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NITRIC OXIDE FOR THE ADJUNCTIVE TREATMENT OF SEVERE MALARIA: HYPOTHESIS AND RATIONALE

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Abstract

We hypothesize that supplemental inhaled nitric oxide (iNO) will improve outcomes in children with severe malaria receiving standard antimalarial therapy. The rationale for the hypothesized efficacy of iNO rests on: (1) biological plausibility, based on known actions of NO in modulating endothelial activation; (2) pre-clinical efficacy data from animal models of experimental cerebral malaria; and (3) a human trial of the NO precursor L-arginine, which improved endothelial function in adults with severe malaria. iNO is an attractive new candidate for the adjunctive treatment of severe malaria, given its proven therapeutic efficacy in animal studies, track record of safety in clinical practice and numerous clinical trials, inexpensive manufacturing costs, and ease of administration in settings with limited healthcare infrastructure. We plan to test this hypothesis in a randomized controlled trial (ClinicalTrials.gov Identifier: NCT01255215).

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CONFLICT OF INTEREST STATEMENT CM is Chief Scientific Officer of Nitric Solutions Inc., developer of nitric oxide (NO) based medical products.

KCK, WCL, and ALC are listed as inventors on a patent owned by University Health Network (Toronto) related to Ang-2 as a biomarker for infectious diseases that compromise endothelial integrity.

All other authors: no conflicts.

INTRODUCTION

Malaria causes approximately 800,000 deaths annually, mostly among children in sub-Saharan Africa [1]. Although the use of artemisinin-based antimalarial therapy has improved outcomes in severe malaria, the mortality rates remain high [2]. Adjunctive therapies that target the underlying pathophysiology of severe malaria may further reduce morbidity and mortality in severe malaria [3]. Nitric oxide (NO) is an attractive, as yet untested, potential adjunctive treatment for severe malaria because it modulates endothelial activation, a critical pathway in the pathogenesis of severe malaria. Based on promising pre-clinical data from animal models [4] and a human trial using the NO precursor L-arginine [5], together with its established record of safety in clinical practice, a clinical trial evaluating nitric oxide for the adjunctive treatment of severe malaria is warranted.

The most common life-threatening clinical syndromes associated with *P. falciparum* infection in children are cerebral malaria (CM), respiratory distress with metabolic acidosis, and severe malarial anemia [6]. Critical pathogenic mechanisms in these severe malaria syndromes represent potential targets for adjunctive therapies, including nitric oxide. CM presents as a diffuse symmetrical encephalopathy with altered level of consciousness and/or repeated seizures [6]. The pathogenesis of CM is incompletely understood but may be due to one or more of the following mechanisms: sequestration of parasitized erythrocytes within the cerebral microvasculature [7-9], reduction in microvascular flow [10], metabolic alterations including hypoglycemia and hypoxia, host inflammatory response [11-14], blood-brain barrier dysfunction [15, 16], and cerebral edema [17, 18]. In African children with severe malaria, lactic acidosis secondary to impaired tissue perfusion appears to explain most cases of respiratory distress, characterized by deep (Kussmaul) respirations. Respiratory distress therefore represents a manifestation of decompensated shock, frequently associated with multi-system organ failure and widespread endothelial dysfunction. Severe malarial anemia is caused by both increased destruction of parasitized and non-parasitized erythrocytes, as well as impaired hematopoiesis [19]. Our hypothesis focuses on two of the three common manifestations of severe pediatric malaria: cerebral malaria and respiratory distress with metabolic acidosis. We postulate that nitric oxide may modulate the deleterious host responses that characterize these syndromes, including systemic inflammation and endothelial dysfunction.

THE HYPOTHESIS

We hypothesize that Ugandan children with severe malaria will benefit from adjunctive iNO in addition to standard anti-malarial therapy. We will test this hypothesis by comparing the change of angiopoietin-2 (Ang-2), an objective and quantitative biomarker of malaria severity, measured longitudinally over the hospital admission, between two parallel groups randomized to receive iNO or placebo (primary outcome). We will also compare relevant clinical outcomes including therapeutic efficacy, safety and neurocognitive outcome (secondary outcomes). The secondary hypotheses are that adjunctive iNO will reduce mortality, accelerate recovery times, and shorten length of hospital stay in severe malaria. Furthermore, we hypothesize that iNO will ameliorate biomarkers of host response to severe malaria including whole blood lactate without affecting parasite clearance. We hypothesize that iNO will reduce the rate of adverse neurocognitive sequelae following severe malaria. Finally, we hypothesize that adjunctive iNO will be safe and well tolerated in children treated for severe malaria. We plan to test these hypotheses in a resource-constrained field setting in Uganda.

RATIONALE

Endogenous NO synthesis and regulation

NO is a gaseous free radical that is endogenously produced by the conversion of L-arginine and molecular oxygen to L-citrulline by members of the NOS family of enzymes. Three isoforms have been described to date: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Tissue expression of nNOS is primarily in neurons of the brain and peripheral nervous system and eNOS is expressed mainly in epithelial cells, although these isoforms also operate in the immune system [4]. Active NOS is a tetramer of two NOS proteins and two calmodulin molecules. Cofactors for the enzyme include (6R)-tetrahydrobiopterin (BH₄), FAD, FMN and iron protoporphyrin IX (haem).

Both nNOS and eNOS are constitutively expressed and are inactive in resting cells and are regulated by intracellular calcium flux. Increased free intracellular calcium stabilizes the interaction of calmodulin to nNOS or eNOS and stimulates the production of NO. This form of regulation leads to transient and short-lasting production of NO, which functions in neuronal signalling and vasodilation. In contrast, iNOS is found in most resting cells, has calcium-independent activity due to tight binding to calmodulin even at low levels of intracellular calcium, and produces high levels of NO for prolonged periods of time. Although all three NOS isoforms have similar NO production rates ($\sim 1 \mu\text{M min}^{-1}\text{mg}^{-1}$), iNOS is responsible for high-level production of NO by phagocytes because it is highly expressed after activation [20]. Expression of iNOS in inflammatory and tissue cells is upregulated by exposure to microbial products such as lipopolysaccharide (LPS) and dsDNA or cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferon- γ (IFN- γ) [20, 21]. Enzyme levels are regulated at the level of transcription as well as mRNA stability [20].

Physiologic actions of NO

NO is a highly reactive molecule due to its unpaired electron, mediating a range of effects through three main molecular mechanisms. First, NO reacts readily with transition metals such as iron, copper and zinc, thereby modifying the function of numerous enzymes. Second, NO reacts with thiol groups (e.g., cysteine residues) abundantly present on many proteins to produce S-nitrosothiols, and alters the function of several proteins including p21^{ras}, coxsackievirus protease A2, as well as transcription factors, kinases involved in signalling cascades, caspases, ion channels and metabolic proteins [21]. Third, nitric oxide reacts rapidly with superoxide anion (O₂⁻) to produce peroxynitrite which is a powerful oxidant, capable of modifying proteins, lipids and nucleic acids. Peroxynitrite plays an important microbicidal role; however, excessive peroxynitrite formation may lead to cytotoxicity through nitration of proteins and inhibition of mitochondrial respiration.

NO has a half-life of several seconds. It can readily diffuse across cell membranes into neighbouring cells, acting as an intercellular messenger. In addition, NO may produce effects distant from its site of production transported by vehicles such as low-molecular weight S-nitrosothiols, S-nitrosylated proteins including haemoglobin and albumin, and nitrosyl-metal complexes which liberate NO spontaneously or after cleavage by ectoenzymes [20].

The activity of NO *in vivo* can be monitored indirectly by measurement of the stable byproducts of NO oxidation, nitrite (NO₂⁻) and nitrate (NO₃⁻), collectively termed NO_x. Dietary factors, exercise and renal insufficiency affect levels of NO_x in body fluids such as plasma and urine, and need to be controlled in comparative studies. Other measures of NO activity are functional (e.g., the NO-mediated reactive hyperaemia peripheral arterial

tonometry (RH-PAT) index), or biochemical (e.g., levels of cGMP, a downstream signalling molecule) [22].

One important molecular target of NO is soluble guanylate cyclase (sGC), an enzyme containing a heme moiety with ferrous iron. Formation of a ferrous-nitrosyl-heme complex alters the porphyrin ring structure and leads to activation of sGC with a 400-500 fold increase in the rate of cGMP synthesis [21]. Intracellular signalling by cGMP is mediated to a large extent by cGMP dependent protein kinase (PKG) which promotes smooth muscle relaxation, as well as platelet and neutrophil activation.

Through its action on numerous target molecules, NO is involved in a broad range of physiologic processes. NO was identified originally as the endothelium-derived relaxation factor that mediates vasodilation [23]. Subsequent studies have demonstrated a role for NO in platelet aggregation [24], endothelial-cell activation [25], apoptosis, inflammation, chemotaxis, neurotransmission and antimicrobial defense.

NO in infectious disease

NO plays a complex and versatile role in the pathogenesis and control of infectious diseases. Protective and toxic effects of NO are frequently seen in parallel in the setting of infection because of its variety of molecular targets, widespread production by diverse cell types, and broad capacity for intra- and intercellular signalling [20].

NO has direct microbicidal activity against numerous viral, bacterial and parasitic agents. The mechanism of action may involve mutation of DNA; inhibition of DNA repair and synthesis; inhibition of protein synthesis; alteration of proteins by S-nitrosylation, ADP-ribosylation or tyrosine nitration; inactivation of iron, copper or zinc-dependent enzymes; and peroxidation of lipid membranes [20]. Peroxynitrite (ONOO^-), a reaction product of NO and superoxide anion (O_2^-), may mediate these effects. Highlighting the central role of ONOO^- in host defense, successful human pathogens, including *M. tuberculosis* and *S. typhimurium*, possess counteracting peroxiredoxins that detoxify ONOO^- to nitrite [26].

NO attenuates neutrophil respiratory burst and neutrophil-derived oxidative stress [27], and reduces neutrophil rolling and adhesion in microvascular endothelial cells [28]. In adaptive immune responses, NO inhibits T-cell and B-cell proliferation [29]. NO alters cytokine responses, down-regulating pro-inflammatory IL-1, IL-2, TNF and IFN- γ and increasing the production of IL-4, IL-13, and transforming growth factor- β [20].

NO in malaria

NO plays a role at multiple stages of malaria infection, beginning with the innate defences of the *Anopheles* mosquito vector, where NO antagonizes the *Plasmodium* parasite [30]. On the other hand, by reversibly binding to salivary proteins (nitrophorins), NO facilitates the mosquito blood meal by enhancing vasodilation and antagonizing hemostasis [31].

Despite its potent microbicidal properties against a number of human pathogens [20], NO does not appear to have a direct effect against *Plasmodium* species. Exogenous NO did not inhibit the growth of *P. falciparum* *in vitro*, even at saturating concentrations (2 mM) [32]. Byproducts of NO metabolism, including NO_2 , NO_3^- and the nitrosothiol derivatives of cysteine and glutathione, exhibit anti-parasitic activity *in vitro* [32], albeit at concentrations 2 to 3 orders of magnitude higher than those in human plasma during malaria infection [33]. In experimental murine infection *in vivo*, some studies have found no effect of iNOS deficiency or pharmacologic inhibition of NOS on *P. berghei* [34, 35], *P. chabaudi* [36-39] and *P. yoelii* [40] parasitemia, suggesting a minimal direct anti-parasitic role of endogenous NO, despite important modifying effects on host disease severity [38, 40]. Likewise, in

human observational studies, NO_x levels are not consistently associated with parasitemia, although they are inversely correlated with disease severity [41, 42].

NO dampens endothelial activation

The vascular endothelium plays a critical role in the pathogenesis of cerebral malaria. Parasitized erythrocytes (PEs) adhere to the microvascular endothelium resulting in sequestration and vascular obstruction, impaired perfusion and tissue hypoxia [43]. Autopsy studies in fatal cerebral malaria reveal sequestration of PEs in the capillaries and post-capillary venules of multiple organs [8]. Cytoadherence is mediated through constitutive and cytokine-inducible receptors on the endothelial cells, including intercellular cell adhesion molecule-1 (ICAM-1) [44]. NO decreases endothelial cell adhesion molecule expression [45], and has been shown to reduce the adherence of PEs to endothelial cells *in vitro* under flow conditions [46].

Severe malaria is characterized by marked activation of the microvascular endothelium. One prominent feature of endothelial activation is the exocytosis of intracellular Weibel-Palade bodies (WPB), causing the release of von Willebrand factor (vWF) [47, 48] and angiopoietin-2 (Ang-2) [49, 50] into the circulation. Ang-2 functions as an autocrine regulator by sensitizing the endothelium to the effects of TNF, resulting in increased adhesion receptor expression [51]. NO inhibits the exocytosis of WPB contents through S-nitrosylation of critical regulatory enzymes [25]. Thus, Ang-2 serves as critical regulator of endothelial activation.

Elevated Ang-2 levels are associated with poor clinical outcome in severe malaria [49, 50] and Ang-2 has been used to follow disease progression and recovery in previous studies of malaria [49]. Among survivors of severe malaria, Ang-2 levels have been shown to decrease linearly during recovery at a mean rate of 2700 pg/mL per 24h [49]. Thus, Ang-2 is an objective, quantitative marker of disease severity, validated for longitudinal follow-up of patients with malaria. As a result, measurement of Ang-2 levels will allow us to test our hypothesis with precision, and we have chosen Ang-2 as the primary outcome of our clinical trial and have

NO reduces inflammatory injury in the pulmonary vascular bed

Pulmonary involvement in adults with severe malaria is associated with high mortality and represents another manifestation of severe malaria for which iNO may be beneficial [52, 53]. Mechanisms of lung injury in severe malaria in adults share common features with acute lung injury/adult respiratory distress syndrome (ALI/ARDS) in sepsis. Increased permeability of the capillary-alveolar barrier is considered to be the principal functional abnormality underlying ALI/ARDS due to malaria and other causes. Other key events include erythrocyte sequestration and host inflammatory response to parasite products released into the circulation [54]. Lung histopathologic and ultrastructural studies from adults with fatal *P. falciparum* infection have found septal and interstitial edema, monocytes and parasitized erythrocytes (PE) adherent to the capillary microvasculature, and endothelial cell cytoplasmic swelling [8, 55]. Similarly, in a murine model of experimental malaria-induced lung injury, disruption of the capillary-alveolar membrane barrier and septal inflammation was observed [56]. Thus, the pulmonary vascular endothelium plays a central role in ALI during malaria infection, as the site of PE and leukocyte adhesion, and the target of parasite-induced inflammation. Endogenous NO inhibits inflammatory injury in murine ALI models, as evidenced by increased vascular leak and pathology in iNOS^{-/-} mice [57, 58]. Some studies have documented that iNO decreases pulmonary capillary pressure through selective vasodilatory effects on post-capillary venules [59], and reduces pulmonary edema in patients with ALI [60]. Furthermore, in experimental models of ALI, mice lacking

inducible nitric oxide synthase had fewer neutrophils sequestered in the pulmonary vasculature [61], and inhaled NO reduced the accumulation of neutrophils in the pulmonary vasculature and air space [62]. Similar effects of inhaled nitric oxide on leukocyte kinetics are observed outside the lung in rodent models of severe sepsis [63]. iNO may therefore be a useful adjunct for the treatment of adults with ALI secondary to severe malaria.

On the other hand, ALI appears to be rare in children with *P. falciparum* infection. Respiratory distress in children with severe malaria is more often associated with metabolic acidosis, and represents respiratory compensation for primary lactic acidosis related to impaired tissue perfusion. In children hospitalized with malaria in sub-Saharan Africa, metabolic acidosis and hypovolemia are common presenting signs [64, 65], fluid replacement rather than fluid restriction restores cardiopulmonary homeostasis [65], and echocardiography frequently reveals tachycardia, low stroke volume index, and high inferior vena cava collapsibility index, which improve with fluid replacement therapy [66]. Of note, lactic acidosis is a prognostic marker for mortality in children with severe malaria [67], and its association with respiratory distress represents a final common pathway of decompensated shock, cardiopulmonary insufficiency and impending death. Respiratory distress with deep (Kussmaul) breathing in children with severe malaria carries a mortality rate higher than cerebral malaria [68].

Despite these important differences in the pathophysiology underlying respiratory distress in adults and children with severe malaria, we hypothesize that iNO may also benefit African children with respiratory distress/metabolic acidosis. We speculate that the local action of iNO on the endothelium of the pulmonary vascular bed and/or more distal effects on the endothelium systemically, may modulate the cascade of inflammation and reduce the associated systemic inflammatory response syndrome.

Action of iNO beyond the pulmonary vasculature

While iNO has been primarily used for the treatment of pulmonary disease, it produces pharmacologic activity outside the pulmonary vasculature through well elucidated mechanisms of blood NO transport. Gaseous nitric oxide in inspired air readily diffuses across the alveolar-capillary membrane to reach the pulmonary vasculature. Although NO is short-lived in biological fluids by virtue of its unpaired electron, reaction products of NO with blood act to conserve its biological activity and transduce an endocrine function [69]. Numerous species subserve this activity, including S-nitroso-albumin, nitrite, iron-nitrosylated hemoglobin, S-nitroso-hemoglobin, plasma haptoglobin-hemoglobin complexes, nitrated lipids, N-nitrosamine, and other iron-nitrosyl complexes [69-71]. A physiologic example is the regulation of systemic vasomotor tone through blood NO transport to smooth muscle targets distal from its site of production. In clinical trials, NO administered via the inhalational route produces systemic effects in the treatment of sickle cell disease vaso-occlusive crisis [72, 73]. Likewise, inhaled NO exerts distant effects in the cerebral vasculature and brain parenchyma. iNO is neuroprotective in animal models of brain injury [74, 75]. Furthermore, in humans, iNO has been shown to decrease the risk of severe intraventricular hemorrhage or periventricular leukomalacia [76], and improve long-term neurocognitive outcomes in premature neonates [77]. These precedents suggest that iNO may also produce systemic and cerebral end-organ effects in the setting of severe malaria.

Severe malaria syndromes are characterized by low nitric oxide bioavailability

In an animal model, reduced NO bioavailability contributed to the pathogenesis of ECM [4]. NO supplementation with either a NO donor (dipropyleneetriamine NONOate) or NO gas provided marked protection against severe disease [4]. Our laboratory has generated

evidence that inhaled NO decreases inflammation, rescues blood-brain barrier dysfunction and reduces parasite sequestration in the brain (Serghides, Kim, et al., unpublished data). Likewise, African children with severe malaria have impaired production of NO [78], low levels of mononuclear cell iNOS expression [78], and low plasma levels of the NOS substrate arginine [79].

Natural variation in the genes encoding the NOS enzymes in human populations lends additional evidence for a protective role of endogenous NO against severe malaria syndromes. Polymorphisms in the promoter region of the iNOS gene at positions -954 (G-->C) and -1173 (T-->C), located at a putative gene repressor binding site [80], are associated with higher NOS enzymatic activity [80] and higher plasma and urine levels of NO_x [41]. These polymorphisms are common in African but not Asian populations [80-83] and are associated with protection from severe malaria in several reports [41, 80, 84]. Another iNOS promoter polymorphism consisting of CCTTT_(n) pentanucleotide microsatellite repeats 2.5kb upstream from the iNOS transcription start site has been associated with susceptibility to malaria, although findings from published reports are inconsistent, with long forms of the allele associated with severe disease in some studies [81, 83, 85] but not others [86]. Genetic variation in other NOS isoforms has also been shown to influence the risk of cerebral malaria. A single amino acid substitution at position 298 (Glu-->Asp) in eNOS was associated with increased plasma levels of NO_x and protection from cerebral malaria in Indian adults [87]. A single nucleotide polymorphism at position -84 (G-->A) of the nNOS gene is responsible for decreased basal transcriptional level, and is associated with increased risk of cerebral malaria in Indian adults [88]. Taken together, these findings from human population genetic studies indicate that nitric oxide synthase gene polymorphisms affect susceptibility to malaria via alterations in NO production and lend support to a protective role for NO against severe malaria syndromes. Findings from the published reports are summarized in Table 1.

Inhaled nitric oxide in clinical practice and clinical trials

NO is approved for use by the US FDA for the treatment of neonates with hypoxic respiratory failure [89]. NO has been used in numerous clinical trials involving older children and adults, reviewed in Table 2. One meta-analysis of 12 trials including 1237 patients with acute lung injury/acute respiratory distress syndrome demonstrated that iNO has an excellent safety profile, although renal dysfunction may occur in critically ill adult ICU patients [90].

Unlike NO donors such as L-arginine, nitroglycerine and sildenafil, iNO has not been reported to cause systemic vasodilation, electrolyte disturbance or effects on blood glucose. Furthermore, unlike NO donors, inhaled NO does not require functional endothelial cell NO synthase. NOS may be compromised in ill patients and therefore there may be a deficit in their ability to generate NO from these donor molecules. It has been used in a wide variety of clinical settings in children and adults, including acute respiratory distress syndrome, pulmonary hypertension, and pregnancy-induced pulmonary hypertension [91]. Outside the current licensing context, NO may be cheaply manufactured, and may be easily administered by mask with minimal infrastructure. These pragmatic considerations make iNO an appealing candidate adjuvant, appropriate for resource-constrained malaria-endemic areas. Although iNO is not routinely used in clinical practice in most hospitals in sub-Saharan Africa and most clinicians in this setting will not be immediately familiar with its use, the ease of administration with cylinder and mask, in a manner similar to oxygen supplementation, may allow for rapid uptake and scale-up of iNO in the African context, should it prove effective.

EVALUATION OF THE HYPOTHESIS

To test our hypothesis, we plan to conduct a randomized controlled trial of adjunctive iNO among Ugandan children with severe malaria. Details of the experimental plan (trial protocol) are outlined in the US NIH trials registry, clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT01255215), and are summarized briefly below.

Study Design

The study will be a prospective, parallel arm, randomized, placebo-controlled, blinded clinical trial of adjunctive continuous inhaled nitric oxide at 80 ppm versus placebo (both arms in addition to standard anti-malarial therapy), among children aged 1-10 years of age with severe malaria.

Inclusion criteria

1. Age 1-10 years
2. Positive malaria rapid diagnostic test
3. Features of severe malaria [6]: repeated seizures, prostration, impaired consciousness, or respiratory distress
4. Willing and able to complete follow up schedules for the study – 14 day and 6 months after hospital discharge

Exclusion criteria

1. Baseline methemoglobinemia (>2%)
2. Known chronic illness: renal, cardiac, or hepatic disease, diabetes, epilepsy, cerebral palsy, or clinical AIDS
3. Severe malnutrition
4. Severe malarial anemia (Hb <50g/L) without other signs of severe malaria.

Study setting

The study will be conducted at a single pediatric hospital in Jinja, Uganda. Uganda is a low-income country with a severely resource-constrained healthcare system. Malaria transmission is moderate and seasonal in Jinja, which lies on the northern shore of Lake Victoria in the central area of Uganda near the capital, Kampala. Jinja Regional Referral hospital admits at least 175 children with severe malaria annually (excluding cases of severe malarial anemia), representing over 30% of all admissions. *P. falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine is widespread in the country (34% to 67%) [92].

Treatment groups

Participants in the intervention group will receive iNO at a concentration of 80 ppm, in addition to Ugandan standard of care of severe malaria, which includes a potent antimalarial agent (either parenteral quinine or artesunate). iNO will be administered continuously via non-rebreather face mask for a maximum period of 72 hours, but may be discontinued earlier if a patient recovers and no longer tolerates the face mask. An air compressor will be used to deliver continuous flow of vehicle air. Participants in the control group will receive a continuous flow of room air delivered using an air compressor (indistinguishable in odour and appearance from the mixture of 80 ppm iNO), in addition to Ugandan standard of care of severe malaria.

Randomization and blinding methods

Simple randomization will be employed, using a computer-generated randomization list. Treatment allocation will be recorded on paper and kept in sequentially numbered sealed opaque envelopes, which will be drawn for each randomized participant by an unblinded investigator who is not responsible for patient care, laboratory or data analysis.

Trial participants, their parents/guardians, and all study personnel involved in the clinical management, assessment of outcomes, and laboratory testing will be blinded to the treatment assignment. An unblinded investigator not involved in patient care will be responsible for the administration, monitoring and recording of iNO, NO₂, and methemoglobin levels. Cylinders containing NO will be attached to the ventilation circuit in all patients, but the flow of NO will be controlled according to treatment arm assignment in a blinded manner. Laboratory analysis for Ang-2 levels (primary outcome) and all other parameter will occur in a manner blinded to treatment allocation.

Outcome measures

The longitudinal change in serum Ang-2 concentration over the first 72 hours of hospital admission will be the primary efficacy endpoint. Ang-2 is an objective, quantitative marker of disease severity, validated for longitudinal follow-up of patients with malaria [49, 50]. A mixed-effects linear model will be used to compare the change in Ang-2 over time between treatment arms.

Secondary outcomes of the trial will include relevant clinical, laboratory and neurocognitive endpoints. We will compare the following clinical endpoints, consistent with other therapeutic trials for malaria [2, 93, 94]: mortality at 48 hours and 14 days after admission; recovery times (time to fever resolution, time to sit unsupported); and length of hospital stay. Parasitological outcomes including time to parasite clearance and parasite recrudescence/re-infection at 14 day follow-up will also be compared between treatment groups. Biomarkers of disease severity (in addition to Ang-2, the primary study endpoint), including whole blood lactate, will also be followed. Lactate is produced by the anaerobic metabolism of glucose in the absence of adequate tissue oxygenation, and elevated lactate levels represent a final common pathway of tissue hypoxia and decompensated shock, the forerunner of cardiovascular collapse and death. We will measure lactate as an independent biomarker of disease severity during the clinical trial. Biomarkers of endothelial activation, inflammation, and coagulopathy will also be followed as they may provide additional insight into the pathways and processes altered in cerebral malaria and modulated by iNO delivery. Finally, neurocognitive outcomes in children with severe malaria will be followed in order to determine if adjunctive iNO may have a neuroprotective effect. The overall cognitive deficit at 6 months after discharge will be assessed by performing neuropsychological tests as previously described [95].

CONSEQUENCES OF THE HYPOTHESIS

Advances in malaria therapeutics have broad potential for global impact because of the large number of deaths attributable to severe malaria [1]. Residual mortality remains high despite potent antiparasitic treatment, underscoring the need for adjunctive therapies that target critical pathways in malaria pathogenesis [2]. Previously tested adjunctive treatment strategies for severe malaria include immunomodulation, iron chelation, reduction of oxidative stress, anticoagulation, volume expansion, reduction of intracranial pressure, and prevention of seizure activity [96]. Only volume expansion with albumin has been associated with a mortality benefit [96-98]. In this context, iNO, if demonstrated to be effective, would represent an important advance, filling a gap in the existing armamentarium against malaria. Given its low manufacturing cost and applicability in resource-constrained

settings, NO could be rapidly scaled up to reach peripheral zones where severe malaria is most prevalent. Thus, our hypothesis that supplemental inhaled nitric oxide (iNO) will improve outcomes in children with severe malaria receiving standard antimalarial therapy, if borne out by experimental evidence from randomized controlled trials, has the potential to alter clinical practice and save lives.

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Research Highlights

- Severe malaria carries high mortality despite the best available anti-malarial therapy.
- The pathogenesis involves endothelial activation and release of Angiopoietin-2 (Ang-2).
- Nitric oxide (NO) inhibits Ang-2 release and promotes endothelial quiescence.
- We hypothesize that inhaled NO will improve outcomes in children with severe malaria.

Polymorphisms in nitric oxide synthase genes and their association with malaria disease severity.

Table 1

NOS gene	polymorphism	Population studied	Effect on nitric oxide levels	Susceptibility to malaria	Refs
iNOS promoter	1173 C-->T	Tanzanian children	Increased NOx	Protection against cerebral malaria and severe malarial anaemia	[41]
	954 G-->C	Children in Gabon	7-fold higher baseline NOS activity	Reduced number of malarial attacks Reduced disease severity	[80, 84]
	CCTTT _n repeat	Indian and Thai adults, Ghanaian children	NR	Longer repeats associated with severe malaria	[81, 83, 85]
		Gambian children	NR	Shorter repeats associated with severe malaria	[86]
eNOS		Asymptomatic children and adults, Papua New Guinea	No association between CCTTT repeat number and plasma NOx levels	No association with severe malaria	[41, 82]
		Indian adults	Increased NOx	Protection from cerebral malaria	[87]
	298 G-->A				
nNOS	84 G-->A	Indian adults	Decreased basal transcriptional activity	Increased risk of cerebral malaria	[88]

NR; not reported

Table 2

Randomized clinical trials of inhaled nitric oxide.

Reference	Study design	Number of patients	Age	Target condition	Dose and delivery route	Adverse events
NINOS. <u>NEJM</u> . 1997.[99]	prospective multicentre controlled RCT	235 (iNO n=114, nitrogen gas n=121)	full-term and nearly full-term infants	hypoxic respiratory failure	inhaled, 20-80 ppm	Concentration of iNO reduced in 11 patients because of methHb (5-10%)
Davidson et al. <u>Pediatrics</u> . 1998 [100]	prospective multicentre controlled RCT	155 (iNO n=114, nitrogen gas n=41)	term infants	persistent pulmonary hypertension (PPHN)	inhaled, 5, 20 and 80 ppm	Elevated methHb (>7%) in 13/37=35% patients receiving 80ppm; and nitrogen dioxide (NO ₂ >3%) in 7/37=19% patients receiving 80ppm
Dobyns et al. <u>J Pediatr</u> . 1999.[101]	prospective, multicentre, placebo-controlled RCT	108 (placebo gas n=55, iNO n=53)	children, median age 2.5y	respiratory failure	inhaled at 10pp from 3-7 days after entry	methHb none, NO ₂ high none, no difference in ICU-dependent therapies
Michael JR et al. <u>Am J Respir Crit Care Med</u> . 1998.[102]	prospective, multicentre RCT	40 (no treatment n=20, increasing iNO n=20)	adults and children age 1-79y	between 12h and 25d after ARDS developed	inhaled at 5, 10, 15, 20 ppm x6h each for 72h	bleeding (1/20 required blood tx, 1/20 ICH after thrombolytic - both iNO groups); no methHb
Dellinger RP et al. <u>Crit Care Med</u> . 1998.[103]	prospective, phase II, multicentre, placebo-controlled RCT	177 (placebo gas n=57, iNO n=120)	>18yo	non-pregnant, with ARDS not >72h before randomis'n	inhaled, at doses 1.25, 5, 20, 40, 80 ppm x28d or until extubation	methHb >5% if giving 40+ ppm, hypotension, renal failure,coagulopathy; pneumothorax; all events not sig different between groups
Troncy et al. <u>Am J Respir Crit Care Med</u> . 1998.[104]	prospective, single centre RCT: pilot	30 (no trt n=15, iNO n=15)	adults	acute hypoxic respiratory failure	inhaled, increasing doses 2.5, 5, 10, 20, 30, 40 ppm	no methHb
Lundin S et al. <u>Int Care Med</u> . 1999.[105]	prospective, multicentre, open phase III RCT	268 (iNO n=180, placebo n=93)	adults	ALI with uni- or bilateral lung infiltrates, ventilated 18-96h with high O ₂	Inhaled, 1-40ppm given at lowest effective dose for up to 30d	incr sCr >300 NO 5/80; control 2/74; renal replacement thx NO 23/84 vs control 10/79, RR 2.16. Other events more in NO grp: circ failure, encephalopathy, sepsis. No difference in platelet/coagulopathy
Gerlach H et al. <u>Am J Respir Crit Care Med</u> . 2003.[106]	prospective, single centre RCT	40 (no treatment n=20, iNO 10ppm n=20)	adults 18-64y	ARDS, ventilated >48h with FiO ₂ >60%, high PEEP, pulm cap wedge P <18mmHg	10 ppm during inspiration	no methHb, no NO ₂ incr; no bleeding, no difference in additional organ dysfxn

Reference	Study design	Number of patients	Age	Target condition	Dose and delivery route	Adverse events
Taylor RW et al. JAMA . 2004;[107]	prospective multicentre placebo-controlled RCT	385 (iNO n=192, nitrogen gas n=193)	adults	mod-severe ARDS, onset within 72h of randomization, no sepsis	inhaled, 5ppm	no diff. in all events; no metHb, no NO2 incr; sCr incr in NO 22/192 vs control 14/193

metHb: methemoglobin; sCr: serum creatinine; ARDS: adult respiratory distress syndrome