma and urine are reduced in CM.

**Methods.** This randomized trial assessed the efficacy of inhaled NO versus nitrogen (N<sub>2</sub>) as an adjunctive treatment for CM patients receiving intravenous artesunate. We hypothesized that patients treated with NO would have a greater increase of the malaria biomarker, plasma angiotensin-converting enzyme-1 (Ang-1) after 48 hours of treatment.

**Results.** Ninety-two children with CM were randomized to receive either inhaled 80 part per million NO or N<sub>2</sub> for 48 or more hours. Plasma Ang-1 levels increased in both treatment groups, but there was no difference between the groups at 48 hours (*P* = not significant [NS]). Plasma Ang-2 and cytokine levels (tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , interleukin [IL]-1 $\beta$ , IL-6, IL-10, and monocyte chemoattractant protein-1) decreased between inclusion and 48 hours in both treatment groups, but there was no difference between the groups (*P* = NS). Nitric oxide metabolite levels—blood methemoglobin and plasma nitrate—increased in patients treated with NO (both *P* < .05). Seven patients in the N<sub>2</sub> group and 4 patients in the NO group died. Five patients in the N<sub>2</sub> group and 6 in the NO group had neurological sequelae at hospital discharge.

**Conclusions.** Breathing NO as an adjunctive treatment for CM for a minimum of 48 hours was safe, increased blood methemoglobin and plasma nitrate levels, but did not result in a greater increase of plasma Ang-1 levels at 48 hours.

**Keywords.** cerebral malaria; methemoglobin; nitric oxide; *Plasmodium falciparum*.

Malaria kills more than half a million people per year, and most are children in sub-Saharan Africa [1]. Cerebral

malaria (CM) is a catastrophic sequel of *Plasmodium falciparum* malaria, with a mortality rate of approximately 20% [2], causing long-term neurological defects in 10% to 24% of survivors [3,4]. The most effective antiparasitic therapy is intravenous (IV) artesunate, which reduced mortality from 21% to 18% in African children with CM [2]. Several adjunctive therapies have been tested, but none have proven to be effective [5].

Nitric oxide (NO) is an intercellular messenger molecule synthesized by various cell types, including vascular endothelial cells. Nitric oxide is a vasodilator and inhibits platelet aggregation, among numerous other effects [6]. Malaria severity was correlated with reduced levels of biochemical markers of NO production [7].

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Physiological markers of reduced NO bioavailability—reduced brachial artery postischemic vasodilation [8, 9] and increased pulmonary arterial pressures [10]—were correlated with increased cell-free hemoglobin levels in patients with severe malaria, reflecting the NO-scavenging property of cell-free hemoglobin.

Angiotensin (Ang)-1 and its antagonist Ang-2 regulate endothelial activation, and serum, plasma, or whole blood levels have been studied as biomarkers of CM severity and disease progression. Angiotensin-1 levels were reduced and Ang-2 levels were elevated in pediatric CM patients compared with healthy children [11] and uncomplicated malaria patients [12, 13]. Significantly elevated levels of Ang-2 were found in fatal cases of CM and severe malaria [13].

Breathing low concentrations of NO gas reduces pulmonary arterial pressures without producing systemic hypotension in animal models and children [14, 15], and inhaled NO has been safely used to treat persistent pulmonary hypertension and other pulmonary disorders [16, 17] in neonates and infants for over 20 years [18]. In *Plasmodium berghei* ANKA (PbA)-infected mice (a murine CM model), breathing 40 or 80 parts per million (ppm) of inhaled NO [19, 20] improved survival rates, reduced plasma inflammatory markers, and increased plasma Ang-1 levels. In other studies, inhaled NO reduced cerebral injury in murine models of stroke [21, 22] and traumatic brain injury [23].

Based on the well established clinical safety profile of inhaled NO and its efficacy in experimental models of CM, we conducted an open-label randomized trial of breathing 80 ppm NO versus nitrogen (N<sub>2</sub>) as an adjunctive therapy to IV artesunate in pediatric CM patients in Uganda. The primary endpoint was to compare the increase of plasma Ang-1 levels over the first 48 hours of treatment in patients breathing either NO or N<sub>2</sub>. Nitric oxide delivery and safety were monitored and assessed by measuring methemoglobin% (metHb%) and NO metabolites. Although the study was too small to detect significant changes, in-hospital mortality, time to recovery from coma, and neurologic sequelae for up to 6 months after discharge were also determined.

## METHODS

### Study Participants

We screened children aged 2 months to 12 years, weighing 5–20 kg, at the tertiary care Mbarara Regional Referral Hospital (MRRH) Pediatric Ward and the Holy Innocents Children's Hospital. We enrolled patients with a Blantyre coma score (BCS) [24] <3/5 (for children aged <4 years) or Glasgow coma score (GCS) <7/15 (for children aged 4–12 years), with a *Plasmodium* infection confirmed by thick blood smear (see [Supplementary Box 1](#) for additional inclusion and exclusion criteria). Potential study patients were transferred to the research unit at MRRH, and Ugandan research staff obtained informed consent

from first-degree relatives, using materials written in English and Runyankole (the local language). See the [Supplement](#) for the full description of the institutions and supporting companies involved.

### Study Design and Randomization

This was a randomized open-label, phase II clinical trial comparing breathing air or oxygen supplemented with inhaled NO (80 ppm [by volume] in N<sub>2</sub>) or N<sub>2</sub> in children with CM. Nitrogen has been used as an inert gas control in other trials of inhaled NO [25]. After consent was obtained, we randomized patients to either study arm using off-site computer-generated random allocation. Sealed envelopes contained the patient treatment designation and were opened after consent and enrollment.

Survival was defined as alive at the time of hospital discharge. Patients were examined for neurological sequelae at the time of discharge and at follow-up neurological assessments at 1, 3, and 6 months after discharge.

### Study Oversight

This study was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association, and was approved by the institutional review boards of the following institutions: Massachusetts General Hospital, Mbarara University of Science and Technology, the Ugandan National Council for Science and Technology, the Comité de Protection des Personnes France, and Médecins Sans Frontières International. The US Food and Drug Administration was notified, but an Investigational New Drug approval was not required. The Ugandan National Drug Authority approved the study. A data and safety monitoring board was convened biannually, for a total of 3 meetings, to review the unblinded safety data based on serious adverse events and mortality. The trial was registered under the ClinicalTrials.gov: NCT01388842.

### Study Procedures

The patient's vital signs and coma score were assessed and logged every 2 hours. Transcutaneous pulse oxygen saturation (SpO<sub>2</sub>), methemoglobin (SpMET), hemoglobin levels, and pulse rate were continuously monitored noninvasively with a Radical 7 co-oximeter (Masimo, Irvine, CA). When elevated transcutaneous SpMET levels were detected, blood metHb% was measured with an Avox 4000 spectrophotometer (ITC, Cambridge, MA) for confirmation. Whole-blood hemoglobin (g/dL) levels were measured with a Hemo Control Hemoglobin Analyzer (EKF Diagnostics, Cardiff, UK). If the metHb% level was 8.5% or greater, the inhaled gas dose was reduced by 50% and then reduced by an additional 25% of the original dose if the metHb% did not decrease below 8.5% within 1–2 hours.

Blood was sampled by venipuncture at enrollment (15 minutes before initiating study gas treatment, time 0) and at 12, 24,

48, 72, and 120 hours after commencing treatment with study gas. Blood-based measurements included the following: hemoglobin, complete blood count, parasite smears, and plasma levels of creatinine, lactate, Ang-1 and Ang-2, cytokines (tumor necrosis factor [TNF]- $\alpha$ , interferon [IFN]- $\gamma$ , interleukin [IL]-1 $\beta$ , IL-6, IL-10, and monocyte chemoattractant protein [MCP]-1), S100b, and NO metabolites (nitrate, nitrite, *N*-nitrosamines [RNNO], *S*-nitrosothiols [RSNO]). A total of 10 mL of blood was sampled for research analyses from each patient over the 5-day study period. See the [Supplement](#) for a description of specific laboratory procedures.

### Gas Administration

Study gas was delivered to the patient via a nasal cannula with a metered dosing apparatus specifically developed for investigational use (INOPulse<sup>TM</sup>, Ikaria, Inc., NJ) [25]. The gas volume, and thus the gas dose to be delivered, was determined with a nomogram, incorporating the child's weight (to estimate tidal volume) and respiratory rate (see the [Supplement](#) for further description). Because the INOPulse was limited to 10 mL of gas injection per breath, to avoid under-dosing NO gas we were restricted to enrolling children weighing less than 20 kg. Enrolled patients received study gas for a targeted minimum of 48 hours and a maximum of 120 hours. All patients with a SpO<sub>2</sub>  $\leq$  94% were given supplemental oxygen by facemask.

If, during the course of treatment, a patient's respiratory rate (and therefore alveolar minute ventilation) changed, his/her INOPulse gas dose was adjusted using the nomogram. If the patient regained full consciousness (BCS 5/5 or GCS 15/15) and had

received treatment gas for greater than 48 hours, or if the patient completed 120 hours of gas treatment regardless of clinical status, the treatment gas was reduced by 50% each hour until turned off.

### Concomitant Treatment

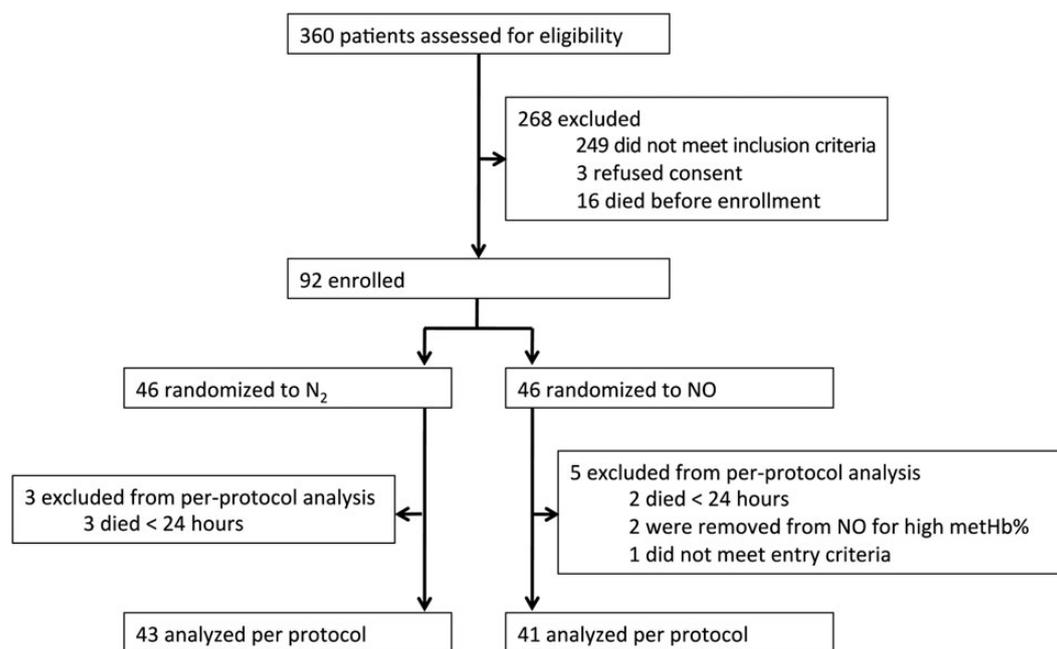
Enrolled patients received IV artesunate per protocol for at least 48 hours and up to 5 days. Patients able to take oral medications were transitioned to a 3-day course of artemether/lumefantrine (Coartem; Novartis Pharma, Switzerland) after 48 hours of IV artesunate treatment (see the [Supplement](#) for additional treatments and interventions).

### Study Endpoints

The primary study endpoint was the increase of plasma Ang-1 levels at 48 hours. The secondary laboratory endpoints included reduction in plasma Ang-2, the Ang-2/Ang-1 ratio, selected plasma cytokine levels and creatinine, and blood lactate. The Ang-2/Ang-1 ratio was calculated for each patient, and the mean of these ratios is reported. Additional secondary endpoints included mortality during hospitalization, time taken to recover from coma, and neurological sequelae occurring up to 6 months after discharge. We evaluated the safety of NO delivery, and we monitored methHb% and NO metabolites to confirm inhalation and uptake of NO.

### Statistical Analysis

We compared proportions with a  $\chi^2$  or Fisher's exact test with 2-sided *P* values. We assessed data within groups using a 2-tailed paired *t* test or Wilcoxon matched-pairs signed-rank test, and we assessed data between groups using a Mann-Whitney *U* test. Data between the N<sub>2</sub> and NO treatment



**Figure 1.** Screening, enrollment, and randomization flow diagram. Abbreviations: methHb%, percentage of methemoglobin; N<sub>2</sub>, nitrogen; NO, nitric oxide.

**Table 1. Baseline Clinical and Laboratory Data of Patients Enrolled in the Treatment Groups\***

Characteristic	N <sub>2</sub> (N = 46)	NO (N = 46)
Female sex, no. (%)	24 (52.2%)	19 (41.3%)
Age, month	33.5 ± 22.4	42.4 ± 23.3
Age <48 month, no. (%)	36 (78.3%)	29 (63.0%)
Age ≥48 month, no. (%)	10 (21.7%)	17 (37.0%)
Weight, kg	11.6 ± 3.7	12.7 ± 3.3
Temperature, °C	38.1 ± 1.1	37.9 ± 1.3
SpO <sub>2</sub> , %	97.8 ± 4.2	98.2 ± 2.0
Blood methHb, % (N)	0.6 ± 0.1 (37)	0.6 ± 0.1 (34)
Heart rate, BPM	147 ± 23	143 ± 25
Respiratory rate, BPM	37 ± 11	38 ± 13
Blood pressure, mmHg		
Systolic (N)	95 ± 11 (45)	98 ± 11 (44)
Diastolic (N)	53 ± 12 (45)	55 ± 10 (44)
Blantyre coma score (N)	1.3 ± 0.7 (36)	1.2 ± 0.8 (29)
Glasgow coma score (N)	5.6 ± 0.7 (10)	5.9 ± 0.5 (17)
History of seizures prior to admission, no. (%)	40 (87.0)	38 (82.6)
Malaria retinopathy (any finding), no. (%)	27 (58.7)	23 (50.0)
WBC, no. ×10 <sup>3</sup> /μL	13.5 ± 7.1	16.8 ± 11.4
Hematocrit, %	23.3 ± 5.3	25.9 ± 6.7
Hemoglobin, g/dL	7.8 ± 2.1	8.7 ± 2.2
Platelets, no. ×10 <sup>3</sup> /μL	93 ± 83	78 ± 68
Parasites, trophozoites, Parasite no. ×10 <sup>3</sup> /μL (N)	61 ± 100	56 ± 130

Abbreviations: BPM, beats per minute; methHb, methemoglobin; N<sub>2</sub>, nitrogen; NO, nitric oxide; NS, not significant; SD, standard deviation; SpO<sub>2</sub>, transcutaneous pulse oxygen saturation; WBC, white blood cells.

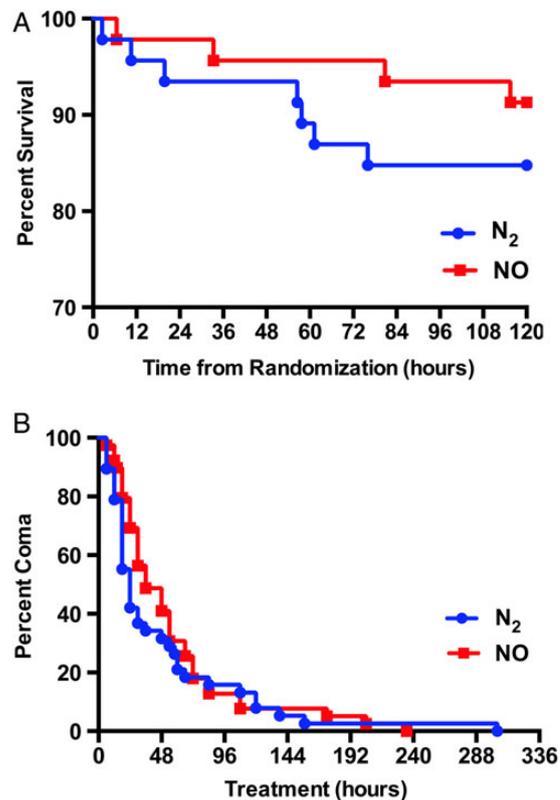
\* All data are mean ± SD. P = NS comparing N<sub>2</sub> to NO.

cohorts, and within treatment cohorts at hours 0 and 48, were compared from patients who received gas for at least 48 hours and those for whom we had paired data. We compared survival and time to coma recovery curves using Kaplan–Meier curves and log-rank test (or Wilcoxon Breslow when appropriate). Statistics and graphs were generated with Stata software (Release 13, StataCorp.) and GraphPad Prism 6 (GraphPad Software, Inc.). The level of statistical significance was set at 0.05. All results are displayed as mean ± standard deviation (SD). The target census for the study was 92 patients (see the [Supplement](#) for a description of how the study was powered).

## RESULTS

### Baseline Characteristics and Clinical Course

Between September 2011 and July 2013, 360 patients with a clinical diagnosis of CM were screened for eligibility. Ninety-two patients were included in the study, and their parent or guardian gave informed consent to enter the trial. Forty-six patients were randomly assigned to each treatment group. Eight



**Figure 2.** (A) Kaplan-Meier curves comparing survival in pediatric patients with cerebral malaria (CM) treated with nitrogen (N<sub>2</sub>) or nitric oxide (NO). P = not significant (NS). (B) Kaplan-Meier curves for resolution of coma in pediatric patients with CM, N<sub>2</sub> vs NO; resolution of coma is defined as Blantyre coma score ≥ 3 or Glasgow coma score of ≥ 7; (N<sub>2</sub> N = 39, NO N = 41). P = NS. Abbreviations: INO, inhaled NO; methHb%, percentage of methemoglobin.

patients enrolled in the study were not included in the per-protocol analysis: 3 patients in the N<sub>2</sub> group and 5 patients in the NO group (Figure 1). There was no difference in baseline characteristics or laboratory values between enrolled NO- and N<sub>2</sub>-treated patients (Table 1).

There was no difference in the duration of treatment with study gas among patients treated ≥48 hours: 60.1 ± 23.3 hours and 63.9 ± 23.9 hours for N<sub>2</sub>- and NO-treated patients, respectively (P = not significant [NS]). For patients receiving NO, the mean inhaled dose for the initial 48 hours of therapy was 0.55 ± 0.12 mg/kg per hour (N = 39) (Figure 2A).

Seventy-nine patients (38 N<sub>2</sub>, 41 NO) returned for examination at 1 month after discharge, 76 patients (37 N<sub>2</sub>, 39 NO) returned at 3 months, and 75 patients (36 N<sub>2</sub>, 39 NO) returned at 6 months. The trial ended when the final enrolled patient completed the 6-month follow-up visit, in February 2014.

### Primary and Secondary Endpoints and Outcomes

Table 2 shows the plasma Ang-1 and Ang-2 levels and the Ang-2/Ang-1 ratio at baseline (hour 0) and 48 hours later (hour 48).

**Table 2. Primary and Secondary Laboratory Outcomes\***

Angiopoietins	Interval	N <sub>2</sub> (N = 42)	NO (N = 39)	Intragroup <i>P</i> Value	Intergroup <i>P</i> Value
Ang-1, pg/dL	Hour 0	2049 ± 2392	1733 ± 2485	N <sub>2</sub> = 0.085 NO = 0.016	.73
	Hour 48	3235 ± 3516	2837 ± 3152		.81
Ang-2, pg/dL	Hour 0	16235 ± 16216	14440 ± 12202	N <sub>2</sub> < 0.0001 NO < 0.0001	.41
	Hour 48	5901 ± 8424	4801 ± 4831		.78
Ang-2/Ang-1 Ratio	Hour 0	19.62 ± 24.29	35.35 ± 119.10	N <sub>2</sub> < 0.0001 NO < 0.0001	.94
	Hour 48	7.80 ± 20.58	5.71 ± 11.35		.96
Cytokines		N <sub>2</sub> (N = 40)	NO (N = 40)		
TNF-α, pg/mL	Hour 0	0.297 ± 0.495	0.311 ± 0.600	N <sub>2</sub> = 0.0005 NO < 0.0001	.90
	Hour 48	0.095 ± 0.410	0.008 ± 0.026		.99
IL-1β, pg/mL	Hour 0	0.359 ± 0.631	0.508 ± 0.910	N <sub>2</sub> = 0.007 NO < 0.0001	.74
	Hour 48	0.151 ± 0.649	0.024 ± 0.074		.93
IL-6, pg/mL	Hour 0	84.63 ± 108.60	99.19 ± 122.30	N <sub>2</sub> < 0.0001 NO < 0.0001	.88
	Hour 48	8.04 ± 27.56	13.46 ± 60.56		.70
S100b		N <sub>2</sub> (N = 37)	NO (N = 38)		
S100b, pg/mL	Hour 0	931.2 ± 813.7	922.7 ± 861.5	N <sub>2</sub> < 0.0001 NO = 0.0001	.80
	Hour 48	283.8 ± 400.4	318.8 ± 477.1		.85

Abbreviations: Ang, angiopoietin; IL, interleukin; N<sub>2</sub>, nitrogen; NO, nitric oxide; SD, standard deviation; TNF, tumor necrosis factor.

\* Plasma levels of Ang-1, Ang-2, cytokines, and S100b, as well as the ratio of plasma Ang-2 levels to plasma Ang-1 levels on enrollment and after 48 hours. All data are mean ± SD.

There was an increase of plasma Ang-1 levels in the NO group over 48 hours ( $P = .016$ ), whereas there was no statistically significant increase in the N<sub>2</sub> group ( $P = .085$ ). However, there was no difference in the change from baseline of plasma Ang-1 levels between the treatment groups. There was a significant decrease in plasma Ang-2 levels and the Ang-2/Ang-1 ratios over the 48 hours of treatment in both groups ( $P < .0001$ ). There was no difference in (1) plasma Ang-2 or (2) the Ang-2/Ang-1 ratio between the treatment groups at either 0 or 48 hours.

In both groups, plasma TNF-α, IL-1β, and IL-6 levels were less at 48 hours than upon enrollment (Table 2), but there was no difference between the treatment groups at 0 or 48 hours ( $P = NS$ ). Similar findings were obtained for plasma levels of IFN-γ, IL-10, and MCP-1 (Supplementary Table 1). Plasma levels of S100b, a biomarker of neural tissue injury [26], decreased after 48 hours in both treatment groups (Table 2), with no difference between treatment groups ( $P = NS$ ).

There was no difference in the time to clear circulating parasites between the N<sub>2</sub> (N = 40) and NO (N = 43) treatment groups: 42.9 ± 26.2 and 40.7 ± 26.1 hours, respectively. Moreover, there was no significant difference in (1) the rate children became afebrile (<38°C) or (2) the change of blood creatinine or lactate levels over 48 hours between the gas treatment groups (see Supplementary Table 2 for creatinine and lactate levels).

There were no clinically significant differences in heart rate, systolic blood pressure, or SpO<sub>2</sub> after initiating treatment in patients receiving NO versus N<sub>2</sub>. In addition, the number of patients affected by severe adverse events (SAEs), or any adverse events (AEs), including cardiopulmonary or respiratory complications, was similar in the 2 treatment groups.

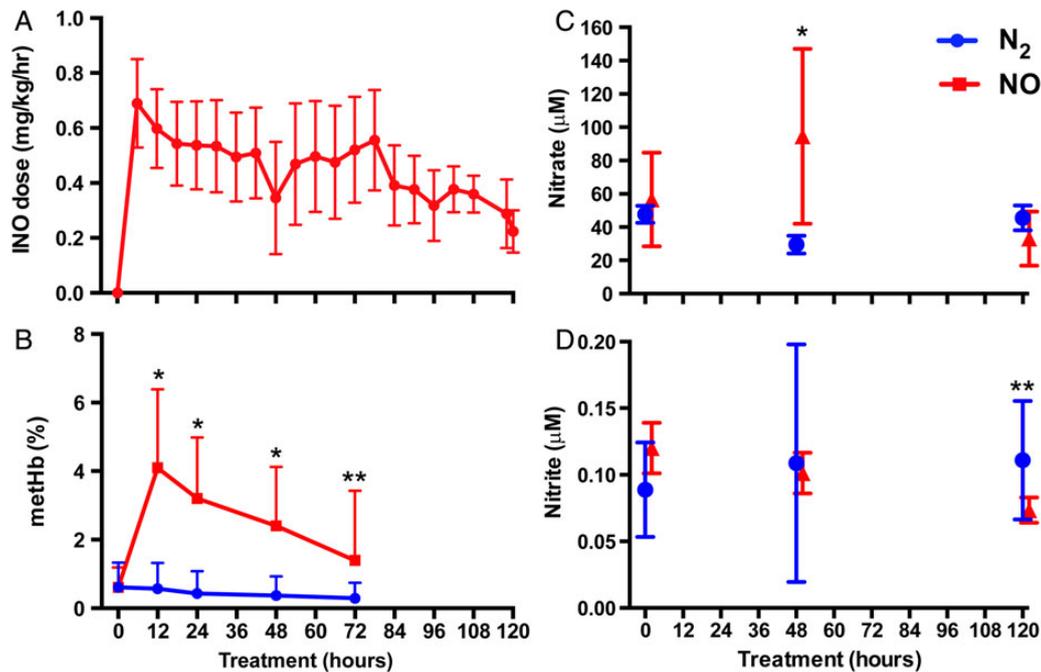
Eleven (12.0%) of 92 enrolled CM patients died while in the hospital (Table 3), 7 of whom were treated with N<sub>2</sub> (15.2%) and 4 of whom were treated with NO (8.7%). Of the patients who were living at the time of hospital discharge (39 N<sub>2</sub>, 42 NO),

**Table 3. Secondary Clinical Outcomes\***

	N <sub>2</sub>	NO
Died before 7 days after enrollment	7 of 46 (15.2%)	4 of 46 (8.7%)
Survived 1 month after discharge	39 of 46 (84.8%)	42 of 46 (91.3%)
Neurological sequelae in 1-month survivors	5 of 39 (12.8%)	6 of 42 (14.3%)
Full recovery in 1-month survivors	34 of 39 (87.2%)	36 of 42 (85.7%)

Abbreviations: N<sub>2</sub>, nitrogen; NO, nitric oxide; NS, not significant.

\* All data are presented as number and percentage of patients in each treatment group.  $P = NS$  comparing N<sub>2</sub> to NO.



**Figure 3.** (A) Delivered dose of nitric oxide (NO) over 120 hours (mg/kg per hour); (B) blood methemoglobin (percentage of methemoglobin [methHb %]) in patients treated with nitrogen (N<sub>2</sub>) and NO; (C) Plasma nitrate (μM) levels; and (D) plasma nitrite (μM) levels in patients treated with N<sub>2</sub> or NO. \**P* < .001 and \*\**P* < .05, NO versus N<sub>2</sub>. All data mean ± standard deviation.

5 of the patients treated with N<sub>2</sub> (12.8%) and 6 of the patients treated with NO (14.3%) exhibited neurological sequelae at the 1-month follow-up visit. Neurological sequelae included ataxia, hemiplegia, hypotonia, seizures, speech dysfunction, and/or behavioral disorders. Of the 11 patients with neurological sequelae at the time of discharge, 3 patients (1 N<sub>2</sub>, 2 NO) resolved their neurological sequelae by the 6-month follow-up visit. Figure 3A shows the Kaplan-Meier survival curve for the 5 days after randomization into the study for patients treated with either N<sub>2</sub> or NO (*P* = NS). There was no difference in the rate of recovery from coma between the NO and N<sub>2</sub> treatment groups (*P* = NS; Figure 3B), with the duration of coma of 48.1 ± 58.2 hours and 55.8 ± 50.1 hours for the N<sub>2</sub> and NO treatment groups, respectively.

Focusing only on those patients with evidence of malarial retinopathy (N = 27, 58.7% N<sub>2</sub> group; N = 23, 50.0% NO group), we observed that there was no difference in the change in (1) Ang-1, Ang-2, Ang-2/Ang-1 ratio between 0 and 48 hours or (2) the survival rate between patients receiving NO or N<sub>2</sub>.

### Nitric Oxide Metabolic Products and Effects

Blood methHb, nitrate, nitrite, RSNO, and RNNO are NO reaction products. Before breathing NO, baseline blood methHb% levels in the N<sub>2</sub> and NO groups were 0.6 ± 0.1% (N = 37) and 0.6 ± 0.1% (N = 34), respectively (*P* = NS) (Figure 2B). In the NO group, blood methHb% levels increased to 4.1 ± 0.4%

(N = 33) at 12 hours, 3.2 ± 0.3% (N = 33) at 24 hours, 2.4 ± 0.3% (N = 33) at 48 hours, and 1.4 ± 0.4% (N = 32) at 72 hours (*P* < .0001 at 12, 24, and 48 hours, and *P* = .03 at hour 72), when compared with patients breathing N<sub>2</sub>, in whom levels of blood methHb% did not change. The decrease in methHb% levels between 24 and 72 hours reflects the reduction or cessation of NO administration, because coma was reversed during recovery. Of note, 6 NO patients transiently developed methHb% levels ≥10%, and a reduction of the inhaled NO dose per protocol led to a rapid decline in methHb% levels in each patient within 2 hours.

There was no difference of baseline nitrate levels in patients treated with N<sub>2</sub> or NO (*P* = NS) (Figure 2C). Nitrate levels decreased in the N<sub>2</sub>-treated group over 48 hours, from 47.7 ± 21.1 to 29.5 ± 22.8 μM (*P* < .05). In contrast, nitrate levels increased over 48 hours with NO treatment from 56.7 ± 28.2 to 94.7 ± 52.5 μM (*P* < .05). Plasma nitrate levels at 48 hours were markedly greater in patients breathing NO versus N<sub>2</sub> (*P* < .01). After discontinuation of NO breathing, nitrate levels returned to baseline by 120 hours. Nitrite levels (Figure 2D) did not change significantly from hour 0 to 48 in either group but decreased from 48 hours to 120 hours in NO-treated patients (*P* < .05). The nitrite levels in the N<sub>2</sub>- and NO-treated patients differed at 120 hours (*P* < .05). Plasma RSNO and RNNO levels were not significantly different between the treatment groups at hours 0, 48, and 120 of treatment (Supplementary Table 2).

## DISCUSSION

This randomized trial compared breathing 80 ppm NO versus N<sub>2</sub> as an in-hospital adjunctive therapy for 92 comatose pediatric patients with CM. There was no significant difference of plasma Ang-1, Ang-2, or cytokine levels between the groups after 48 hours of treatment (Table 2).

Nitric oxide proved to be effective in murine CM models, wherein PbA-infected C57BL/6 mice treated with a systemic NO donor (dipropyleneetriamine NONOate), or breathing 40 ppm NO, had improved survival [19]. Prophylactic treatment with 80 ppm NO in the same model, with and without antimalarial therapy, resulted in a reduced mortality rate, a greater increase of plasma Ang-1 levels, reduced brain mRNA copy numbers of the ratio of Ang-2/Ang-1, and reduced plasma levels of IFN- $\gamma$ , TNF, and MCP-1 [20].

In this small clinical trial, we found an overall mortality rate of 12%, with fewer patients dying breathing NO; however, NO treatment did not significantly reduce the mortality rate. The overall mortality was less than the 18% reported in a recent multicenter study of 5425 children with severe malaria (of whom 1825 had CM) treated with IV artesunate [2]. The overall incidence rate of neurological sequelae in our study was 12%, which is similar to the 10%–25% rate reported in sub-Saharan Africa [3, 4]. We believe NO treatment to be safe because the mortality rate in our study was low, breathing NO did not increase the incidence of neurological sequelae, mean methemoglobin levels remained low (4.1%  $\pm$  0.4%), and the number of patients affected by SAEs and AEs were similar in both groups.

Plasma S100b, a biomarker of injury to astrocytes, oligodendrocytes, and other neural cell types, is increased in patients after traumatic brain injury and cardiac arrest [26–28]. Elevated S100b levels have been measured in adult and pediatric CM patients with seizures, but this was only investigated using cerebrospinal fluid [29, 30]. We measured plasma S100b levels in our CM patients, and, although we found no significant difference in the reduction of the levels between the treatment groups over 48 hours (Table 2), plasma S100b may be useful as a tool for assessing the level of central nervous system injury in future studies involving CM patients.

We delivered NO or N<sub>2</sub> with the INOPulse, a gas injector that was triggered on inspiration. Even though it was not possible to measure the precise NO concentration in the trachea of children receiving NO gas with the INOPulse, we confirmed that NO was inhaled by detecting elevated levels of blood metHb% and plasma NO metabolites (Figure 2B and C). Similar observations were reported by Gladwin et al [25], among adult sickle cell patients breathing 40 or 80 ppm NO using the INOPulse.

We measured plasma NO metabolites in an effort to better understand their changes in CM patients breathing NO, and we observed increased plasma nitrate levels in the NO arm and a reduction of nitrate levels in the N<sub>2</sub> arm after 48 hours

of treatment (Figure 2C). After inhalation, NO has an extremely short half-life with 97% rapidly converted to nitrate and the remainder to stable adducts (S-nitrosylated proteins [eg, S-nitrosothiols or N-nitrosamines], nitrosyl-hemeproteins, and nitrite), which can release NO in systemic vascular beds by various mechanisms [31, 32]. Studies have shown beneficial extrapulmonary effects of inhaled NO in humans, including reduced platelet aggregation [33], reduced myocardial injury after cardiopulmonary bypass [34], increased renal blood flow [35], enhanced liver allograft function in liver transplant recipients [36], and reduced inflammation in lower extremity ischemia-reperfusion from tourniquet use [37].

Despite the possible vasodilatory and anti-inflammatory benefits of inhaled NO described above, including the positive outcomes of NO trials in murine models of CM [11, 12], we did not find a significant difference in the change of plasma Ang-1 or Ang-2 levels after 48 hours of inhaled NO treatment. Plasma angiopoietin levels have been correlated to the severity of illness in severe malaria and CM patients [11, 12]. In the future, it may be valuable to focus the administration of inhaled NO upon CM patients with laboratory evidence of NO scavenging and pulmonary or systemic vasoconstriction. We would suggest studying the subset of CM patients with elevated levels of plasma cell-free hemoglobin and therefore increased NO scavenging and consumption [38]. This subset has shown evidence of increased right heart/pulmonary pressures on echocardiogram [10]. It is also possible that this subset of patients suffer from increased cerebrovascular resistance with reduced cerebral blood flow. Breathing NO oxidizes cell-free hemoglobin to metHb, thereby reducing its ability to scavenge intrinsic NO [39]. Inhaling NO may have greater clinical efficacy in this subgroup of patients.

## CONCLUSIONS

Treatment with NO did not significantly increase plasma Ang-1 levels at 48 hours compared with patients treated with N<sub>2</sub>. This small trial could not demonstrate a reduction of mortality or neurological impairment in children treated with NO. Seven patients in the N<sub>2</sub> group and 4 patients in the NO group died. A larger trial, utilizing measurements of cell-free hemoglobin and right heart pressures to assess pulmonary vasoconstriction, would be required to learn whether breathing NO could lead to improved survival rates in this subgroup of malaria patients.

## Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

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**Disclaimer.** No scientific writer was involved in the development of this manuscript. R. W. C. had full access to all of the data in the study, takes full responsibility for the integrity of the data and accuracy of the data analysis, and is the guarantor.

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**Potential conflicts of interest.** A. B. was an employee of Ikaria, Inc. when she trained the clinical investigators on the use of the drug and the delivery devices provided by Ikaria Inc. W. M. Z. reports that the Massachusetts General Hospital has received royalties on the sales of nitric oxide for inhalation from Linde Corporation (Munich, Germany) and Ikaria (New Jersey), and he has received a portion of these royalties. Ikaria provided free gas and delivery devices for this study but played no other role. Masimo provided the oximeters but played no other role.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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## Supplementary Material

### Box S1. Inclusion and Exclusion Criteria

#### Box 1.

The inclusion criteria for this study were:

- Age 2 months - 12 years, and weight 5 – 20 kg
- Positive malaria antigen test and confirmed by parasites observed on thick blood smear
- Cerebral malaria with Blantyre coma score < 3 for children aged < 4 years, or Glasgow coma score < 7 for children aged 4 – 12 years
- Informed consent from a parent or guardian

Exclusion criteria for this study were:

- Another cause of coma (toxin ingestion or pre-existing severe neurological disease)
- Evidence and/or history of pre-existing brain injury and neurologic impairment
- Impending respiratory failure: patient requiring bag mask ventilation
- Clinical evidence of a coagulopathy: persistent bleeding from nose or mouth or IV sites
- Severe chronic malnutrition with marasmus or Kwashiorkor edema and/or a weight-for-height Z-score of  $\leq -3$  SD (WHO standards)
- Advanced AIDS (wasting, super-infection, cardiomyopathy, and neuropathy secondary to HIV, as defined by WHO clinical stage 4)

## Laboratory Procedures

All laboratory analytical procedures were carried out in a blinded fashion. Serum electrolyte levels were determined at the bedside with an iStat (i-STAT, Abbott Laboratories, Abbott Park, IL, USA). AST and ALT were determined in the laboratory with a Cobas Mira biochemistry spectrophotometry analyzer (Roche Diagnostics, Indianapolis, IN, USA). CBCs were analyzed with an ABX Pentra 60 cytochemistry analyzer (Horiba, Kyoto, Japan). Plasma from blood samples was collected in EDTA and separated by centrifugation at 1730g for 5 minutes at room temperature and stored at  $-80^{\circ}\text{C}$ .

Patients were initially tested for the presence of parasites with the SD Bioline Malaria Ag P.f/Pan (Standard Diagnostics Ref 05FK60, Inc., Suwon City, Republic of Korea) rapid diagnostic test. The presence of parasites was then confirmed by a thick smear and patients were considered negative if no parasites were detected over 100 fields. Parasite species was confirmed by thin blood smear, and counts were determined by thick smear, after staining with 10% Giemsa, and asexual parasites were counted over 200 – 500 leukocytes (depending on parasite density) and converted to parasites/ $\mu\text{L}$  of blood by assuming a blood leukocyte concentration of 8000/ $\mu\text{L}$  whole blood.

Plasma Ang-1 and Ang-2 levels were determined using ELISA assays (R&D Systems, DANG10 and DANG20 kits, Minneapolis, MN, USA). Plasma TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and IL-10 levels were measured using the BD multiplexed enhanced sensitivity bead system (Becton Dickinson, Waltham, MA, USA). Plasma S100B levels were measured at MGH using a human S100B ELISA assay (BioVendor, Heidelberg,

Germany). Results for angiotensin, cytokine, and S100B levels are reported if the patient received study drug for  $\geq 48$  hours, and if there were paired data points at baseline and 48h of treatment.

Plasma levels of NO metabolites (nitrate, nitrite, RSNO, and RNNO) from 14 patients receiving NO and 18 breathing N<sub>2</sub> were measured using high-pressure liquid chromatography and gas-phase chemiluminescence techniques, as described previously [1].

## **GAS DELIVERY DETAILS USING THE INOPulse™**

The INOPulse™ monitors the inspiratory pressure of the patient's breath, and injects a prescribed volume ("pulse") of study gas during the first half of each inhalation, via the nasal cannula. Administering gas via the INOPulse™ minimized both the volume of NO or N<sub>2</sub> gas required for the study and the time NO contacted oxygen, thus reducing formation of nitrogen dioxide. As described in the text, the gas volume to be delivered, and, thus, the dose of NO or N<sub>2</sub> was determined with a nomogram, incorporating the child's weight (to estimate tidal volume) and respiratory rate. When the child inhaled, the INOPulse™ injected 800 ppm of NO into the nasal cannula. We estimate that a ten-fold dilution of the injected gas with alveolar gas volume resulted in an alveolar NO gas concentration of approximately 80 ppm. The delivered NO dose per hour (mg/kg per hr) was calculated by multiplying the estimated inhaled concentration by the estimated minute ventilation and divided by the patient weight in kilograms.

## **Concomitant Treatment And Interventions**

A blood and urine culture, and lumbar puncture were obtained whenever possible from all patients. Repeat urine, blood, and/or cerebral spinal fluid cultures were obtained if there was evidence of bacterial infection.

Fundoscopy was attempted on patients within the first 24 hours of treatment. Cycloplegia was obtained with myadriaticum drops (tropicamide 0.5%) with phenylephrine 5% within the first 24 hours of treatment. Selected aspects of malarial retinopathy were recorded when present, including: hemorrhages, papilledema, macular and peripheral retinal whitening, and vascular changes.

Blood transfusions (10 mL/kg of packed red blood cells or 20 mL/kg of whole blood) were given whenever hemoglobin was  $\leq 7$  g/dL. Children were given ascorbic acid (5 – 10 mg/kg/day) via mouth or nasogastric tube for 5 days [2]. Patients received enteral feedings via a nasogastric tube, when appropriate. Anti-seizure medications, antipyretics, antibiotics, and fluid boluses were given at the discretion of the clinician.

## **How The Study Was Powered**

Using previous data on Ang-1 on children with CM, reported in the literature, a sample size of 92 (46 in each arm) was needed to provide adequate power (80%) with a 2 sided test and alpha of 5% to detect the expected difference in Ang-1 between the two arms, using the Wilcoxon (Mann-Whitney) rank-sum test (nQuery Advisor 6.01).

**For Figure 1.** Detailed reasons for excluding patients from analyses

**N<sub>2</sub>:**

Three patients assigned to the N<sub>2</sub> breathing arm were not treated per protocol: all 3 died prior to 24 hours, including one that was randomized but died just prior to the initiation of treatment.

**NO:**

Five patients assigned to the NO breathing arm were not treated per protocol: 1 patient died prior to 24 hours; 2 patients were removed from treatment prior to 24 hours for high methHb% levels; and 1 was enrolled and randomized at the same time she developed a GCS of 7/15, too high for inclusion, and thus was removed from analysis.

**Supplement Table S1.** Plasma IFN- $\gamma$ , IL-10, and MCP-1 Levels At Enrollment and After 48 Hours. All data mean $\pm$ SD.

Cytokines	Interval	N <sub>2</sub> (N=40)	NO (N=40)	Intra-group p-value	Inter-group p-value
IFN- $\gamma$ , pg/mL	Hr 0	8.02 $\pm$ 20.85	9.15 $\pm$ 19.64		0.20
	Hr 48	1.01 $\pm$ 4.17	0.92 $\pm$ 3.02	N <sub>2</sub> 0.0129; NO < 0.0001	0.65
IL-10, pg/mL	Hr 0	162.2 $\pm$ 209.7	153.6 $\pm$ 183.1		0.77
	Hr 48	8.8 $\pm$ 12.1	9.7 $\pm$ 14.1	N <sub>2</sub> < 0.0001; NO < 0.0001	0.94
		N <sub>2</sub> (N=37)	NO (N=37)		
MCP-1, pg/mL	Hr 0	133.8 $\pm$ 211.6	215.9 $\pm$ 535.1		0.36
	Hr 48	25.3 $\pm$ 34.7	49.6 $\pm$ 68.8	N <sub>2</sub> 0.0039; NO 0.038	0.024

**Supplement Table S2.** Secondary Laboratory Outcomes. Plasma creatinine, blood lactate, and plasma RNNO and RSNO levels. \*Absence of paired data. All data mean±SD.

Lab values	Interval	N <sub>2</sub> (N)	NO (N)	Intra-group p-value from baseline, Hr 0	Inter-group p-value
Creatinine, mg/dL	Hr 0	1.09 ± 0.92 (38)	1.18 ± 1.10 (36)		0.79
	Hr 48	1.54 ± 3.00 (37)	0.80 ± 0.93 (36)	N <sub>2</sub> = 0.139; NO < 0.0001	0.42
	Day 5	0.71 ± 0.84 (33)	0.83 ± 1.33 (34)	N <sub>2</sub> = 0.0058; NO = 0.0037	0.83
Lactate, mg/dL	Hr 0	24.23 ± 12.68 (42)	24.01 ± 17.02 (41)		0.64
	Hr 48	16.84 ± 7.18 (42)	17.62 ± 11.66 (41)	N <sub>2</sub> = 0.0049; NO = 0.029	0.56
	Day 5	23.92 ± 9.08 (36)	20.96 ± 7.91 (39)	N <sub>2</sub> = 0.846; NO = 0.5572	0.19
RNNO, nM	Hr 0	29.4 ± 21.1 (4)	32.0 ± 22.9 (6)		0.86
	Hr 48	15.1 ± 8.0 (4)	15.1 ± 9.3 (5)	N/A*	1.0
	Day 5	14.1 ± 5.7 (5)	10.3 ± 2.6 (5)	N/A*	0.21
RSNO, nM	Hr 0	12.5 ± 8.3 (4)	20.1 ± 23.0 (6)		0.55
	Hr 48	9.1 ± 4.9 (4)	10.3 ± 7.4 (5)	N/A*	0.79
	Day 5	6.6 ± 7.3 (5)	22.8 ± 20.3 (5)	N/A*	0.13

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