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Impaired nitric oxide bioavailability and L-arginine–reversible endothelial dysfunction in adults with falciparum malaria

Tsin W. Yeo,[1] Daniel A. Lampah,[2] Retno Gitawati,[3] Emiliana Tjitra,[3] Enny Kenangalem,[2] Yvette R. McNeil,[1] Christabelle J. Darcy,[1] Donald L. Granger,[4] J. Brice Weinberg,[5] Bert K. Lopansri,[4] Ric N. Price,[1,6] Stephen B. Duffull,[7] David S. Celermajer,[8,9] and Nicholas M. Anstey[1]

[1]International Health Division, Menzies School of Health Research and Charles Darwin University, Darwin NT 0810, Australia
[2]Menzies School of Health Research-National Institute of Health Research and Development Research Program and District Health Authority, Timika, Papua, Indonesia
[3]National Institute of Health Research and Development, Jakarta 10560, Indonesia
[4]Division of Infectious Diseases, University of Utah and Veterans' Affairs Medical Center, Salt Lake City, UT 84132
[5]Division of Hematology-Oncology, Duke University and Veterans' Affairs Medical Centers, Durham, NC 27710
[6]Centre for Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, Churchill Hospital, Oxford OX37LJ, England, UK
[7]School of Pharmacy, University of Otago, Dunedin 9054, New Zealand
[8]Department of Medicine, University of Sydney, Sydney NSW 2006, Australia
[9]Department of Cardiology, Royal Prince Alfred Hospital, Sydney NSW 2050, Australia

Severe falciparum malaria (SM) is associated with tissue ischemia related to cytoadherence of parasitized erythrocytes to microvascular endothelium and reduced levels of NO and its precursor, l-arginine. Endothelial function has not been characterized in SM but can be improved by l-arginine in cardiovascular disease. In an observational study in Indonesia, we measured endothelial function using reactive hyperemia–peripheral arterial tonometry (RH-PAT) in 51 adults with SM, 48 patients with moderately severe falciparum malaria (MSM), and 48 controls. The mean RH-PAT index was lower in SM (1.41; 95% confidence interval [CI] = 1.33–1.47) than in MSM (1.82; 95% CI = 1.7–2.02) and controls (1.93; 95% CI = 1.8–2.06; P < 0.0001). Endothelial dysfunction was associated with elevated blood lactate and measures of hemolysis. Exhaled NO was also lower in SM relative to MSM and controls. In an ascending dose study of intravenous l-arginine in 30 more patients with MSM, l-arginine increased the RH-PAT index by 19% (95% CI = 6–34; P = 0.006) and exhaled NO by 55% (95% CI = 32–73; P < 0.0001) without important side effects. Hypoargininemia and hemolysis likely reduce NO bioavailability. Endothelial dysfunction in malaria is nearly universal in severe disease, is reversible with l-arginine, and likely contributes to its pathogenesis. Clinical trials in SM of adjunctive agents to improve endothelial NO bioavailability, including l-arginine, are warranted.

CORRESPONDENCE
Nicholas M. Anstey:
anstey@menzies.edu.au

Abbreviations used: ANOVA, analysis of variance; ARDS, acute respiratory distress syndrome; CI, confidence interval; HC, healthy controls; HRP2, histidine-rich protein 2; ICAM-1, intercellular adhesion molecule 1; LDH, lactate dehydrogenase; MSM, moderately severe falciparum malaria; NOS, NO synthase; ppb, parts per billion; RH-PAT, reactive hyperemia–peripheral arterial tonometry; SCD, sickle cell disease; SM, severe falciparum malaria.

Case fatality rates in adults with severe falciparum malaria (SM) remain high despite the use of rapidly parasiticidal antimalarial chemotherapy (1). Adjunctive therapies (in addition to antimalarial treatment) have been used in an attempt to reduce mortality, but none have proved effective to date (2). Targeted interventions with novel adjunctive agents require a better understanding of the pathophysiologic processes that occur in SM.

The vascular endothelium plays a central role in the pathogenesis of SM. Parasitized red cells adhere to constitutive and cytokine-inducible receptors on the microvascular endothelium, resulting in sequestration and vascular obstruction, impaired perfusion, and tissue dysoxia in critical organs (3–6). This cytoadherence is associated with in vitro and histopathological evidence of endothelial inflammation and damage (4, 5, 7, 8). However, there have been no studies examining endothelial function in SM in vivo.

Our previous studies in African children with SM demonstrated impaired production of NO (9), impaired mononuclear cell NO synthase type 2 (NOS2) expression (9), and low plasma concentrations of L-arginine (10), the substrate for NO synthesis from NOS. In an animal model of

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cerebral malaria, reduced NO availability is associated with increased mortality and NO replacement improves survival (11). In vitro, NO reduces the expression of cytokine-inducible adhesion molecules on endothelial cells (12) and decreases cytoadherence of parasitized erythrocytes to the microvascular endothelium (13). Impaired in vivo endothelial NO production in malaria is likely to exacerbate these processes.

An additional mechanism of reduced NO availability has recently been described in disease states with intravascular hemolysis (14). Erythrocyte rupture results in increased cell-free hemoglobin and plasma arginase (15, 16), leading to increased NO consumption and plasma L-arginine catabolism, respectively, and an overall reduction in NO bioavailability (14). In sickle cell disease (SCD), these mechanisms are thought to contribute to endothelial dysfunction and mortality (16–18). Because hemolysis is found in malaria, these processes may also contribute to NO deficiency, endothelial dysfunction, and pathogenesis in SM; however, human studies relative to this issue are lacking.

Endothelial function is characterized by the ability of vessels to dilate in response to increased shear stress or chemical agonists and is inversely related to endothelial activation (19). Impaired endothelial function is found in chronic diseases such as hypercholesterolemia (19) and lysinuric protein intolerance (an inherited deficiency of L-arginine uptake) (20), both of which improve with L-arginine therapy (20, 21). It is unknown whether restoration of plasma L-arginine concentrations can improve endothelial function in acute infections such as malaria.

Our first hypothesis was that endothelial function, exhaled NO, and plasma L-arginine concentrations would be reduced in adults with falciparum malaria in proportion to disease severity, and that measures of hemolysis would be associated with endothelial dysfunction. In stage 1 of this study, we therefore compared each of these parameters among patients with and without SM. Our second hypothesis was that L-arginine infusion in acute malaria would improve endothelial function. In stage 2, we undertook a single ascending dose study of L-arginine infusion in hospitalized patients with moderately severe falciparum malaria (MSM) to demonstrate safety and "proof of concept" of L-arginine infusion as a potential adjunctive therapy targeting the endothelium in malaria.

RESULTS

Stage 1

Subjects. Out of the 158 patients enrolled, 5 were excluded from the SM group because of an alternative diagnoses and 6 were excluded from the control group because of asymptomatic parasitemia (Fig. 1). Baseline characteristics of the remaining 147 patients are shown in Table I. Among the 51 patients with SM, 28 (55%) had coma, 17 (33%) had acute renal failure, 23 (45%) had hyperbilirubinemia with either renal impairment or parasitemia (>100,000 parasitemia/μl), and 30 (59%) had more than one criterion for severe disease (22). In total, 35 (69%) patients received artesunate and 16 (31%) received quinine. All of the 48 patients with MSM were treated with quinine except for one, who received artesunate. There were seven (14%) deaths among SM patients and none in the MSM group. Measurement of exhaled NO was possible in 88% (42 out of 48) of MSM patients and 48% (11 out of 23) of SM patients without coma. Reactive hyperemia–peripheral arterial tonometry (RH-PAT) could be assessed in 99% (145 out of 147), with restlessness precluding reliable measurement in 2 patients with SM.

Endothelial function. The mean RH-PAT index was 1.41 (95% confidence interval [CI] = 1.33–1.47) in patients with SM ($n = 49$), which was significantly lower than in patients with MSM (1.82; 95% CI = 1.7–2.02; $n = 48$) or healthy controls (HC; 1.93; 95% CI = 1.8–2.06; $n = 48$; $P < 0.0001$; Fig. 2 A). There was no significant difference between the HC and MSM groups ($P = 0.49$). Overall, the proportion of

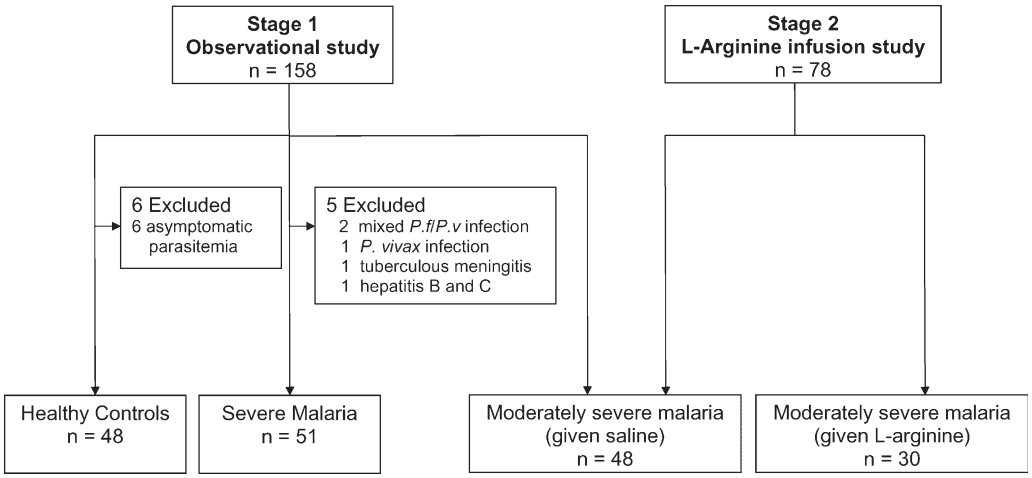

Figure 1. Study profile of patients recruited in stages 1 and 2 of the study. Stage 1 was an observational study to compare endothelial function among individuals with SM, MSM, and HC. Stage 2 was intervention study to measure the effect of L-arginine or saline infusion on RH-PAT index and exhaled NO in MSM.

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Table I. Baseline characteristics of stage 1 patients according to clinical status

	HC	MSM	SM
Number	48	48	51
Age (y): mean (range)	27 (18–42)	28 (18–56)	29 (18–56)
Males: no. (%)	31 (65)	32 (67)	37 (72)
Weight (kg): mean (range)	59 (42–73)	58 (43–77)	57 (45–85)
Ethnicity: no. (%) Papuan highlander*	42 (87)	37 (77)	27 (53)
Current smoker: no. (%)	23 (48)	19 (40)	22 (43)
Exsmoker: no. (%)*	3 (6)	7 (14)	8 (15)
Days of fever before presentation: median (IQR)‡	NA	2 (1-5)	4 (1-7)
Systolic blood pressure (mmHg): mean (range)‡	130 (96–136)	114 (88–152)	106 (60–154)
Hypertensive on enrollment (%)	0	2	2
Pulse rate (beats/min): mean (range)‡	68 (44–104)	86 (56–118)	97 (61–138)
Respiratory rate (breaths/min): mean (range)‡	20 (18–26)	25 (14–42)	30 (16–60)
Temperature (°C): mean (range)‡	35.6 (35–36.7)	36.5 (34.1–39.8)	37.1 (34.8–40.3)
Coma: no. (%)*	0	0	28 (55)
Time (h) from start of antimalarial therapy to physiological testing: mean (95% CI)‡	NA	4.5 (3–6)	8 (6–10)

*, $P < 0.01$, calculated from a χ^2 test comparing SM, MSM (stage 1), and HC. ‡, $P < 0.01$, calculated from ANOVA comparing SM, MSM (stage 1), and HC; or where no data in HC, calculated from a two-sided *t* test comparing SM and MSM (stage 1). IQR, interquartile range; NA, not applicable.

subjects with impaired endothelial function (RH-PAT < 1.67) (23) was significantly higher in SM patients (46 out of 49; 94%) compared with that in patients with MSM (17 out of 48; 35%) or HC (14 out of 48; 29%; $P < 0.0001$; Fig. 2 A). In patients with SM, there was no significant difference in the RH-PAT index among patients according to severity criteria ($P = 0.32$) (22) or mortality ($P = 0.66$).

Baseline concentrations of plasma L-arginine and biomarkers of disease severity. Plasma L-arginine concentrations were lower in both the MSM (42 μmol/liter; 95% CI = 37–45) and SM groups (49 μmol/liter; 95% CI = 43–55) relative to controls (77 μmol/liter; 95% CI = 68–85; $P < 0.0001$; Table II and Fig. 2 B). In patients with SM, there was no significant difference in mean L-arginine concentrations among different severity criteria ($P = 0.15$). There was no significant difference in the plasma L-arginine/ornithine ratios among groups ($P = 0.14$; Table II). Compared with patients with MSM, patients with SM had higher mean concentrations of lactate (2.89 mmol/liter [95% CI = 2.34–3.44] vs. 1.36 mmol/liter [95% CI = 1.17–1.55]; $P < 0.0001$; Table II), soluble intercellular adhesion molecule 1 (ICAM-1; 937 pg/ml [95% CI = 795–1,080] vs. 518 pg/ml [95% CI = 470–566]; $P < 0.0001$; Table II), and E-selectin (152 pg/ml [95% CI = 113–192] vs. 94 pg/ml [95% CI = 83–106]; $P < 0.0001$; Table II). Plasma histidine-rich protein 2 (HRP2), a measure of total parasite biomass, was also significantly higher in patients with severe disease ($P < 0.0001$; Table II).

Baseline measures of hemolysis. Plasma haptoglobin concentrations were below the lower limit of detection (10 mg/dl) in 92% (45 out of 49) of patients with SM compared with 45% (22 out of 48) of those with MSM (relative risk = 2; 95% CI = 1.46–2.76; $P < 0.0001$; Table II). Plasma lactate dehydrogenase (LDH) concentrations increased with disease severity ($P < 0.0001$; Table II); in pairwise comparisons, the mean plasma LDH concentration was significantly higher in patients with SM (1,667 IU/liter; 95% CI = 1,439–1,882]) than in MSM patients (660 IU/liter; 95% CI = 563–757; $P < 0.001$) or HC (447 IU/liter; 95% CI = 373–522; $P < 0.001$). In patients with SM, mean LDH concentrations were significantly higher in those with a fatal outcome (2,394 IU/liter; 95% CI = 1,834–3,054) than in survivors (1,526 IU/liter; 95% CI = 1,306–1,747; $P = 0.009$). Plasma LDH was also positively

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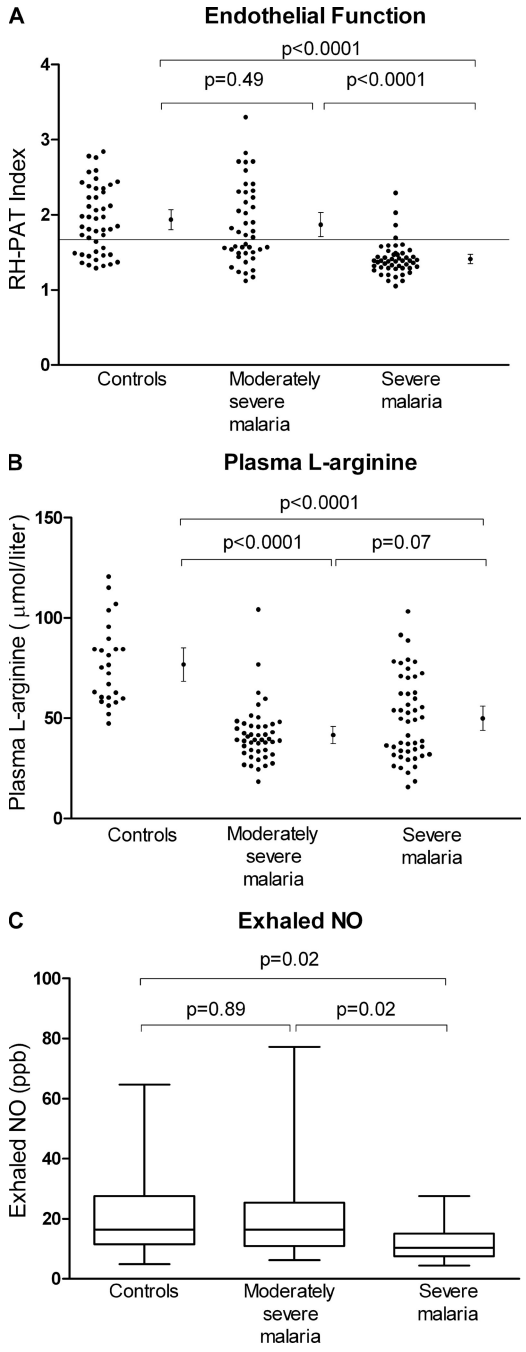

Figure 2. RH-PAT index, plasma L-arginine concentrations, and exhaled NO in each study group at enrollment. (A) Comparison of RH-PAT index at enrollment among disease categories ($P < 0.0001$ by ANOVA). Dots and error bars indicate means and 95% CIs. Horizontal line indicates an RH-PAT index of 1.67; values below this represent impaired endothelial function (reference 23). (B) Comparison of plasma L-arginine concentrations among disease categories ($P < 0.0001$ by ANOVA). Dots and bars indicate means and 95% CIs. (C) Comparison of exhaled NO concentrations at enrollment among disease categories ($P = 0.049$ by the Kruskal-Wallis test). Central line and box indicate the median and interquartile range. Whiskers indicate range.

correlated with both plasma arginase activity ($r = 0.4$; $P < 0.001$) and plasma ICAM-1 ($r = 0.75$; $P < 0.001$) and E-selectin ($r = 0.47$; $P < 0.001$), and these correlates remained after stratifying for disease severity. Plasma arginase activity increased with disease severity ($P = 0.0002$; Table II) and was weakly associated with plasma ICAM-1 ($r = 0.27$; $P = 0.01$) and E-selectin ($r = 0.25$; $P = 0.02$) but not with plasma L-arginine concentrations.

Relationship between endothelial function and biomarkers of disease severity and hemolysis. Among all malaria patients ($n = 99$), the RH-PAT index was negatively correlated with plasma HRP2 (parasite biomass; $r = -0.38$; $P < 0.0001$), lactate concentrations ($r = -0.34$; $P = 0.001$), plasma LDH concentrations ($r = -0.41$; $P < 0.001$), plasma arginase activity ($r = -0.3$; $P = 0.004$), and plasma-soluble ICAM-1 concentrations ($r = -0.45$; $P < 0.0001$), but not exhaled NO, hemoglobin, bilirubin, E-selectin, L-arginine, or the L-arginine/ornithine ratio. After stratifying by disease severity, blood lactate and plasma LDH concentrations remained correlated with the RH-PAT index. In the 26 patients with MSM who had haptoglobin concentrations in the measurable range, there was a significant association between plasma haptoglobin concentrations and the RH-PAT index ($r = 0.62$; $P = 0.003$).

Exhaled NO. Median exhaled NO was lower in the SM group (10.5 parts per billion [ppb]; range = 4.4–27.5; $n = 11$) compared with the MSM (16.5 ppb; range = 6.2–77.1; $n = 42$) and HC (16.5 ppb; range = 4.9–67.1; $n = 48$) groups ($P = 0.049$; Fig. 2 C). After stratifying by disease category, there was no association between exhaled NO and plasma L-arginine concentration or any measure of hemolysis.

Stage 2

Subjects. A total of 30 patients received L-arginine infusion intravenously at doses of 3 ($n = 10$), 6 ($n = 10$), and 12 g ($n = 10$). Their baseline clinical and physiological characteristics did not differ significantly from the stage 1 patients with MSM who received an identical volume of saline (Table III).

Effect of L-arginine infusion on plasma L-arginine and RH-PAT. Overall, plasma L-arginine concentrations increased in all subjects receiving L-arginine. The mean L-arginine concentration increased from 37 μmol/liter (95% CI = 33–43) to 853 μmol/liter (95% CI = 628–1,078; $P < 0.0001$), with maximum values observed at the end of the 30-min infusion. The increases in peak L-arginine concentrations were dose related, with the mean after 3, 6, and 12 g being 288 (95% CI = 172–405), 809 (95% CI = 592–1,027), and 1,310 μmol/liter (95% CI = 911–1,709), respectively. There was no significant change in plasma L-arginine after saline infusion ($P = 0.89$).

After L-arginine infusion at any dose ($n = 30$), the overall mean RH-PAT index rose from 1.76 (95% CI = 1.62–1.9) to 2.05 (95% CI = 1.84–2.26), a 19% (95% CI = 6–34) increase ($P = 0.006$; Fig. 3 A). The proportion of patients with an abnormal RH-PAT (<1.67) decreased from 47% (14 out of 30) to

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Table II. Laboratory and physiological results of stage 1 patients according to clinical status

	HC	MSM	SM
Number	48	48	51
White blood cell count ($\times 10^3$ μl^{-1}): mean (95% CI)‡	ND	5.9 (2.6–10.8)	9.4 (3.2–17.3)
Hemoglobin (g/liter): mean (range)‡	ND	128 (7.1–16.7)	108 (6–16.3)
Plasma L-arginine (μmol/liter): mean (95% CI)‡	77 (68–85)	42 (37–45)	49 (43–55)
Plasma ornithine (μmol/liter): mean (95% CI)‡	71 (62–80)	50 (39–60)	60 (43–77)
L-arginine/ornithine ratio: mean (95% CI)	1.15 (1–1.29)	0.99 (0.84–1.14)	1.03 (0.89–1.17)
Plasma arginase activity (μmol/ml/h): median (IQR)**	0.14 (0.06–0.19)	0.18 (0.12–0.24)	0.24 (0.17–0.31)
Lactate concentration (mmol/liter): mean (95% CI)‡	NA	1.36 (1.1–1.5)	2.89 (2.3–3.4)
Haptoglobin undetectable (<10mg/dl): no. (%)‡	ND	22/48 (45)	45/49 (92)
Parasite density (μl^{-1}) geometric mean (range)‡	0	13,297 (850–127,350)	35,067 (125–725,340)
HRP2 concentration (log_e ng/ml): mean (range)‡	NA	5.78 (1.7–8.79)	7.53 (1–10.98)
LDH (IU/liter): mean (95% CI)‡	447 (373–522)	660 (563–757)	1667 (1,439–1,882)
Soluble ICAM-1 (pg/ml): mean (95% CI)‡	NA	518 (470–566)	937 (795–1,080)
Soluble E-selectin (pg/ml): mean (95% CI)‡	NA	94 (83–106)	152 (113–192)
RH-PAT index: mean (95% CI)‡	1.93 (1.8–2.06)	1.82 (1.7–2.02)	1.41 (1.33–1.47)
Exhaled NO (ppb): median (IQR)**	16.5 (11.6–26.6)	16.5 (10.9–25.2)	10.5 (7.5–15)

‡, $P < 0.01$, calculated from ANOVA or a χ^2 test comparing SM, MSM (stage 1), and HC; or where no data in HC, calculated from a two-sided *t* test comparing SM and MSM (stage 1). **, $P < 0.05$, calculated from a Kruskal-Wallis test comparing SM, MSM (stage 1), and HC. IQR, interquartile range; NA, not applicable; ND, not done.

23% (7 out of 30; P = 0.01). In the control group, there was no overall change in the RH-PAT index after infusion of saline (P = 0.76; Fig. 3 A). The difference in mean relative increase in the RH-PAT index after L-arginine infusion compared with that after saline administration was 16% (95% CI = 1–32; P = 0.04).

In the a priori–selected subgroup of 14 subjects with baseline endothelial dysfunction (RH-PAT index < 1.67) who received L-arginine infusion, the mean RH-PAT index rose from 1.46 (95% CI = 1.37–1.55) to 1.99 (95% CI = 1.61–2.38), a relative increase of 38% (95% CI = 11–65; P = 0.004). Among patients with baseline impairment of endothelial function, there was a significant dose–response with L-arginine infusion, with a mean increase in the RH-PAT index of 12% (95% CI = from −1 to 25) in those receiving 3 g (n = 4), 24% (95% CI = from −5 to 54) receiving 6 g (n = 6), and 78% (95% CI = from −16 to 173) receiving 12 g (n = 4; P = 0.03; Fig. 3 B).

In patients with an RH-PAT index >1.67, there was no significant change in RH-PAT before and after L-arginine (5% mean relative change; 95% CI = from −7 to 17; P = 0.5), and no significant difference compared with those who received saline (−5% mean relative change; 95% CI = from −20 to 9; P = 0.2). In these patients, there was also no significant difference in the proportion in whom the RH-PAT index fell below 1.67 after L-arginine (12%) compared with saline (9%; P = 0.7).

Effect of L-arginine infusion on exhaled NO. Paired measurements were available for 27 patients after L-arginine infusion at any dose, with 3 patients unable to perform the procedure. The mean exhaled NO increased from 21.2 ppb (95% CI = 15.9–26.4) to 31.8 ppb (95% CI = 23.9–9.7), an increase of 55% (95% CI = 32–73; P < 0.0001; Fig. 3 C). There was no significant change in exhaled NO after saline infusion (P = 0.1; Fig. 3 C). The mean relative increase in exhaled NO after L-arginine infusion was 37% higher (95% CI = 15–59) when compared with saline administration (P = 0.002).

Safety. L-arginine infusion was well tolerated. Two patients experienced mild pain at the infusion site, but there were no other adverse effects or clinically important changes in vital signs.

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Table III. Baseline characteristics of the stage 2 patients with MSM given either saline or L-arginine

	Saline infusion group[a]	L-arginine infusion group
Number	48	30
Age (y): mean (range)	28 (18–56)	28 (18–54)
Males: no. (%)	32 (67)	20 (67)
Weight (kg): mean (range)	58 (43–77)	58 (42–70)
Ethnicity: no. (%) Papuan highlander	37 (77)	23 (77)
Current smoker: no. (%)	19 (40)	11 (37)
Exsmoker: no. (%)	7 (14)	4 (13)
Days of fever before presentation: median (IQR)	2 (1–5)	2 (1–4)
Systolic blood pressure (mmHg): mean (range)	114 (88–152)	109 (90–138)
Hypertensive on enrollment (%)	2	0
Pulse rate (beats/min): mean (range)	86 (56–118)	80 (54–116)
Respiratory rate (breaths/min): mean (range)	25 (14–42)	24 (18–32)
Temperature (°C): mean (range)	36.5 (34.1–39.8)	37 (34.8–40.2)
White blood cell count ($\times 10^3$ μl^{-1}): mean (range)	5.9 (2.6–10.8)	6.2 (2.3–11.7)
Hemoglobin (g/liter): mean (95% CI)	128 (7.1–16.7)	123 (7.5–17)
Plasma L-arginine (μmol/liter): mean (95% CI)	42 (37–45)	37 (33–43)
Lactate concentration (mmol/liter): mean (95% CI)	1.29 (1.1–1.5)	1.5 (1.2–1.8)
RH-PAT index: mean (95% CI)	1.82 (1.7–2.02)	1.76 (1.62–1.9)
Exhaled NO (ppb): mean (range)	20.5 (6.2–77.1)	21.2 (4.2–51.3)
Parasite density (μl^{-1}): geometric mean (range)	16,297 (850–227,350)	17,221 (890–281,864)
HRP2 concentration ($\log_e$ ng/ml): mean (range)	5.78 (1.7–8.79)	5.76 (1.34–8.79)
Time (h) from start of antimalarial therapy to physiological testing: mean (95% CI)	4.5 (3–6)	6 (4.5–7.5)

There was no significant difference ($P < 0.05$) between groups for any of the variables.
[a]The patients with MSM given saline were those enrolled in stage 1 of the study.

Mean changes in systolic blood pressure before and after infusion of 3 g (−0.4 mmHg; 95% CI = from −5 to 6), 6 g (2 mmHg; 95% CI = from −7 to 11), and 12 g (−3 mmHg; 95% CI = from −13 to 8) were not clinically significant. Mean changes in pulse rate before and after infusion of 3 g (−1 beat/min; 95% CI = from −9 to 7), 6 g (−4 beats/min; 95% CI = from −6 to 14), and 12 g (1 beat/min; 95% CI = from −19 to 21) were also not clinically significant. There were also no clinically significant changes in concentrations of blood glucose or electrolytes (potassium, phosphate, bicarbonate, chloride, and pH).

DISCUSSION

Autopsy studies in fatal cases of malaria have described cytoadherence of parasitized red cells to endothelium, endothelial inflammation, and endothelial damage (5). Our study has shown impairment of endothelium-dependent vasodilatation

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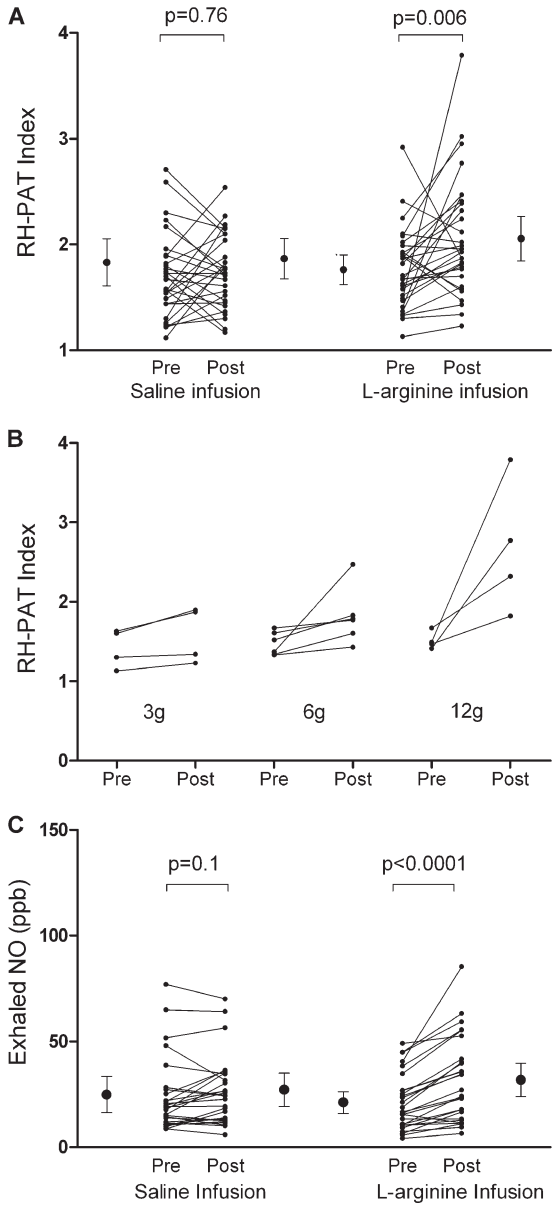

Figure 3. Change in RH-PAT index and exhaled NO after L-arginine infusion. (A) Change in RH-PAT index in MSM after infusion of 100 ml saline (3%; 95% CI = from −1 to 12) or L-arginine (19%; 95% CI = 8–33). p-values refer to paired comparisons before and after infusion. Dots and bars indicate means and 95% CIs. (B) Dose-related change in RH-PAT index after intravenous L-arginine infusion in the a priori–defined subset of patients with MSM who had baseline endothelial dysfunction (RH-PAT index <1.67; $n = 14$; $P = 0.03$). Lines show RH-PAT index before and after intravenous L-arginine at doses of 3, 6, and 12 g. In the 16 patients without baseline impairment of endothelial function (RH-PAT index >1.67), there was no significant change in RH-PAT after L-arginine infusion. (C) Change in exhaled NO concentration in MSM after infusion of 100 ml saline (18%; 95% CI = from −2 to 28) or L-arginine (55%; 95% CI = 39–71). p-values refer to paired comparisons before and after infusion. Dots and bars indicate means and 95% CIs.

and a decrease in organ-specific NO production in nearly all adults with SM. This near-universal endothelial dysfunction in SM and its significant improvement after infusion of L-arginine (but not saline) in patients with MSM demonstrate that the endothelium can be targeted for adjunctive treatment in falciparum malaria.

Our measure of endothelial function, RH-PAT, is at least 50% dependent on endothelial NO production (24). The endothelial dysfunction present in SM and its reversibility with L-arginine in MSM suggests a deficiency of NO availability in a cell type central to the pathophysiology of SM. Furthermore, the association between endothelial dysfunction and both soluble ICAM-1 and total parasite biomass supports the hypothesis that impaired NO production in SM contributes to enhanced expression of endothelial adhesion molecules and the exacerbation of cytoadherence and sequestration. In myocarditis, coronary vessel endothelial ICAM-1 expression is also inversely associated with NO-dependent endothelial function (25).

Plasma L-arginine concentrations, exhaled NO levels, and endothelial function were all significantly reduced in Indonesian adults with SM, which was consistent with the reduction in systemic NO production and hypoargininemia found in our previous studies in African children with cerebral malaria (9, 10). Although data in severe malarial anemia are lacking, our findings suggest that hypoargininemia and low NO bioavailability are characteristic of SM across different age groups, transmission intensities, ethnic groups, and disease manifestations. The increase in exhaled NO and endothelial function after L-arginine infusion in MSM supports our earlier hypothesis that hypoargininemia contributes to impaired NO bioavailability in SM (10).

The rapid reversibility of endothelial dysfunction with L-arginine infusion in MSM suggests that in addition to the physical obstruction found in small vessels, there may also be a functional defect in perfusion resulting from an impaired ability of small vessels to appropriately dilate in response to ischemia. Although our study measured dysfunction in the endothelial cells of small digital vessels, it is likely that similar impairments occur in the more distal microvasculature at sites of parasitized red cell sequestration. In SM, metabolic acidosis is proportional to disease severity and is an important risk factor for death (26). The acidosis is thought to result from a combination of obstructed microvascular flow and impaired tissue oxygen delivery (26) and/or utilization (27). In our study, the degree of endothelial dysfunction correlated with blood lactate and acidosis; this supports the hypothesis that endothelial dysfunction and NO deficiency contribute to impaired microvascular flow and functional tissue hypoxia. In sepsis, another state of acute L-arginine deficiency, impairment in microcirculatory flow is reversible after infusion of the NO-donor nitroglycerin (28). Collectively, these observations give a rational basis for future functional studies of microvascular perfusion and reversibility with L-arginine in severe SM.

Endothelial dysfunction was significantly greater in SM compared with MSM despite comparable degrees of hypoargininemia in these groups. Possible reasons for this include increased endothelial NO quenching in severe disease because of

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a greater degree of hemolysis, greater hypoargininemia-related superoxide production, and/or more microvascular sequestration of parasitized red cells than in MSM. In patients with SCD, hemolysis leads to an increase in plasma cell–free hemoglobin and arginase, resulting in NO scavenging and metabolism of plasma L-arginine, respectively, each of which reduces NO bioavailability (14, 17). In SCD, plasma LDH (from lysed erythrocytes) and reduced L-arginine/ornithine ratio (reflecting plasma arginase activity) are associated with up-regulation of endothelial adhesion receptors, L-arginine–reversible endothelial dysfunction, and early mortality (15–18). Our study suggests that similar mechanisms may be operating in SM. LDH was highest in SM and was associated with endothelial dysfunction, increased expression of plasma endothelial adhesion molecules, and increased mortality.

Greater endothelial dysfunction in SM may also relate to the dependence of endothelial NO production on the activity of the cationic amino acid transporters responsible for cellular uptake of extracellular L-arginine (29). As in African children with cerebral malaria (10), the mean plasma L-arginine concentration in adults with SM was below the half-saturating concentration (Km) of the cationic amino acid transporters (100–150 μmol/liter), making it likely that hypoargininemia limits L-arginine transport into cells and NO production in severe disease in both adults and children. With low L-arginine levels, NOS generates superoxide rather than NO, resulting in oxidant-mediated cellular damage (30, 31), with NO quenching by superoxide further reducing NO bioavailability. Because patients with SM may have increased expression of NOS2 in endothelial cells (32), increased endothelial superoxide generation and local quenching of NO may contribute to the greater endothelial dysfunction found in SM. In addition, the greater dysfunction in SM may result from parasite-mediated effects on endothelial cells associated with a larger biomass of parasitized red cells adherent to the endothelium. Sequestration of parasitized red cells may be both a cause and an effect of endothelial dysfunction.

Possible causes of hypoargininaemia in SM include decreased L-arginine synthesis and increased catabolism caused by the increased activity of plasma arginase (33) and/or cytokine-inducible arginase in endothelial cells or immune cells (34, 35), processes that may also occur in other inflammatory conditions. As well as impairing endothelial function, hypoargininemia and NO deficiency may contribute to pathology in SM through other mechanisms. NO inhibits platelet activation (36), and deficiency may therefore exacerbate platelet-mediated processes linked to the microvascular pathology of SM, including endothelial cell activation (37) and apoptosis and platelet-mediated clumping (38). Because NO is also a determinant of red cell deformability (39), deficiency of NO may exacerbate the impaired red cell deformability found in SM (40), further compromising microvascular perfusion.

The extremely short half-life of NO has made measurement of NO production technically challenging in malaria, with most studies measuring concentrations of NO metabolites in body fluids. Results have been difficult to interpret because of the failure to control for dietary nitrate ingestion, nitrate retention in renal failure, and perturbations in extracellular fluid volumes (41, 42). Experiments that have controlled for these variables have shown reduced concentrations of NO metabolites and impaired expression of NOS in circulating mononuclear cells in SM (9). Although not possible to measure in those with coma, our real-time measurement of exhaled NO has provided the first direct measurement of NO in malaria and demonstrates impaired organ-specific NO production in severe disease. We speculate that the impaired pulmonary NO production we demonstrate in this study in SM may increase the risk of developing acute respiratory distress syndrome (ARDS), a syndrome that usually commences 1–6 d after starting antimalarial treatment and is a major cause of death in SM (43) Pulmonary NO is reduced in patients with ARDS in other diseases (44), and NO production has been associated with improved outcomes from acute lung injury (45). In animal experiments, disruption of extracellular L-arginine uptake is associated with decreased NO production and increased lung inflammation (46). By increasing pulmonary NO, L-arginine therapy could potentially reduce the risk of developing ARDS in SM.

Our study has several limitations. The allocation of patients to receive either L-arginine or saline was not randomized. However, there were no differences in baseline characteristics between those receiving saline and L-arginine, and neither of the primary outcome measures was subjective. We could not exclude concurrent infections, particularly in SM. However, the low frequency of secondary bacteremia in SM in adults (47) makes it unlikely that concurrent bacterial infection could explain the near-universal impairment of endothelial function in SM. Exhaled NO may not reflect systemic NO production (48, 49), and we could not directly measure intracellular endothelial NO concentrations. Although RH-PAT response is at least 50% NO dependent, we were unable to exclude other mechanisms for endothelial dysfunction in SM, such as concurrent deficiency of endothelium-derived hyperpolarizing factor and prostacyclin (19). However, the increase in both endothelial function and exhaled NO after L-arginine infusion suggests an ability to increase NO bioavailability in at least two cell types. Our LDH results are comparable to previous studies in malaria (50). We cannot exclude a contribution to plasma LDH levels from extraerythrocytic sources, but the relative contribution from parasite LDH is likely to be minor. Finally, we cannot exclude effects of other factors (e.g., a high fat diet [19] or diurnal variation [51]) on endothelial function, but the impact of these confounding factors is likely to be low in patients acutely ill with malaria.

The improvement in endothelial function after L-arginine infusion in MSM was greatest in those with baseline impairment of endothelial function. With endothelial dysfunction occurring in nearly all patients with SM, the potential benefit of L-arginine infusion in SM may be greater than that we have demonstrated in MSM. L-arginine infusion was used in patients with MSM in this study because of the need to collect pharmacokinetic data and to demonstrate

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safety and proof of concept before proceeding to studies in severe disease.

In conclusion, SM is characterized by hypoargininemia, hemolysis, decreased pulmonary NO, and impairment of endothelial function, with each potentially contributing to its pathogenesis. Given its central role in malaria pathophysiology, the endothelium may be an important therapeutic target for adjunctive treatment. Short-term parenteral L-arginine therapy is safe in MSM and increases pulmonary NO production. Its efficacy in improving endothelial function suggests that hypoargininemia and decreased NO bioavailability contribute to endothelial dysfunction. Our work provides firm proof of concept to warrant clinical trials of adjunctive agents such as L-arginine to improve endothelial NO bioavailability in SM.

MATERIALS AND METHODS

Study design

Stage 1: relationship between endothelial function and disease severity. An initial prospective cross-sectional observational study was conducted to compare endothelial function, plasma L-arginine concentrations, exhaled NO, and biochemical markers among patients with MSM and SM and healthy individuals (Fig. 1). Patients were enrolled between February 2005 and April 2006.

Stage 1 patients. The study was conducted at Mitra Masyarakat Hospital in Timika, Papua, Indonesia, a region with unstable transmission of both *Plasmodium falciparum* and *P. vivax* (52). Three groups of adults ≥18 yr old were enrolled from the emergency department or outpatient clinic and differentiated as follows: (a) MSM, defined as fever or history of fever in the past 48 h, with >1,000 asexual *P. falciparum* parasites per microliter (a parasitemia threshold predicting clinical disease in Papua) (53), no other etiology identified, and requiring inpatient parenteral therapy because of the inability to tolerate oral therapy (54) but exhibiting no World Health Organization (WHO) warning signs or criteria for SM (22); (b) SM, defined as the presence of *P. falciparum* parasitaemia and ≥1 modified WHO criteria of severity (22, 43), a Glasgow coma score <11, renal failure (creatinine >265 μmol/liter after rehydration or urine output of <400 ml per 24 h), hyperbilirubinaemia (total bilirubin >50 μmol/liter) with either renal impairment (creatinine >130 μmol/liter) or parasitaemia (>100,000 parasites per microliter), blackwater fever, hypoglycemia (whole blood glucose <2.2 mmol/liter), respiratory distress (respiratory rate >32 breaths/min), acidosis (venous bicarbonate <15 mmol/liter), shock (systolic blood pressure <80 mmHg after fluid resuscitation with cold peripheries), and/or hyperparasitaemia (>10% parasitized red cells); and (c) HC, defined as unrelated hospital visitors, subjectively well with no history of fever in the preceding 48 h, no parasitaemia, no concurrent illness or medication, and not having smoked within the preceding 12 h. Exclusion criteria included the following: pregnant or breastfeeding women; patients treated with parenteral antimalarials for >18 h; mixed *P. falciparum*/*P. vivax* infections; diabetes; known cardiac, renal, or hepatic disease; concurrent infection; concurrent medication; hemoglobin <60g/liter; and among MSM, those with systolic blood pressure <100 mmHg, a baseline venous bicarbonate level <20 mmol/liter, potassium ≥4.2 mmol/liter, glucose <4 mmol/liter, or chloride >106 mmol/liter. 20 patients with SM were initially randomized to either intravenous quinine or artesunate as part of a multicenter clinical trial (1) . Based on the results of the study and an ensuing national policy change, all patients subsequently received intravenous artesunate. Individuals with MSM were treated with intravenous quinine in accordance with national guidelines. Both groups also received doxycycline or clindamycin. Supportive care, including antibiotics and fluid administration, was provided at the discretion of the treating physicians, who were independent of the study.

Ethical approval was obtained from the Health Research Ethics Committees of the National Institute of Health Research and Development, (Indonesia) and the Menzies School of Health Research (Australia). Written informed consent was obtained from patients or attending relatives in Indonesian or a local language, when necessary.

Stage 1 clinical observations. At enrollment, all patients underwent a standardized medical history, physical examination, venous blood collection, and measurement of endothelial function and exhaled NO. Blood pressure was measured with an automated sphygmomanometer and axillary temperature with a digital thermometer.

Laboratory methods. Hemoglobin and white blood cell counts were measured by a counter (T890; Beckman Coulter), and routine biochemistry, acid-base parameters, and lactate were measured using a bedside biochemical analyzer (i-STAT-1; i-STAT Corp.). Parasite counts were determined by Giemsa-stained thick and thin fields and were cross-checked by an experienced microscopist. Plasma was separated within 30 min of collection by centrifugation and stored at −70°C. Amino acids were extracted from 50 μl of plasma after the addition of 50 μl of internal standard (norleucine) and 200 μl of cold ethanol. Deproteinized plasma was derivitized with AccQFluor reagent (Waters), and amino acids were measured by HPLC (Shimadzu) using a method modified from van Wandelen and Cohen (55). Plasma concentrations of the endothelial activation markers soluble ICAM-1 and E-selectin were assayed by ELISA (R&D Systems). To quantitate total parasite biomass, plasma HRP2 was measured by ELISA, as previously described (56). Purified HRP2 was provided by D. Sullivan (Johns Hopkins University, Baltimore, MD). Plasma haptoglobin and LDH were measure by ELISA and a calorimetric assay, respectively (Roche Diagnostics). Plasma arginase activity was measured using a radiometric assay, as previously described, and reported as micromole/milliliter/hour (16).

Endothelial function. RH-PAT (Itamar) is a recently validated noninvasive method of assessing endothelial function. RH-PAT correlates with the more labor-intensive flow-mediated dilatation method (57) and endothelial function in other vascular beds (57). At least 50% of RH is dependent on endothelial NO production (24). Finger probes measure digital volume changes detected by a pressure transducer. PAT was measured before and after a 5-min ischemic stress, generating an RH-PAT index, normalized to the control arm (57). All studies were performed in a quiet air-conditioned room at 25°C after a 20-min equilibration time. In 10 patients with SM, tests were performed in a high dependency unit at similar temperatures. To internally validate endothelial function measurements, RH-PAT indices were repeated 0.5–0.75 h after initial measurements in 37 HC. The reproducibility coefficient was 0.59 (58), comparable with previous results (59) and those obtained with the flow-mediated dilatation method (51).

Exhaled NO. Fractional concentration of exhaled NO in ppb was measured using American Thoracic Society guidelines with an NO analyzer (NiOX; Aerocrine AB) at a flow rate of 250 ml/sec (60).

Stage 2: effect of L-arginine infusion on endothelial function. A single ascending dose study of L-arginine infusion was undertaken in 30 additional subjects with MSM (Fig. 1). This stage was designed to assess safety, pharmacokinetics, and proof of mechanism and concept that L-arginine infusion increases NO production and improves endothelial function.

Stage 2 patients. 30 additional patients with MSM were enrolled for L-arginine infusion, with the same inclusion and exclusion criteria as in stage 1 and the additional exclusion criterion of known allergy to L-arginine. Based on the known variability of endothelial function (59), this sample size gave an 80% power to detect a 10% increase in RH-PAT index after L-arginine infusion. A control group comprised the 48 patients with MSM from stage 1 (Fig. 1). These patients underwent identical assessments before and after a 30-min infusion of 100 ml of normal saline.

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L-arginine infusion. In a sequential single ascending dose design, L-arginine hydrochloride (Pharmalab) was diluted in 100 ml of normal saline (or sufficient to provide a concentration ≤10% wt/vol) and administered intravenously by an infusion pump over 30 min at doses of 3 ($n = 10$), 6 ($n = 10$), and 12 g ($n = 10$). Patients received intravenous quinine and clinical care as in stage 1.

Stage 2 clinical, physiologic, and biochemical observations. All patients underwent the same baseline measurements as in stage 1. To assess safety, patients were monitored with serial assessments of symptoms, vital signs, and biochemistry (blood potassium, glucose, bicarbonate, pH, chloride, and phosphate) until discharge. Exhaled NO and plasma L-arginine were measured immediately before and after infusion. RH-PAT measurement was completed within 10 min before infusion and within 20 min after infusion.

Stage 2 outcome measures. The primary outcome measures were the changes in RH-PAT index and exhaled NO after L-arginine infusion. Changes in RH-PAT index and exhaled NO in stage 2 MSM patients given L-arginine were compared with changes seen in patients with MSM in stage 1 who had been given an identical volume of normal saline. Primary safety outcomes were changes in hemodynamic measures and electrolytes.

Secondary outcome measures were the change in RH-PAT index in the a priori subgroup of patients with baseline impairment of endothelial function (predefined as an index of <1.67) (23) and evidence for dose dependency of the change in RH-PAT index among all patients and among those with baseline impairment of endothelial function.

Statistical methods

For cross-sectional analyses, continuous variables with a normal distribution were compared among groups by analysis of variance (ANOVA) or the Student's *t* test. The Mann-Whitney U test or the Kruskal-Wallis test was used when data were not normally distributed. Pearson's method was used to estimate correlation between normally distributed continuous variables. Comparison of paired proportions and paired continuous variables used McNemar's χ^2 test and the paired *t* test, respectively. All analyses were performed with Stata software (version 8.2; Statacorp.). A two-sided value of $P < 0.05$ was considered significant.

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N.M. Anstey, D.L. Granger, and J.B. Weinberg are named as inventors in a US patent for the use of L-arginine as treatment for SM but have transferred all of their rights to their respective institutional malaria research collaborations. This patent was issued for US rights only, and no rights are being sought in other countries. The authors have no other competing financial interests.

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