Increased Asymmetric Dimethylarginine in Severe Falciparum Malaria: Association with Impaired Nitric Oxide Bioavailability and Fatal Outcome

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Abstract

Asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is a predictor of mortality in critical illness. Severe malaria (SM) is associated with decreased NO bioavailability, but the contribution of ADMA to the pathogenesis of impaired NO bioavailability and adverse outcomes in malaria is unknown. In adults with and without falciparum malaria, we tested the hypotheses that plasma ADMA would be: 1) increased in proportion to disease severity, 2) associated with impaired vascular and pulmonary NO bioavailability and 3) independently associated with increased mortality. We assessed plasma dimethylarginines, exhaled NO concentrations and endothelial function in 49 patients with SM, 78 with moderately severe malaria (MSM) and 19 healthy controls (HC). Repeat ADMA and endothelial function measurements were performed in patients with SM. Multivariable regression was used to assess the effect of ADMA on mortality and NO bioavailability. Plasma ADMA was increased in SM patients (0.85 μM; 95% CI 0.74–0.96) compared to those with MSM (0.54 μM; 95% CI 0.5–0.56) and HCs (0.64 μM; 95% CI 0.58–0.70; p < 0.001). ADMA was an independent predictor of mortality in SM patients with each micromolar elevation increasing the odds of death 18 fold (95% CI 2.0–181; p = 0.01). ADMA was independently associated with decreased exhaled NO (r2 = −0.31) and endothelial function (r2 = −0.32) in all malaria patients, and with reduced exhaled NO (r2 = −0.72) in those with SM. ADMA is increased in SM and associated with decreased vascular and pulmonary NO bioavailability. Inhibition of NOS by ADMA may contribute to increased mortality in severe malaria.

Introduction

*P. falciparum* causes ~1 million deaths annually [1,2]. Despite rapid parasite clearance with the anti-parasitic drug artesunate, the mortality rate in severe malaria remains high [3,4]. Endothelial activation, parasite sequestration, impaired microvascular perfusion and dysregulated inflammatory responses are all thought to contribute to severe and fatal malaria [5–9]. Increased understanding of these pathogenic mechanisms may identify targets for adjunctive therapies to further improve outcomes.

Severe malaria is associated with impaired nitric oxide (NO) bioavailability and blood mononuclear cell NO synthase (NOS) type 2 expression in both children [10,11] and adults [6]. The concentrations of L-arginine, the substrate for NO production by all three NOS isoforms [12], are low in children and adults with severe malaria and likely contribute to the decreased NO production found in severe disease [6,10,13]. However, in adults with moderately severe malaria, L-arginine concentrations are at least as low as those seen with severe malaria, yet there is no impairment of vascular and pulmonary NO bioavailability as found in severe disease [6]. This suggests that factors other than substrate limitation contribute to impaired NO bioavailability in severe malaria.

Asymmetrical dimethylarginine (ADMA) is a non-specific endogenous NOS inhibitor which decreases vascular function in cardiovascular and renal disease [14,15]. Protein-arginine-meth-
Severe falciparum malaria is associated with impaired microvascular perfusion, lung injury and decreased bioavailability of nitric oxide (NO), but the causes of these processes are not fully understood. Asymmetrical dimethylarginine (ADMA), a competitive endogenous inhibitor of nitric oxide synthase (NOS), is an independent predictor of mortality in other critical illnesses, and can impair vascular function in chronic disease. ADMA can be produced by both the host and malaria parasites. The major novel findings of this study in malaria are that ADMA is an independent predictor of death in falciparum malaria, and is associated with decreased availability of nitric oxide in at least two organ systems affected by malaria parasites, the lining of blood vessels and the lungs. This study contributes to knowledge of regulation and availability of pulmonary and endothelial NO in critical illness and identifies pathogenic processes which may contribute to death in severe malaria. Therapies which increase the availability of NO or which reduce ADMA levels may have potential for adjunctive therapy of severe malaria.

Acute lung injury is a common but little-studied complication of severe falciparum malaria associated with high mortality [21]. In sepsis and critical illness, acute lung injury and mortality are associated with decreased total and pulmonary NO [22,23]. Pulmonary diffusion capacity and exhaled NO concentrations are both reduced in severe malaria [6,24], however the causative factors have not been identified. The role of ADMA in impairing pulmonary NO bioavailability in severe malaria, or indeed any critical illness, is not known.

In a prospective longitudinal study of Indonesian adults with malaria, we evaluated the hypotheses that concentrations of methylated arginines are independently associated with a) disease severity, b) reduced exhaled NO and vascular NO bioavailability and c) increased mortality.

### Results

#### Patients

We measured asymmetrical dimethylarginine (ADMA), symmetrical dimethylarginine (SDMA) and L-arginine in 49 patients with severe malaria, 78 with moderately severe malaria and 19 healthy controls. In the SM patients, 20 patients had only one criterion for severe disease (coma in 14, hyperbilirubinemia in 4, respiratory distress in 2) with the remainder having >1 criteria. In total, 34 of the patients with SM were treated with intravenous artesunate and the remaining 15 received intravenous quinine [6]. All of the 78 MSM patients were treated with quinine with the exception of one who received artesunate. Exhaled NO concentrations (FeNO) could not be measured in those with coma, but were possible in 48% (11/23) of non-comatose SM patients, 88% (69/78) of MSM patients and 100% (19/19) of HCs. RH-PAT index was measured in all patients with malaria as well as HC. There were eight deaths among the patients with SM, and none in the MSM patients. Repeat RH-PAT and venous blood measurements were only performed in one and four of the eight fatal cases respectively. Baseline characteristics of study participants are summarized in Table 1.

### ADMA, SDMA, L-arginine/ADMA ratio, ADMA/SDMA ratio and clinical disease

ADMA and SDMA concentrations were increased in SM patients (0.85 μM; 95% CI 0.74–0.96 and 1.67 μM; 95% CI 1.24–2.09 respectively) compared to those with MSM (0.54 μM; 95% CI 0.37–0.71).

#### Table 1. Baseline Characteristics of Patients According to Clinical Status.

|                     | Healthy controls | Moderately-severe malaria | Severe malaria |
|---------------------|------------------|---------------------------|---------------|
| Number              | 19               | 78                        | 49            |
| Age; mean (range), y| 26 (18–40)       | 28 (18–56)                | 29 (18–56)    |
| Males, No. (%)      | 13 (68)          | 32 (67)                   | 36 (74)       |
| Weight; mean (range), kg | 60 (50–85)     | 58 (43–77)                | 57 (45–70)    |
| Ethnicity, No. (%)  | Papuan highlander* | 59 (77)                       | 27 (55)       |
| Current smoker, No. (%) | 9 (47)        | 31 (40)                   | 22 (45)       |
| Days of fever before presentation; median (IQR) | Nil | 2 (1–5)                   | 4 (1–7)       |
| Systolic blood pressure; mean (range), mmHg† | 128 (112–138)  | 110 (80–134)              | 105 (60–154)  |
| Pulse rate; mean (range), beats/minute‡ | 67 (48–91)     | 81 (54–118)               | 98 (61–138)   |
| Temperature; mean (range), °C | 35.6 (35.1–36.8) | 36.5 (34.8–40.2) | 37.2 (34.8–40.3) |

* p<0.01 calculated by χ² test.
† p<0.01 calculated by ANOVA or two sided t test.
‡ IQR, interquartile range.

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Table 2. Baseline Laboratory and Physiological Measurements According to Clinical Status.

|                          | Healthy controls | Moderately-severe malaria | Severe malaria |
|--------------------------|------------------|---------------------------|---------------|
| **Number**               | 19               | 78                        | 49            |
| **White blood cell count** | mean (95% CI), x 10^3 /µL | ND                         | 5.9 (5.4–6.9) | 9.5 (8.5–10.5) |
| **Hemoglobin; mean (range), g/dL** | ND                | 121 (70–170)              | 109 (60–163)  |
| **Plasma cell-free hemoglobin; median (IQR), µM** | 1.3 (0.74–2.4)    | 2.6 (1.3–4.5)             | 5.4 (3.2–7.4) |
| **Exhaled NO concentrations; median (IQR), ppb** | 16.0 (9.5–19.3)   | 16.1 (10.7–25.3)          | 10.5 (7.5–15) |
| **RH-PAT Index; mean (95%CI)** | 1.87 (1.58–2.17) | 1.82 (1.71–1.93)          | 1.37 (1.32–1.42) |
| **Plasma creatinine; mean (95%CI), µmol/L** | ND                | 88 (82–94)                | 286 (207–365) |
| **Plasma total bilirubin; mean (95%CI), µmol/L** | ND                | 16.7 (12.9–20.4)          | 95.2 (47.7–142.6) |
| **Lactate concentration; mean (95%CI), mmol/L** | ND                | 1.4 (1.2–1.6)             | 2.93 (2.3–3.5) |
| **Parasite density, geometric mean (range), µl^−1** | ND                | 14,900 (850–127,000)      | 35,100 (125–725,000) |
| **HRP2 concentration; mean (range), loge ng/ml** | ND                | 5.75 (1.34–8.79)          | 8.08 (1–10.98) |
| **Soluble ICAM-1; mean (95%CI), pg/ml** | ND                | 569 (516–623)             | 938 (792–1084) |
| **Soluble E-selectin; mean (95%CI), pg/ml** | ND                | 106 (95–118)              | 153 (113–193) |
| **Plasma Angiopoietin-2; mean (95%CI), pg/ml** | ND                | 2,800 (2,000–3,500)       | 6,500 (5,000–8,000) |
| **Plasma ADMA; mean (95%CI), µmol/L** | ND                | 0.64 (0.58–0.70)          | 0.55 (0.5–0.56) |
| **Plasma SDMA; mean (95%CI), µmol/L** | ND                | 0.53 (0.47–0.59)          | 0.58 (0.54–0.63) |
| **Plasma L-arginine concentration; mean (95%CI), µmol/L** | ND                | 77 (65–88)                | 41 (37–44) |
| **L-arginine/ADMA ratio; mean (95%CI)** | ND                | 121 (107–135)             | 78 (71–86) |
| **L-arginine/SDMA ratio; mean (95%CI)** | ND                | 0.14 (0.08–0.17)          | 0.20 (0.14–0.24) |

* p < 0.01 calculated by ANOVA (overall) or two sided t test. ND, not measured.
† p < 0.05 calculated by Kruskal-Wallis test.
IQR, Interquartile Range.

There was no significant correlation between ADMA or SDMA with E-selectin, and none between the L-arginine/ADMA ratio and markers of endothelial activation. Patients with SM had significantly elevated plasma concentrations of Ang-2, ICAM-1 and E-selectin compared to those with MSM and HC; Table 2. Angiopoietin-2 and ICAM-1 were significantly correlated with both ADMA (r = 0.48 and 0.42 respectively; p<0.001; Table 3) and SDMA (r = 0.54 and 0.52; p<0.001), and this was also apparent in the subgroup of SM patients; Table 3. ADMA remained independently associated with Ang-2 and ICAM-1 after adjusting for confounding factors, including creatinine, plasma hemoglobin, parasite biomass and disease severity. There was no significant correlation between ADMA or SDMA with E-selectin, and none between the L-arginine/ADMA ratio and Ang-2, ICAM-1 and E-selectin.

ADMA, SDMA, L-arginine/ADMA ratio and biomarkers of severity

The plasma creatinine, total bilirubin, P. falciparum histidine rich protein 2 (HRP2) and venous lactate were increased in SM compared to MSM (Table 2). In all patients with malaria, there were correlations between ADMA and SDMA with creatinine (r = 0.45; p<0.001; r = 0.69; p<0.001; Table 3), total bilirubin and cell free hemoglobin (r = 0.40). In contrast, the L-arginine/ADMA ratio was not associated with the RH-PAT index. Longitudinally there was no association between the RH-PAT index and ADMA concentration or L-arginine/ADMA ratio in SM patients.
ADMA, SDMA, L-arginine/ADMA ratio and mortality

In SM patients, ADMA and SDMA concentrations were significantly higher in the 8 patients who died (1.28 μM; 95% CI 0.88–1.74 and 3.76 μM; 95% CI 1.88–5.56, respectively) compared to the 41 survivors (0.77 μM; 95% CI 0.64–0.84 and 1.27 μM; 95% CI 0.99–1.54, respectively; p < 0.001; Figure 1A and 1B).

Each micromolar increase in ADMA and SDMA concentrations was associated with an 18-fold (OR 18.8, 95% CI 2.0–181; p = 0.01) and three-fold (OR 3.0, 95% CI 1.5–6.2; p = 0.002) increased risk of death, respectively. ADMA but not SDMA remained a significant risk factor for death after adjusting for other confounding factors, such as Ang-2, creatinine, parasite biomass, bilirubin, base deficit and lactate. A final model predicting a fatal outcome included ADMA, Ang-2, HRP2 and creatinine (Table 4). The L-arginine/ADMA ratio was not associated with risk of death.

The prognostic value of ADMA in predicting a fatal outcome was measured by the area under the receiver operating curve (ROC). ADMA (AUROC 0.85; 95% CI 0.71–0.99; Figure 2) was comparable to other reliable prognostic indicators, including Ang-2 (AUROC 0.84; 95% CI 0.71–0.96), HRP2 (AUROC 0.86; 95% CI 0.73–0.94), base deficit (AUROC 0.73; 95% CI 0.53–0.92), and TNF (AUROC 0.71; 95% CI 0.43–0.98), and a better predictor of fatal outcome than venous blood lactate (AUROC 0.63; 95% CI 0.41–0.83; p = 0.003).

Longitudinal Course of ADMA, SDMA and L-arginine/ADMA ratio

In patients with severe malaria, there was no significant change in ADMA (Figure 3A) or SDMA concentrations during the course of hospitalization among the overall group, survivors or those with a fatal outcome. Among survivors, there was a daily increase in L-arginine/ADMA (β = 9.1, p < 0.001; Figure 3B) but no increase in those who died.

Table 3. Correlation Coefficients (r) for ADMA and Physiological Measures/Biomarkers of Severity.

|                      | All malaria patients | Severe malaria |
|----------------------|----------------------|---------------|
|                      | Correlation (r) p df | Correlation (r) p df |
| ADMA                 | RH-PAT Index         | --0.32 0.001 139 | --0.22 0.18 48 |
|                      | Exhaled NO           | --0.31 0.003 79  | --0.72 0.01 10 |
|                      | Lactate              | 0.30 0.01 124   | 0.1 0.3 48    |
|                      | HRP2                 | 0.40 <0.001 109 | 0.42 0.004 44 |
|                      | ICAM-1               | 0.42 <0.001 109 | 0.61 <0.001 47 |
|                      | E-selectin           | 0.12 0.4 116    | 0.20 0.10 47  |
|                      | Ang-2                | 0.48 <0.001 140 | 0.56 <0.001 48 |
|                      | TNF                  | 0.29 0.06 39    |               |
|                      | Creatinine           | 0.45 <0.001 117 | 0.55 <0.001 47 |
|                      | Total Bilirubin      | 0.32 <0.001 115 | 0.58 <0.001 47 |

*df = degrees of freedom.

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Discussion

ADMA is increased in severe falciparum malaria and is an independent predictor of mortality. Indeed ADMA was a better predictor of death than blood lactate, previously shown to be a reliable prognostic indicator of increased mortality in severe malaria [26]. Our study demonstrated that elevated plasma ADMA concentrations are independently associated with decreased exhaled NO concentrations, impaired vascular NO bioavailability, increased endothelial activation and parasite burdens.

Table 4. Factors Associated with Death in Severe Malaria Patients.

| Risk Factors         | Univariate Model | Final Model |
|----------------------|------------------|-------------|
|                      | Odds Ratio       | 95% CI      | P          | Odds Ratio       | 95% CI      | P          |
| ADMA (µmol/L)        | 18.8             | 2.0–181     | 0.010      | 229             | 2.9–2675    | 0.025      |
| Creatinine (µmol/L)  | 1.004            | 1.001–1.007 | 0.005      | 1.006           | 1.001–1.01  | 0.025      |
| Angiopoietin 2 (pg/ml) | 1.00058         | 1.00003–1.00109 | 0.024     | 1.00009         | 1.000002–1.0008 | 0.048     |
| HRP2 (ng/ml)         | 1.00082          | 1.00009–1.001| 0.01       | 1.0001          | 1.00002–1.002 | 0.017      |
| Standard Base Deficit| 1.19             | 1.01–1.4    | 0.037      | 0.93            | 0.64–1.35   | 0.659      |
| Lactate (mmol/L)     | 1.32             | 1.02–1.41   | 0.044      | 1.29            | 0.51–3.3    | 0.485      |
| Total Bilirubin (µmol/L) | 1.006        | 1.001–1.009 | 0.015      | 1.003           | 0.98–1.1    | 0.21       |

Figure 2. Top panel is the nonparametric receiver operating curve (ROC) assessing asymmetrical dimethylarginine (AUROC, 0.85; 95%CI 0.71–0.99) and the bottom panel venous lactate (AUROC 0.63; 95%CI 0.41–0.83) as prognostic markers for mortality in severe malaria (p = 0.003).

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Figure 3. Longitudinal course of plasma ADMA concentrations and L-arginine/ADMA ratio in patients with severe malaria. Mean values (circles) and 95%CI (bars) are displayed at each time point. Values from day 5–14 indicate mean of all values obtained during this period. X axis values show time from start of anti-malarial therapy. Numbers indicate patients examined during each time period.

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bile. To our knowledge this is the first demonstration of a relationship between increased ADMA and impaired exhaled NO in any critical illness. Taken together, these findings suggest that ADMA, an endogenous inhibitor of all three nitric oxide synthase (NOS) isoforms, reduces NO bioavailability in at least two organ systems and may contribute to increased mortality in falciparum malaria.

In critically ill patients, elevated ADMA concentrations are likely to result from increased production and reduced elimination. The elevation in both ADMA and SDMA may result from increased host production of methylated arginines in severe malaria. The majority of circulating ADMA is taken up by the liver before being metabolized by dimethylarginine-dimethylaminohydrolase-1 (DDAH-1); approximately 20% is excreted unchanged in the urine [16]. Hepatic blood flow is known to be significantly impaired in severe malaria [27]. The correlation of bilirubin and creatinine with increased ADMA, suggests that similar to sepsis [28], decreased hepatic and renal elimination may also increase ADMA concentrations in severe malaria. The large parasite biomass in severe malaria may also be a potential source of ADMA, with Plasmodium falciparum possessing protein arginine methyltransferases capable of producing ADMA [20]. The significant independence correlation between parasite biomass and ADMA on admission suggests this may be occurring in vivo, although the persistently elevated levels in severe malaria after commencement of anti-malarial therapy suggest the importance of altered host production and clearance in the post-treatment period. Increased clearance due to increases in either hepatic blood flow or DDAH activity may explain the decreased ADMA concentrations in patients with moderately severe malaria. There are no clinical studies to date documenting increased DDAH activity in mild inflammatory diseases, but hepatic blood flow is known to be significantly increased in acute uncomplicated falciparum malaria compared to patients with severe disease and healthy individuals [27]. The converse may also be true, with lower plasma ADMA concentrations potentially increasing vascular NO bioavailability in moderately severe malaria [14], and possibly contributing to elevated hepatic blood flow.

The loss of DDAH-1 function in a murine model of sepsis increased ADMA, reduced NO signaling, and worsened vascular pathophysiology including endothelial function [29]. In human severe sepsis, a polymorphism in the DDAH-2 enzyme increased ADMA levels which were associated with increased severity of organ failure and early septic shock [30]. Recently, a genome-wide association study in children found that a polymorphism in the gene encoding DDAH-1 was associated with an increased likelihood of severe malaria [19]. These studies indicate that altered DDAH function may be a contributor to organ damage and increased mortality in severe malaria as well as in other critical illnesses.

SDMA does not inhibit NOS, but competes with plasma L-arginine for intracellular uptake by the cationic amino acid transporters (CAT). Unlike ADMA, it is not metabolized by DDAH and is almost exclusively eliminated by the kidneys [16]. In chronic disease SDMA has recently been shown to be an independent predictor for major cardiovascular events in certain chronic diseases [31]. We find that in malaria, SDMA concentrations are associated with mortality and decreased vascular bioavailability on univariate analysis, but not after adjusting for renal function. While the association between SDMA and disease severity is likely to reflect the degree of renal impairment and SDMA retention, it is possible that retained SDMA may also contribute to decreased NO bioavailability in severe malaria.

In critically ill adults with organ failure and severe sepsis, ADMA concentrations are associated with increased all-cause mortality and the severity of organ failure [17,30]. Investigators have hypothesized that this may result from non-selective inhibition by ADMA of all three isoforms of NOS, particularly homeostatic NOS3 (endothelial NOS) [18]. This is similar to the postulated mechanism to explain the increased mortality with use of N^4^-monomethyl-arginine (NMMA), another non-specific NOS inhibitor, in a phase 3 clinical trial of sepsis [32]. In falciparum malaria, systemic NO production is impaired in severe disease and hypoargininemia is likely to be a contributing cause [6,10,11,13]. Exhaled and vascular NO are both reduced in adults with severe malaria [6], but not in moderately severe malaria (MSM) despite similar degrees of hypoargininemia [6]. This may be explained by the higher ADMA in severe malaria and a greater competitive inhibition of NOS in SM compared to MSM, similar to clinical studies of healthy volunteers in which ADMA infusion reduced blood flow [15,33]. In mouse studies, ADMA infusion alone reduces splenic blood perfusion, but when combined with hypoargininemia, causes a reduction in renal, hepatic and splenic blood flow with organ damage [34]. Regulation of microcirculatory flow is dependent on pre-capillary arteriolar vasodilatory responses which in turn are critically dependent on NO production [35], with both likely to be decreased by ADMA in SM. By decreasing functional capillary density, ADMA could further impair microcirculatory function already compromised by parasite sequestration in capillaries and post-capillary venules [36].

We have previously shown that hemolysis-related NO quenching by cell-free hemoglobin is associated with reduced vascular NO bioavailability in severe malaria [25]. In malaria, increased ADMA and cell-free hemoglobin were independently related to endothelial dysfunction, suggesting that inhibition of NOS and NO quenching both reduce vascular NO bioavailability.

NO has multiple regulatory functions that maintain endothelial quiescence in vivo, including inhibition of endothelial Weibel-Palade body (WPB) exocytosis and ICAM-1 expression [37,38]. Plasma concentrations of angiopoietin-2 (Ang-2), an angiogenic factor stored in WPBs, predict increased mortality in malaria [7], and ICAM-1 is a major endothelial adhesion receptor mediating cytoadherence of parasitized red cells and microvascular sequestration [5]. We demonstrate that ADMA levels correlate with increased Ang-2, but the association between ADMA and increased mortality is independent of Ang-2, suggesting effects of NO inhibition in addition to increased WPB exocytosis.

Acute lung injury is a complication of severe malaria in adults associated with a high mortality rate [21,24]. Gas transfer at the alveolar-capillary membrane and exhaled NO are both decreased in severe falciparum malaria [6,24]. In an animal model of sepsis–associated pulmonary injury, non-selective NOS inhibition causes increased lung edema [39]. Clinical studies have shown decreased pulmonary NO concentrations in patients with acute respiratory distress syndrome, as well as an association between decreased NO production and a worse outcome in acute lung injury [22,23]. The lung is a major source of ADMA and increased concentrations are associated with pulmonary arterial hypertension [40,41]. In severe malaria, both ADMA and parasite biomass are strongly inversely associated with exhaled NO concentrations, suggesting that both factors impair pulmonary NO production in severe disease.

There are several limitations in our study. Measurement of exhaled NO was not possible in patients with coma and was possible in only half of severe malaria patients without coma. Our results may therefore not reflect the relationship between ADMA and exhaled NO in all syndromes of severe malaria. In patients
who died, only 4 of 8 had at least one repeat blood sample, and
the longitudinal data may not truly reflect the course of the
methylated arginines in fatal cases. RH-PAT index is at least 50%
dependent on endothelial NO release [42], but we cannot
exclude an effect of ADMA on other vasodilators such as
prostacyclin and endothelium-derived hyperpolarizing factor.
Although we have measured plasma ADMA concentrations,
the effects of ADMA are intracellular. Nevertheless, in vitro studies
with endothelial cells have shown that increasing extracellular
ADMA results in five-fold increases in intracellular concentra-
tions. This suggest that intracellular concentrations of ADMA in
severe malaria may be higher, and may be adequate for
meaningful inhibition of for all three NOS isoforms (IC50 ~2-
5μM [43]. The observational nature of the study does not allow
us to conclude with certainty a direct role for ADMA in the
pathophysiology of severe malaria. While the association of
ADMA with mortality may reflect impaired renal and hepatic
function, it remained significant after adjusting for these factors in
a multivariable model. Furthermore, increased ADMA from
impaired hepatic and/or renal clearance is not just a marker of
organ dysfunction in critical illness, with retained ADMA having
functional consequences on NOS activity.

In summary, the endogenous non-selective NOS inhibitor
ADMA is elevated in SM and is an independent risk factor for
mortality. ADMA is also associated with decreased FeNO and
vascular NO bioavailability, as well as increased endothelial
activation and parasite biomass. Therapies which increase NO
bioavailability or which diminish ADMA levels represent rational
approaches for interventional trials of adjunctive therapy in severe
malaria.

Methods

Study site and patients

The study was conducted at Mitra Masyarakat Hospital,
Timika, Papua, Indonesia, a region with unstable transmission
of multidrug resistant malaria [44,45]. Written informed consent
was obtained from all patients, if they were comatose or too ill,
consent was obtained from relatives. The Ethics Committees of the
National Institute of Health Research and Development, Indone-
sia, and Menzies School of Health Research, Australia approved
the study.

Patients were ≥18 years old with moderately-severe (MSM) or
severe (SM) Plasmodium falciparum malaria without P. vivax infection
and with a hemoglobin level >60 g/L who had been prospectively
enrolled in a study of endothelial dysfunction and exhaled NO [6].
Previous results from this study group have been published
[6,7,13,25]. Briefly, SM was defined as P. falciparum parasitemia
and ≥1 modified WHO criterion of severity (excluding severe
anemia). MSM was defined as fever within the preceding
48 hours, >1,000 asexual P. falciparum parasites/μL, no WHO
warning signs or severe malaria criteria and a requirement
for inpatient parenteral therapy because of inability to tolerate oral
treatment. Healthy controls (HC) were non-related hospital
visitors with no history of fever in last 48 hours, intercurrent
illness or smoking in last 12 hours, or evidence of parasitemia [6].

Standardized history and physical examination were documented.
Heparinized blood was collected daily, centrifuged within 30
minutes of collection and plasma stored at −70°C. Parasite counts
were determined by microscopy. Hemoglobin, biochemistry, acid-
base parameters and lactate were measured with a bedside analyser
(i-STAT Corp). Patients were treated with anti-malarials
and antibiotics using standard national protocols as previously
described [6].

L-arginine, asymmetrical dimethylarginine and
symmetrical dimethylarginine

Solid phase extraction (SPE) of amino acids was followed by
derivatization with Acq-Fluor and separation on a Gemini-NX
column at pH 9 [46]. The SPE method gives absolute recoveries
of >80% for ADMA and symmetrical dimethylarginine (SDMA)
and average relative recoveries of 102% for ADMA and 101% for
SDMA. The HPLC method gives intra-assay RSDs of 2.1% and
2.3% and inter-assay RSDs of 2.7% and 3.1% for ADMA and
SDMA respectively [46].

Cell-free hemoglobin, cytokines, endothelial activation,
arginase and L-arginine

Plasma concentrations of cell-free hemoglobin and the endo-
thalial activation markers, ICAM-1, E-selectin and angiopoietin-2
were measured by ELISA as previously reported in this population
[6,7,25]. Total parasite biomass was quantified by measuring
plasma histidine rich protein-2 (HRP2) by ELISA [6], plasma
arginase by a radiometric method [6] and plasma TNF
concentrations by flow cytometry, as previously reported [7].

Endothelial function and pulmonary nitric oxide

Endothelial function was measured non-invasively using
peripheral arterial tonometry (EndoPAT) by the change in digital
pulse wave amplitude in response to reactive hyperemia, giving a
RH-PAT Index as reported previously [6]. The RH-PAT index is
at least 50% dependent on endothelial NO production [42].
Endothelial function was measured daily until death or discharge,
or until the RH-PAT index was above an a priori cutoff (1.67) for
two consecutive days [13]. Fractional concentrations of exhaled
NO were measured by NO analysyr (Aerocrine), as previously
described, using American Thoracic Society guidelines and a flow
crate of 250 ml/sec [6].

Statistical methods

Statistical analysis was performed with STATA 9.2 software.
Intergroup differences were compared by ANOVA or Kruskal-
Wallis test, where appropriate. Pearson’s or Spearman’s correla-
tion coefficients were determined depending on normality of
distributions. Multiple stepwise linear regression was used to adjust
for confounding variables. Longitudinal associations were assessed
by mixed effects modeling using generalized estimating equations.
Logistic regression was used to determine the association between
death and ADMA concentrations. Variables hypothesized, as well
as those shown in previous publications [6,7,25], to contribute to
mortality, pulmonary NO and endothelial pathology were
included in a multiple regression model if p<0.05 on univariate
analysis and retained if they remained significant. Goodness-of-fit
was assessed by the Hosmer-Lemeshow goodness of fit test and
independent variables tested for interactions. The prognostic
utility of continuous variables was measured using the area under
the receiver operating curves (ROCs) and its 95% confidence
intervals were calculated. A two-sided value of p<0.05 was
considered significant.

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Author Contributions
Conceived and designed the experiments: TWY ET RG DKL BKL JBW RNP SBD NMA. Performed the experiments: TWY ET RG CJD CJ EK YRM NMA. Analyzed the data: TWY RNP NMA. Wrote the paper: TWY DAL ET RG CJD CJ EK YRM DKL BKL JBW RNP SBD NMA.

References
1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434: 214–217.
2. World Health Organization (2008) World Malaria Report.
3. Day N, Boodrop AM (2007) The Management of Patients with Severe Malaria. Am J Trop Med Hyg 77(Suppl 6): 29–35.
4. The SEACSUMAT Trial Group (2005) Artemesinum versus quinine for treatment of severe falciparum malaria: a randomised trial. Lancet 366: 717–725.
5. Turner GD, Morrison H, Jones M, Davis TM, Looareesuwan S, et al. (1994) An angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe falciparum malaria. Proc Natl Acad Sci U S A 101: 17097–17102.
6. Yeo TW, Lamph DA, Gitawati R, Tjitra E, Kenangalem E, et al. (2007) Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria. J Exp Med 204: 2693–2704.
7. Yeo TW, Lamph DA, Gitawati R, Tjitra E, Kenangalem E, et al. (2008) Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe falciparum malaria. Proc Natl Acad Sci U S A 105: 17097–17102.
8. Minigo G, Woodberry T, Peza KA, Salvati E, Tjitra E, et al. (2009) Paracrine-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. PLoS Pathog 5: e1000402. doi: 10.1371/journal.ppat.1000402.
9. Levelgrove FE, Tangsukkdeel O, Opoka RO, Laferi EJ, Rajcans N, et al. (2009) Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children. PLoS ONE 4: e4912. doi: 10.1371/journal.pone.004912.
10. Lopezari BK, Anstey NM, Weinberg JB, Stansfeld CJ, Hobbs MR, et al. (2009) Low plasma arginine concentrations in children with cerebral malaria and decreased nitric oxide production. Lancet 361: 676–678.
11. Anstey NM, Weinberg JB, Hassanali MY, Mwaikambo ED, Manyenga D, et al. (2007) Higher urine nitric oxide is associated with improved outcomes in patients with the acute respiratory distress syndrome. Am J Respir Crit Care Med 175: 256–262.
12. Maguire GP, Handjojo T, Pain MC, Kenangalem E, Price RN, et al. (2005) Lung injury in uncomplicated and severe falciparum malaria: a longitudinal study in papua, Indonesia. J Infect Dis 192: 1966–1974.
13. Richer MC, Bouwman RH, Teerlink T, Siroen MP, de Vries TP, et al. (2008) The prominent role of the liver in the elimination of asymmetric dimethylarginine (ADMA) and the consequences of impaired hepatic function. JPEP J Parenter Enter Nutr 32: 613–621.
14. Leiper J, Nandi M, Torondel B, Murray-Rust J, Malaki M, et al. (2007) Disruption of methylarginine metabolism impairs vascular homeostasis. Nat Med 13: 198–203.
15. O'Dwyer MJ, Dempsey F, Crowley V, Kelleher DP, MacManus R, et al. (2006) Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study. Crit Care 10: R139.
16. Wang Z, Tang WH, Cho L, Brennan DM, Hazen SL (2005) Targeted Metabolic Evaluation of Arginyl Methylation and Cardiovascular Risks. Potential Mechanisms Beyond Nitric Oxide Synthase Inhibition. Arterioscler Thromb Vasc Biol 16(3) e467–473. doi:10.1161/01.ATV.0000171340.38396.3c. Published online 2005 Apr 25.
17. Richer MC, van Lagalgaen BA, Teerlink T, Wiesinlin W, Bloemena E, et al. (2009) Low arginine/asymmetric dimethylarginine ratio deteriorates systemic hemodynamics and organ blood flow in a rat model. Crit Care Med 37: 2019–2027.
18. Vaughn MW, Kuo L, Liao JC (1998) Effective diffusion distance of nitric oxide in the microcirculation. Am J Physiol 274: H1705–1714.
19. Dondorp AM, Pongsonrat E, White NJ (2004) Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. Acta Trop 89: 309–317.
20. Lowenstein CJ, Morrell CN, Yamakuchi M (2005) Regulation of Weibel-Palade body cxoxcytosine. Trends Cardiovasc Med 15: 302–309.
21. DeCastro R, Llibby P, Persijn GR, Thrommel VJ, Rajvashash TR, et al. (1997) Nitric oxide decreases cytokine-induced endothelial activation. Circulation 95: 60–68.
22. Hinder F, Stubb DE, Van Aken H, Waurick R, Boochee M, et al. (1999) Role of nitric oxide in sepsis-associated pulmonary edema. Am J Respir Crit Care Med 159: 252–257.
23. Bilas P, Zakrzewska D, Kowalska K, Leiper J, Gunther A, et al. (2007) Analysis of methylarginine metabolism in the cardiovascular system identifies the lung as a major source of ADMA. Am J Physiol Lung Cell Mol Physiol 292: L18–24.
24. Kielstein JT, Bode-Boger SM, Heese G, Martens-Lobenhoffer J, Takacs A, et al. (2005) Asymmetric dimethylarginine in idiopathic pulmonary arterial hypertension. Arterioscler Thromb Vasc Biol 25: 1411–1417.
25. Norhia A, Gerhardt-Hermans M, Greager MA, Hurley S, Mitra D, et al. (2006) Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. J Appl Physiol 101: 545–549.
26. Anthony S, Leiper J, Vallance P (2005) Endogenous production of nitric oxide synthase inhibitors. Vasc Med 10 Suppl 1: S73–81.
27. Nijveldt RJ, Teerlink T, Van Der Hoven B, Siroen MP, Kuik DJ, et al. (2003) Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality. Clin Nutr 22: 25–30.
28. Jallow M, Teo YY, Mai NT, Chau TT, Loc PP, et al. (2000) The pathophysiological and prognostic significance of acidosis in severe adult malaria. Crit Care Med 28: 1833–1840.
29. Molyneux ME, Looareesuwan S, Menzies IS, Grainger SL, Phillips RE, et al. (1989) Reduced hepatic blood flow and intestinal malabsorption in severe falciparum malaria. Am J Trop Med Hyg 40: 470–476.
30. Lovgrove FE, Tangsukkadeel O, Opoka RO, Laferi EJ, Rajcans N, et al. (2009) Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and decreased nitric oxide production/nitric oxide synthase type 2 expression. J Exp Med 198: 537–567.
31. Hibbs J, Vaven Z, Tanner R (1987) L-arginine is required for the expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J Immunol 138: 550–557.
32. Lopezari BK, Anstey NM, Weinberg JB, Stansfeld CJ, Hobbs MR, et al. (2009) Low plasma arginine concentrations in children with cerebral malaria and decreased nitric oxide production. Lancet 361: 676–678.
33. Richer MC, van Lagalgaen BA, Teerlink T, Wiesinlin W, Bloemena E, et al. (2009) Low arginine/asymmetric dimethylarginine ratio deteriorates systemic hemodynamics and organ blood flow in a rat model. Crit Care Med 37: 2019–2027.
34. Kielstein JT, Bode-Boger SM, Heese G, Martens-Lobenhoffer J, Takacs A, et al. (2005) Asymmetric dimethylarginine in idiopathic pulmonary arterial hypertension. Arterioscler Thromb Vasc Biol 25: 1411–1417.
35. Norhia A, Gerhardt-Hermans M, Greager MA, Hurley S, Mitra D, et al. (2006) Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. J Appl Physiol 101: 545–549.
36. Jones CE, Darcy CJ, Woodberry T, Anstey NM, McNeil YR (2010) HPLC of methylarginine and arginine in small plasma volumes using a Gemini-NX column at high pH. Journal of Chromatography B Analyt Technol Biomed Life Sci 876: 8–12.