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Kinetic and Cross-Sectional Studies on the Genesis of Hypoargininemia in Severe Pediatric *Plasmodium falciparum* Malaria

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ABSTRACT The low bioavailability of nitric oxide (NO) and its precursor, arginine, contributes to the microvascular pathophysiology of severe falciparum malaria. To better characterize the mechanisms underlying hypoargininemia in severe malaria, we measured the plasma concentrations of amino acids involved in *de novo* arginine synthesis in children with uncomplicated falciparum malaria (UM; $n = 61$), children with cerebral falciparum malaria (CM; $n = 45$), and healthy children (HC; $n = 109$). We also administered primed infusions of L-arginine uniformly labeled with $^{13}C_6$ and $^{15}N_4$ to 8 children with severe falciparum malaria (SM; age range, 4 to 9 years) and 7 healthy children (HC; age range, 4 to 8 years) to measure the metabolic flux of arginine, hypothesizing that arginine flux is increased in SM. Using two different tandem mass spectrometric methods, we measured the isotopic enrichment of arginine in plasma obtained at 0, 60, 90, 120, 150, and 180 min during the infusion. The plasma concentrations of glutamine, glutamate, proline, ornithine, citrulline, and arginine were significantly lower in UM and CM than in HC ($P \leq 0.04$ for all pairwise comparisons). Of these, glutamine concentrations were the most markedly decreased: median, 457 μM (interquartile range [IQR], 400 to 508 μM) in HC, 300 μM (IQR, 256 to 365 μM) in UM, and 257 μM (IQR, 195 to 320 μM) in CM. Arginine flux during steady state was not significantly different in SM than in HC by the respective mass spectrometric methods: 93.2 μmol/h/kg of body weight (IQR, 84.4 to 129.3 μmol/h/kg) versus 88.0 μmol/h/kg (IQR, 73.0 to 102.2 μmol/h/kg) ($P = 0.247$) by the two mass spectrometric methods in SM and 93.7 μmol/h/kg (IQR, 79.1 to 117.8 μmol/h/kg) versus 81.0 μmol/h/kg (IQR, 75.9 to 88.6 μmol/h/kg) ($P = 0.165$) by the two mass spectrometric methods in HC. A limited supply of amino acid precursors for arginine synthesis likely contributes to the hypoargininemia and NO insufficiency in falciparum malaria in children.

KEYWORDS *Plasmodium falciparum*, arginine, glutamine, malaria, nitric oxide

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Effective chemotherapeutics and successful public health prevention campaigns have reduced the malaria incidence worldwide (1). However, falciparum malaria remains a major cause of childhood morbidity and mortality, with 45 million disability-adjusted life-years lost (2) and in 2016 over 2,164 million cases, including 445,000 malaria-related deaths (3). The pathogenesis of severe falciparum malaria is characterized by endothelial dysfunction (4), endothelial activation (5–7), and parasite sequestration in the postcapillary venules of the human host (8). All of these processes lead to microvascular obstruction and metabolic acidosis (8, 9). We have previously observed that nitric oxide (NO) production, as measured by determination of the amount of NO metabolites, and vascular NO bioavailability are low in patients with severe malaria (4, 10). This low NO bioavailability likely contributes to the microvascular pathophysiology of severe malaria. NO can prevent or reverse inflammatory cytokine overproduction (11); prevent binding of parasitized red blood cells to endothelial cells (12); decrease adhesion molecule expression (13); and modulate endothelial activation, which is an independent predictor of death in severe malaria (5, 8).

NO synthesis from arginine and molecular oxygen is catalyzed by nitric oxide synthase (NOS) in its three isoforms: NOS3 (present in endothelium); NOS1 (present in nerve cells); and the inducible form, NOS2 (present in multiple cell types). NOS generates NO from the equivalent guanido-nitrogen atoms of arginine, leaving citrulline as the amino acid reaction product. We and others have found that plasma arginine levels are markedly decreased in children with uncomplicated falciparum malaria (UM) and children with severe falciparum malaria (SM) compared to healthy children (HC) (14–18), with hypoargininemia also being observed in adult severe falciparum malaria (4). We have also demonstrated that the clearance of exogenously administered L-arginine is increased in adult falciparum malaria (19, 20). To better characterize our findings of low plasma arginine levels and low NO production in severe malaria, we measured the plasma concentrations of amino acids involved in *de novo* arginine synthesis. *De novo* arginine synthesis involves the absorption of glutamine by small intestinal enterocytes, where glutamine is enzymatically converted to citrulline. Enterocytes then secrete citrulline into the bloodstream, and citrulline is enzymatically converted to arginine in the proximal tubular cells of the kidney (see Fig. 1A for a detailed illustration of the metabolic pathway for arginine *de novo* synthesis). In the current work, we complement these measurements of amino acid precursors for arginine synthesis by conducting a heavy isotope tracer study in which we infuse L-arginine uniformly labeled with $^{13}C_6$ and $^{15}N_4$ to trace the metabolic flux of arginine (i.e., the turnover or the rate of appearance and rate of disposal per unit of time) in children with severe falciparum malaria (SM) and in healthy children (HC) in Dar es Salaam, Tanzania (see Fig. 1B for a schematic overview of arginine metabolism). This infusion tracer study enabled us to measure the appearance of heavy isotope-labeled arginine (A+10) and the downstream amino acids resulting from both hepatic and extrahepatic arginine metabolism (Fig. 1C). In order to demonstrate the analytical validity of our heavy isotope measurements, we employed two independent mass spectrometry (MS) methodologies. Through this controlled, heavy isotope tracer experiment, we tested our hypothesis that low levels of arginine in SM are associated with increased arginine flux.

RESULTS

Arginine amino acid precursor observational study. We enrolled 116 HC, 66 UM, and 52 children with cerebral falciparum malaria (CM). A total of 109 HC, 61 UM, and 45 CM had sufficient plasma for amino acid analysis. The baseline characteristics of the three groups are shown in Table 1. Among the CM, seven died. CM were significantly older than the children in the other two groups, and the time since the last meal was longer in both UM and CM than in HC.

Glutamine levels were significantly lower in UM and CM than in HC (Fig. 2) ($P \leq 0.025$ for all pairwise comparisons). In addition, glutamate levels were lower in children with malaria than in HC (Fig. 2) ($P \leq 0.036$ for all pairwise comparisons).

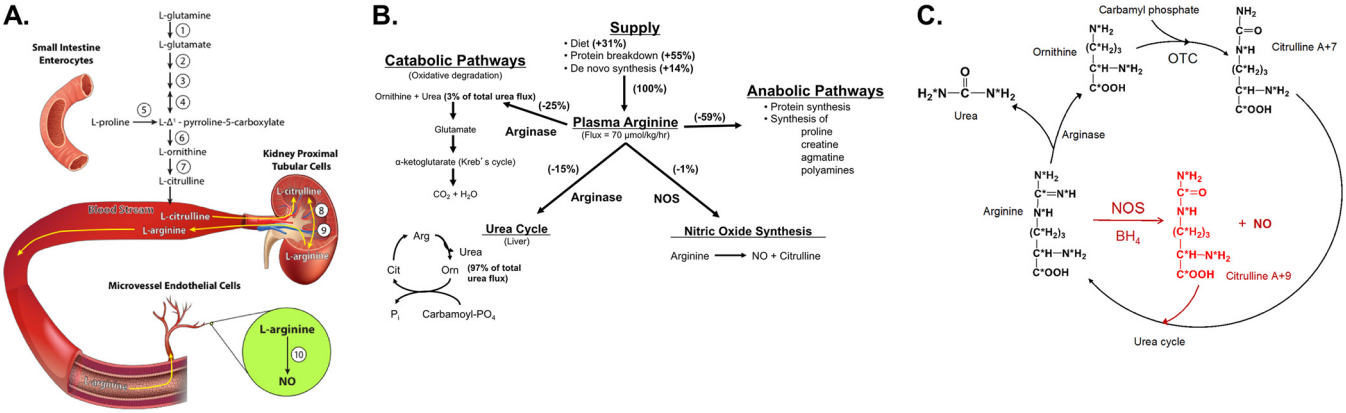

FIG 1 (A) L-Arginine *de novo* synthesis pathways. Reactions 1 to 7 take place in small intestinal enterocytes, reactions 8 and 9 take place in proximal tubular cells of the kidney, and reaction 10 takes place in microvascular endothelial cells in many organs of the body. The definitions of steps 1 to 10 are as follows: 1, glutaminase; 2, pyrroline-5-carboxylase synthetase; 3, pyrroline-5-carboxylase synthetase (a bifunctional enzyme); 4, nonenzymatic reaction (equilibrium favors pyrroline-5-carboxylase): 5, proline oxidase; 6, ornithine aminotransferase; 7, ornithine carbamoyltransferase; 8 argininosuccinate synthetase; 9, argininosuccinate lyase; 10, nitric oxide synthase. (Republished from reference 45 with permission of the publisher.) (B) Arginine balance in the healthy, fed state. The arginine balance during health (fed state) is based upon studies of human adults. The urea synthesis is cyclic, with neither a net loss nor a net gain of amino acids. The *de novo* synthesis of arginine occurs from the metabolism of glutamine in the gut and kidney, and arginine is also derived from proteolysis and the diet. Arginine is disposed of through the synthesis of proteins, proline, creatine, agmatine, and polyamines, as well as oxidation to carbon dioxide and water (42, 45). The action on arginine by arginase can occur within the liver as part of the urea cycle or extrahepatically, in many cell types, as part of arginine oxidative degradation. Arginine succinate synthetase and arginine-succinate lyase participate in arginine recycling. Under homeostatic conditions in the fed state, arginine flux is ~70 μmol/h/kg in adult humans. The numbers in parentheses show the percentage of arginine flux through various metabolic routes (49, 50). (C) Metabolic scheme showing the fate of heavy isotope-labeled l-arginine and its metabolites. The figure illustrates the pathway by which [U-$^{13}C_6$, U-$^{15}N_4$]L-arginine (A+10) produces citrulline (A+9) via the nitric oxide synthase (NOS) pathway and heavy isotope-labeled [U-$^{13}C_6$, U-$^{15}N_4$]L-arginine (A+10) produces ornithine A+7 and citrulline A+7 via the arginase pathway. *, a heavy isotope atom. Accordingly, A represents the unlabeled, most abundant amino acid molecule, and the integer (A+10, A+9, or A+7) signifies the number of heavy isotope atoms on the labeled molecule, as shown by the asterisks. OTC, ornithine transcarbamoylase.

Citrulline, arginine, proline, and ornithine levels were also decreased in those with malaria (Fig. 2) (for all comparisons of HC versus UM and HC versus CM, $P \leq 0.013$; for comparisons of UM versus CM, $P > 0.05$). Compared to the normal reference range of concentrations established in North America, a significant proportion of UM and CM children had low amino acid concentrations for glutamine, proline, ornithine, citrulline, and arginine (Fig. 2 and Table 2). The proportion of UM and CM with amino acid levels

TABLE 1 Baseline clinical characteristics of HC, UM, and CM

Characteristic	Value for[a]: HC (n = 109)	UM (n = 61)	CM (n = 45)	P value[b]
Age (yr)	2.9 (2.6–3.2)	3.6 (3.2–4.0)	4.2 (3.8–4.6)	<0.001[AB]
No. (%) of female patients	44 (45)	30 (49)	17 (37)	0.436[c]
Wt (kg)	12.8 (12.2–13.4)	14.1 (13.2–15.1)	15.3 (14.2–16.4)	<0.001[B]
Time to last food (h)	3.2 (2.9–3.6)	5.8 (4.6–6.9)	6.6 (4.6–8.6)	<0.001[AB]
Temp (°C)	36.7 (36.6–36.7)	39.1 (38.8–39.3)	38.2 (37.9–38.6)	<0.001[ABC]
Heart rate (no. of beats/min)	103 (101–106)	116 (111–121)	131 (125–137)	<0.001[ABC]
Mean arterial blood pressure (mm Hg)	65 (63–66)	67 (65–68)	74 (71–77)	<0.001[BC]
Respiratory rate (no. of breaths/min)	32 (31–33)	37 (35–39)	43 (39–47)	<0.001[ABC]
White blood cell count ($10^3/\mu$l)	10.7 (9.1–12.4)	8.9 (8.1–9.7)	10.0 (8.6–11.5)	0.226
Hemoglobin concn (g/dl)	10.7 (10.4–11.1)	8.3 (7.7–8.9)	7.1 (6.6–7.7)	<0.001[ABC]
Platelet count ($10^3/\mu$l)	301 (269–332)	154 (128–180)	85 (67–103)	<0.001[ABC]
Creatinine concn (mg/dl)[d]	ND[e]	0.3 (0.3–0.4)	0.6 (0.4–0.8)	<0.001
Glucose concn (mg/dl)[d]	ND	119 (99–143)	118 (104–132)	0.936
Parasitemia (no. of parasites/μl)[f]	0	94,451 (32,440–211,264)	42,744 (8,592–131,424)	0.018

[a]Results are presented as means (95% confidence intervals) unless otherwise noted.
[b]The test of significance was performed by one-way ANOVA with the Bonferroni correction for comparisons across all groups unless otherwise noted. A, Bonferroni correction P value of <0.05 for HC-versus-UM comparison; B, Bonferroni correction P value of <0.05 for HC-versus-CM comparison; C, Bonferroni correction P value of <0.05 for UM-versus-CM comparison.
[c]Chi-square test of proportions.
[d]Creatinine and glucose concentrations were obtained for only 27 UM and 43 CM patients, and Student's t test was the test of significance used.
[e]ND, not determined.
[f]Results are presented as the median (interquartile range), and the Wilcoxon rank-sum test was the test of significance.

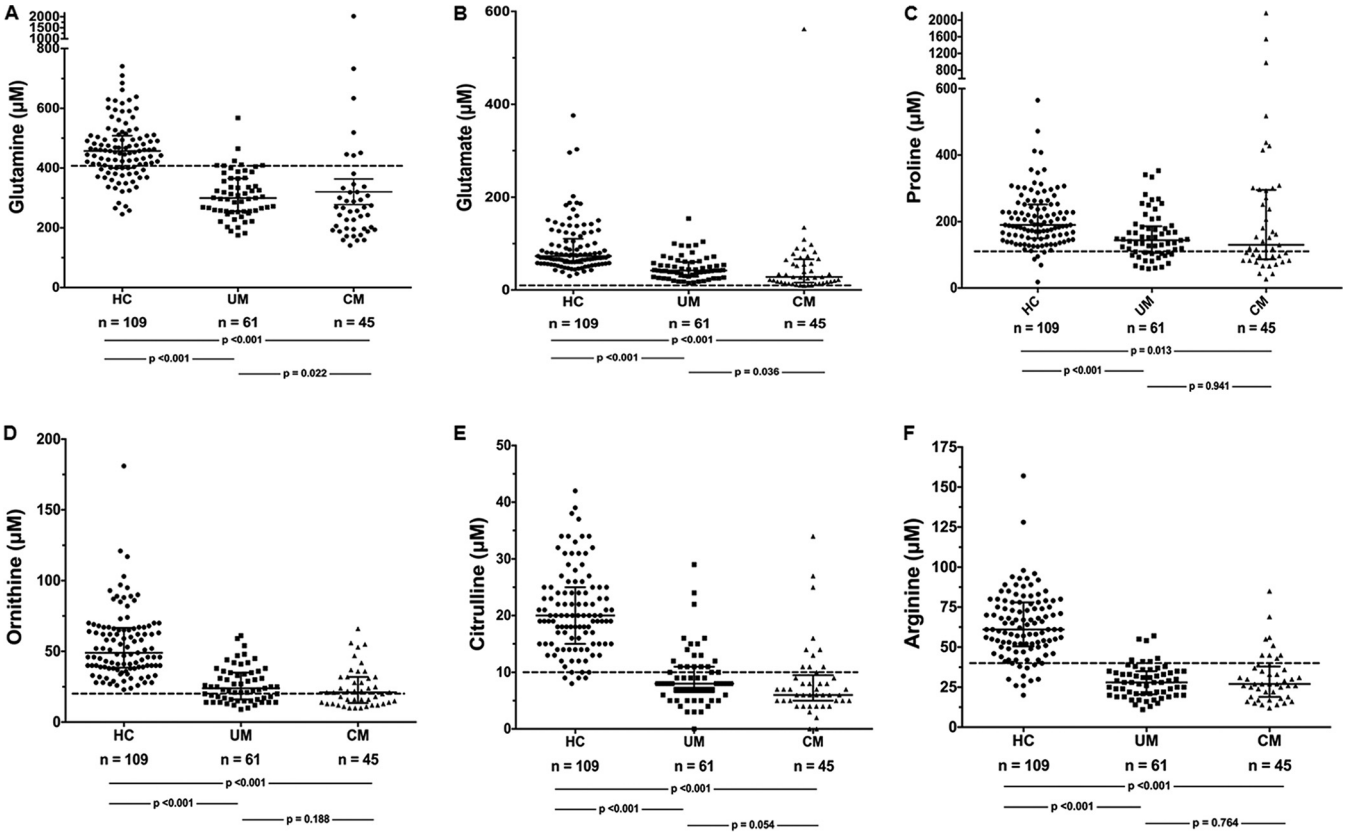

FIG 2 Plasma concentrations of arginine and its amino acid precursors among healthy children (HC), children with uncomplicated malaria (UM), and children with cerebral malaria (CM). Plasma amino acid levels are presented as the median (horizontal line) with the interquartile range (vertical line with whiskers). The dashed line represents the lower limit of normal taken from the established pediatric reference range (34). (A) The median levels of glutamine were 457 μM (IQR, 400 to 508 μM) for HC, 300 μM (IQR, 256 to 365 μM) for UM, and 257 μM (IQR, 195 to 320 μM) for CM. (B) The median levels of glutamate were 73 μM (IQR, 58 to 105 μM) for HC, 42 (IQR, 29 to 60 μM) for UM, and 28 μM (IQR, 17 to 65 μM) for CM. (C) The median levels of proline were 190 μM (IQR, 151 to 252 μM) for HC, 144 μM (IQR, 107 to 184 μM) for UM, and 130 μM (IQR, 90 to 294 μM) for CM. (D) The median levels of ornithine were 49 μM (IQR, 39 to 66 μM) for HC, 24 μM (IQR, 16 to 35 μM) for UM, and 21 μM (IQR, 14 to 32 μM) for CM. (E) The median levels of citrulline were 20 μM (IQR, 15 to 25 μM) for HC, 8 μM (IQR, 7 to 11 μM) for UM, and 6 μM (IQR, 5 to 9 μM) for CM. (F) The median levels of arginine were 61 μM (IQR, 51 to 78 μM) for HC, 28 μM (IQR, 21 to 35 μM) for UM, and 27 μM (IQR, 19 to 37 μM) for CM. For citrulline (E) and arginine (F), the lower limit displayed is for children ≥12 months of age. Twelve of 109 HC were infants, all of whom had citrulline and arginine measurements above the lower limits of normal for infants (6 μM and 20 μM, respectively). Four of 61 UM were infants, all of whom had a citrulline level above the lower limit of normal for infants; 2 infants that were in the UM group had arginine measurements below the lower limit of normal for infants. The Wilcoxon rank-sum test was the test of significance.

below the lower limit of normal was significantly higher than the proportion of HC (Table 2).

Arginine flux infusion study. For arginine flux studies, we enrolled 10 SM and 10 HC. Of these, 3 HC and 2 SM were excluded from the analysis because of deviations from the infusion protocol (*viz.*, early cessation of the infusion, errors in the volumes

TABLE 2 Numbers of HC, UM, and CM with plasma concentrations below the pediatric normal reference range for arginine and amino acid precursors for *de novo* arginine synthesis

Amino acid	Lower limit of reference range concn[a] (μM)	No. (%) of children			*P* value[b]
		HC (n = 109)	UM (n = 61)	CM (n = 45)	
Glutamine	410	31 (28)	57 (93)	38 (84)	<0.001
Glutamate	10	0 (0)	0 (0)	1 (2)	0.150
Proline	110	5 (5)	18 (30)	18 (40)	<0.001
Ornithine	20	0 (0)	21 (34)	20 (44)	<0.001
Citrulline	6 (<12 mo of age) or 10 (≥12 mo of age)	4 (4)	38 (62)	34 (76)	<0.001
Arginine	20 (<12 mo of age) or 40 (≥12 mo of age)	10 (9)	52 (85)	36 (80)	<0.001

[a]As no established reference range was available for Tanzanian children, the lower limits were taken from the normal ranges for the North American pediatric population established by the reference laboratory that performed the plasma amino acid measurements for this cohort study (34).
[b]The chi-square test was used to compare proportions across all three groups.

TABLE 3 Baseline clinical characteristics of HC and SM included in the arginine flux infusion study

Characteristic	Value[a] for:		*P* value[b]
	HC ($n = 7$)	SM ($n = 8$)	
Age (yr)	7 (5–8)	5 (4–7.5)	0.213
% of female patients	57.1	50.0	0.077[c]
Wt (kg)[d]	21.9 (17.8–26.1)	16.9 (13.7–20.2)	0.039
Time to last food (h)	14 (14–16)	12 (8–12)	0.019
Temp (°C)[d]	36.7 (36.4–37.1)	37.6 (37.8–38.5)	0.055
Heart rate (no. of beats/min)	92 (87–104)	113 (101–160)	0.020
Mean arterial blood pressure (mm Hg)	70 (69–73)	70 (68–72)	0.859
Respiratory rate (no. of breaths/min)	24 (22–26)	41 (34–53)	0.003
White blood cell count ($10^3/\mu$l)	5.3 (4.1–6.6)	7.4 (4.6–10.3)	0.247
Hemoglobin concn (g/dl)[d]	11.2 (10.3–12.2)	7.7 (6.0–9.3)	0.001
Platelet count ($10^3/\mu$l)[d]	299 (81–517)	65 (46–83)	0.023
Creatinine concn (mg/dl)[e]	0.4 (0.3–0.4)	0.6 (0.3–0.6)	0.291
Lactate concn (mmol/liter)[e]	2.2 (2.2–2.3)	2.4 (1.6–3.3)	0.684
Glucose concn (mg/dl)[e]	91 (76–99)	87 (55–102)	0.567
Parasitemia (no. of parasites/μl)	0	305,445 (262,760–422,680)	NA

[a]Values represent the median (IQR), unless indicated otherwise.
[b]The Wilcoxon rank-sum test was the test of significance unless otherwise noted. NA, not applicable.
[c]Chi-square test of proportions.
[d]The data represent the mean (95% confidence intervals), and *P* values were determined by Student's *t* test of significance.
[e]Data are for 5 HC and 7 SM. The sample size was insufficient for the remaining participants.

infused, and missed blood draws at stipulated time intervals). Among the SM, two had metabolic acidosis, one had severe anemia, five had hyperparasitemia, and none had cerebral falciparum malaria. Baseline clinical characteristics are presented in Table 3. HC had higher weights ($P = 0.003$) and a longer duration of fasting ($P = 0.005$) than SM. There was no difference in the median lactate concentration between SM and HC. No deaths occurred among SM, and no adverse events occurred in either group.

As in the observational study, SM had significantly lower median concentrations of plasma arginine and plasma citrulline at enrollment than HC (Table 4) ($P = 0.039$ and $P = 0.002$, respectively), while ornithine levels were not significantly different. Figure 3A to D shows the mole fractions of heavy isotope-labeled arginine (A+10) at each time point for each individual HC and SM during the labeled arginine infusion, as measured by both MS methods. The mean steady-state mole fractions for the clinical group (HC and SM) at each sampling time interval are shown in Fig. 3E to H. The mean mole fractions for each HC and SM (the average of each child's mole fraction measurements at the steady-state time points) are depicted in Fig. 4: median by MS method 1, 0.074 (interquartile range [IQR], 0.065 to 0.089) for HC versus 0.0722 (IQR, 0.051 to 0.077) for SM ($P = 0.247$); median by MS method 2, 0.079 (IQR, 0.073 to 0.087) for HC versus 0.071 (IQR, 0.057 to 0.083) for SM ($P = 0.247$). From these data, we calculated the mean arginine flux (in micromoles per hour per kilogram of body weight). The median arginine fluxes for HC and SM were not significantly different by either MS method: 88.0 μmol/h/kg (IQR, 73.0 to 102.2 μmol/h/kg) for HC and 93.2 μmol/h/kg (IQR, 84.4 to 129.3 μmol/h/kg) for SM ($P = 0.247$) by MS method 1 and 81.0 μmol/h/kg (IQR, 75.9 to 88.6 μmol/h/kg) for HC and 93.7 μmol/h/kg (IQR, 79.1 to 117.8 μmol/h/kg) for SM ($P = 0.165$) by MS method 2 (Fig. 5).

TABLE 4 Plasma amino acid concentrations in HC and SM enrolled in heavy isotope-labeled arginine infusion study

Amino acid	Median (IQR) concn (μM)		*P* value[a]
	HC ($n = 7$)	SM ($n = 8$)	
Arginine	78 (63–79)	43 (29–57)	0.039
Ornithine	35 (29–47)	24 (17–35)	0.200
Citrulline	16 (12–22)	7 (6–9)	0.002

[a]The Wilcoxon rank-sum test was used as the test of significance.

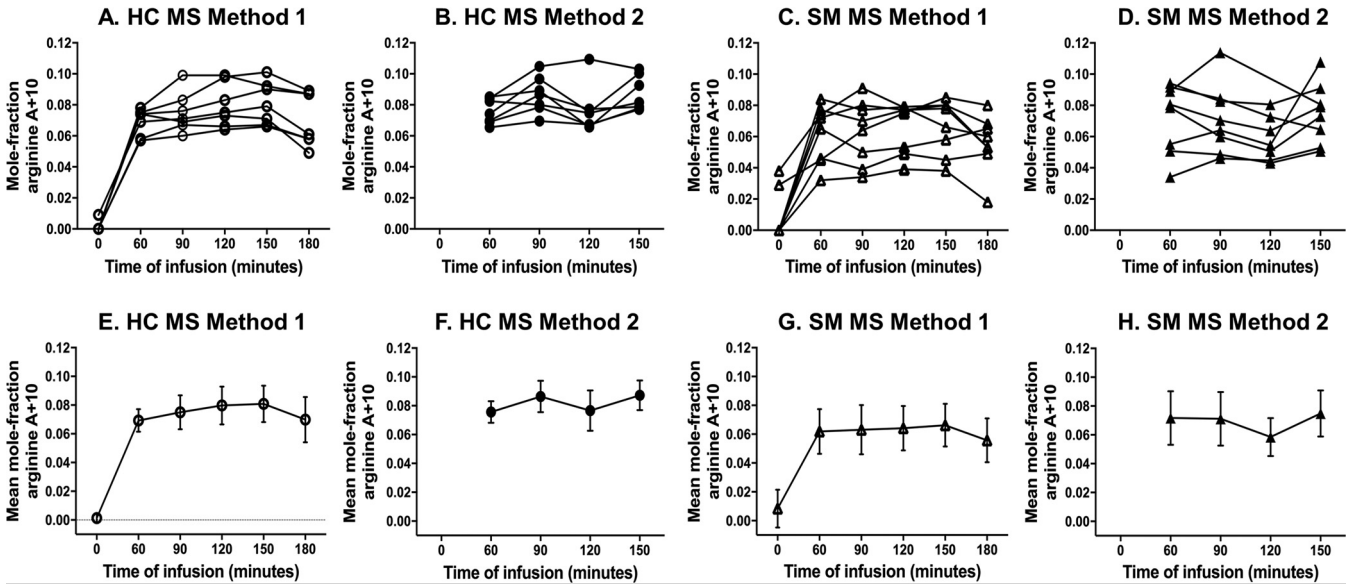

FIG 3 Plasma mole fractions of labeled arginine during infusion of the heavy isotope tracer among healthy children (HC) and children with severe malaria (SM). (A to D) The mole fraction of arginine A+10 for each of the 7 HC and for each of the 8 SM was measured at time intervals during arginine A+10 infusion. (A) Mole fraction measurements by MS method 1 for each HC before the priming dose and at 30-min intervals through 180 min. Note that 1 HC inadvertently received a fraction of the priming dose briefly before the first blood sample was taken, and thus, the time zero mole fraction value was above zero. (B) Mole fraction measurements by MS method 2 for each HC at time points of 60 to 150 min. (C) Mole fraction measurements by MS method 1 for each SM before the priming dose and at 30-min intervals through 180 min. Note that 2 SM had inadvertently received a fraction of the priming dose briefly before the first blood sample was taken, and thus, their time zero mole fraction values were above zero. (D) Mole fraction measurements by MS method 2 for each SM at time points 60 to 150 min. (E to H) The mean mole fraction for the respective clinical group (aggregate average for the 7 HC and aggregative average for the 8 SM) at each time point (vertical lines with whiskers represent the 95% confidence interval). (E) Values for HC measured by MS method 1; (F) values for HC measured by MS method 2; (G) values for SM measured by MS method 1; (H) values for SM measured by MS method 2.

We also measured the mole fraction enrichment of the following labeled amino acid metabolites of arginine: ornithine A+7 and citrulline A+7, the products of heavy isotope-labeled arginine metabolized via arginase (arginine A+10 to ornithine A+7) and then ornithine carbamoyltransferase (ornithine A+7 to citrulline A+7), and citrulline A+9, the product of labeled arginine metabolized by NOS. Figures 6, 7, and 8 show the mole fractions for HC and SM and the mean mole fractions of the respective clinical group (HC and SM) at each sampling time interval for ornithine A+7, citrulline A+7, and citrulline A+9, respectively. These data are presented principally to demonstrate that the tracer measurement methodology was successful. Quantitative comparisons of these tracer metabolites between HC and SM were not done because labeled infusions

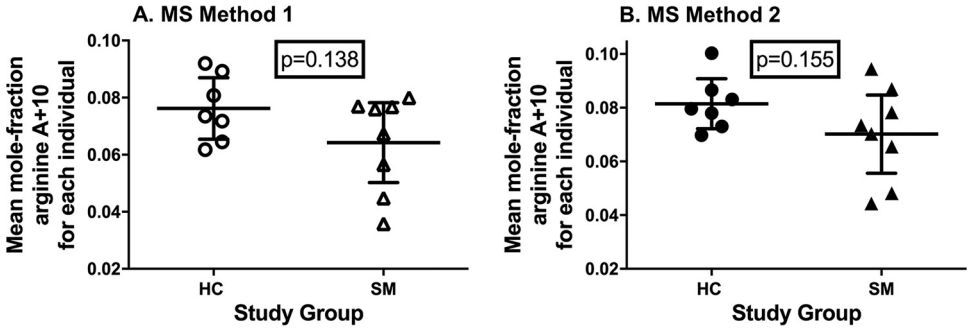

FIG 4 Steady-state mean mole fractions of labeled arginine for each of the healthy children (HC) and for each of the children with severe malaria (SM). Mean mole fractions of labeled arginine A+10 for each HC and each SM measured by MS method 1 (A) and by MS method 2 (B). The symbols represent the average mole fraction measurement for each participant (derived from all measurements from time points of 60 to 150 min for each individual). Horizontal lines display the means for each group, and vertical lines with whiskers display the 95% confidence intervals (CI). Student's *t* test was the test of significance.

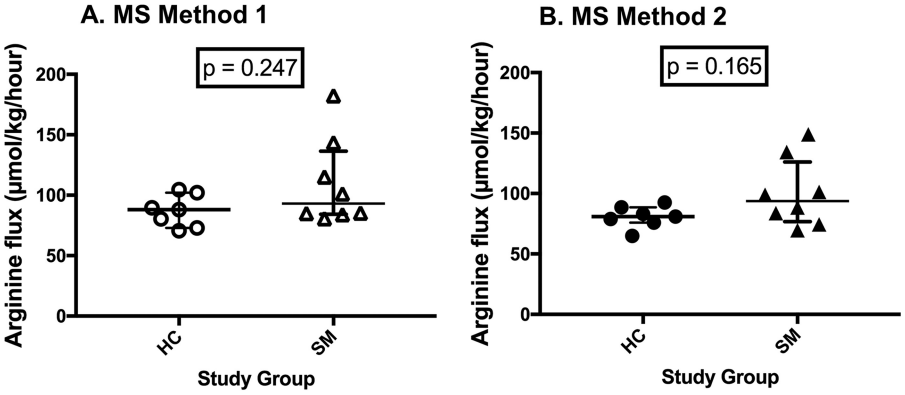

FIG 5 Steady-state arginine flux comparisons between healthy children (HC) and children with severe malaria (SM). The mean arginine flux values for each HC and SM are shown. Arginine flux is in micromoles per hour per kilogram of body weight and was calculated from Fig. 2A data using equation 1 in the Materials and Methods section. Arginine flux values are presented as medians (horizontal lines) with interquartile ranges (vertical lines with whiskers). The *P* value was derived by the Wilcoxon rank-sum test, which was the test of significance.

of citrulline, ornithine, and urea were not part of our infusion protocol. Since we cannot quantitatively measure the flux of these metabolites, quantitative comparisons of these heavy metabolite mole fractions are not meaningful: any differences between HC and SM could reflect differences in upstream metabolism or downstream metabolism, or both.

DISCUSSION

The results obtained from these observational data and the labeled arginine infusion experiment provide further characterization of hypoargininemia (14–17) and low NO

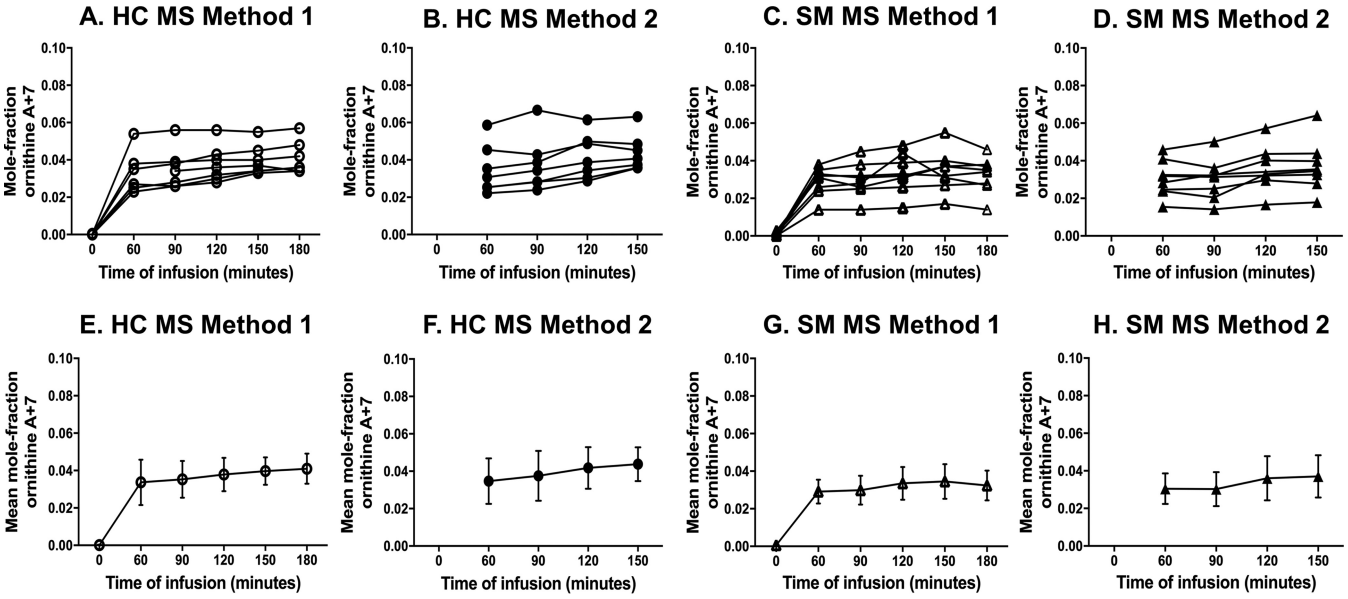

FIG 6 Plasma mole fractions of ornithine A+7 during heavy isotope-labeled arginine infusion among healthy children (HC) and children with severe malaria (SM). Ornithine A+7 represents heavy isotope-labeled arginine A+10 that has been metabolized via the arginase pathway, producing ornithine A+7 and urea (Fig. 1C). (A to D) Mole fraction of ornithine A+7 for each of the 7 HC and for each of the 8 SM measured at time intervals during arginine A+10 infusion. (A) Mole fraction measurements by MS method 1 for each HC before the priming dose and at 30-min intervals through 180 min. (B) Mole fraction measurements by MS method 2 for each HC at time points of 60 to 150 min. (C) Mole fraction measurements by MS method 1 for each SM before the priming dose and at 30-min intervals through 180 min. (D) Mole fraction measurements by MS method 2 for each SM at time points 60 to 150 min. (E to H) Mean mole fraction for the respective clinical group (aggregate average for the 7 HC and aggregative average for the 8 SM) at each time point vertical lines with whiskers represent the 95% confidence interval. (E) Values for HC measured by MS method 1; (F) values for HC measured by MS method 2; (G) values for SM measured by MS method 1; (H) values for SM measured by MS method 2.

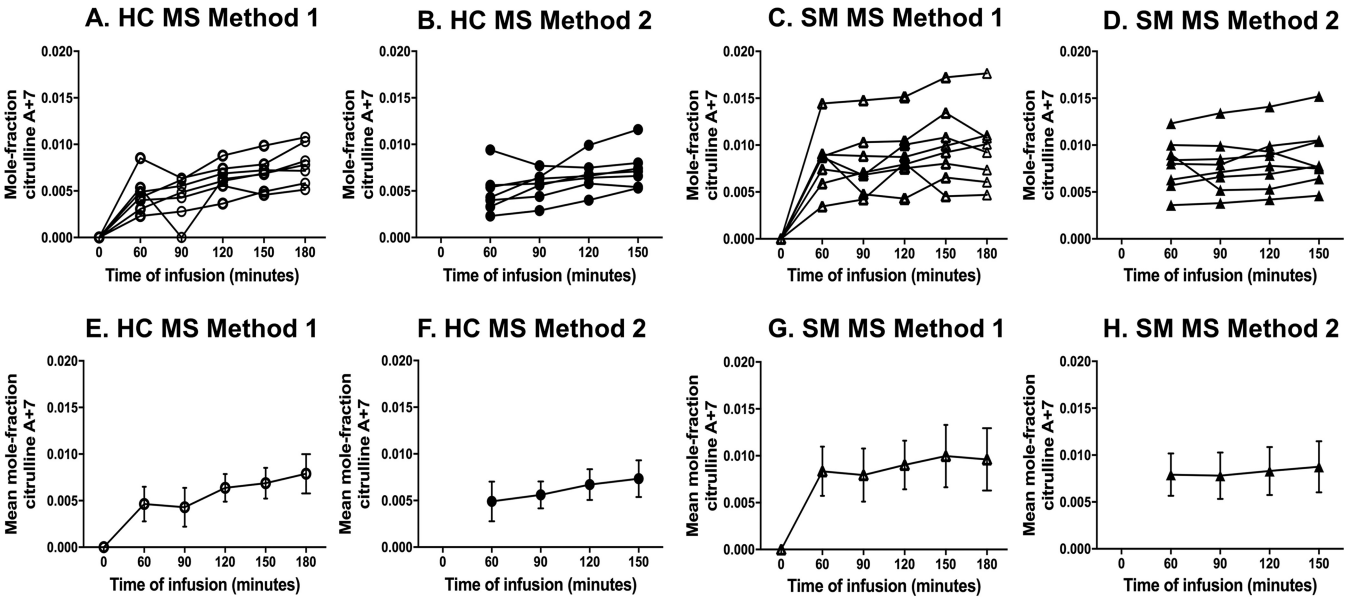

FIG 7 Plasma mole fractions of citrulline A+7 during heavy isotope-labeled arginine infusion among healthy children (HC) and children with severe malaria (SM). Ornithine (A+7) can undergo conversion to citrulline (A+7) via ornithine transcarbamoylase (Fig. 1C). (A to D) Mole fraction of citrulline A+7 for each of the 7 HC and for each of the 8 SM measured at time intervals during arginine A+10 infusion. (A) Mole fraction measurements by MS method 1 for each HC before the priming dose and at 30-min intervals through 180 min. (B) Mole fraction measurements by MS method 2 for each HC at time points of 60 to 150 min. (C) Mole fraction measurements by MS method 1 for each SM before the priming dose and at 30-min intervals through 180 min. (D) Mole fraction measurements by MS method 2 for each SM at time points of 60 to 150 min. (E to H) Mean mole fraction for the respective clinical group (aggregate average for the 7 HC and aggregative average for the 8 SM) at each time point (vertical lines with whiskers represent the 95% confidence interval). (E) Values for HC measured by MS method 1; (F) values for HC measured by MS method 2; (G) values for SM measured by MS method 1; (H) values for SM measured by MS method 2.

bioavailability (4, 10) relative to the pathogenesis of severe falciparum malaria in humans. Based on our previous work that demonstrated increased arginase activity in plasma and peripheral blood mononuclear cells in children with malaria (16), we hypothesized that arginine flux would be increased in children with severe falciparum malaria. If compensatory metabolism failed to increase the supply of arginine to the circulation, then hypoargininemia would result. To investigate this hypothesis, we first measured the plasma concentrations of the amino acid precursors for *de novo* arginine synthesis. In a second cohort, we measured arginine flux in HC and in SM using steady-state isotope dilution to determine enrichment during a constant infusion of labeled arginine. Our results revealed significant depressions in the levels of amino acids along the biosynthetic pathway for arginine synthesis. In addition, our kinetic study in children with severe falciparum malaria showed no significant increase in arginine flux compared to that in HC.

Role of arginine precursors in acute infection. The arginine supply is derived from diet, protein catabolism, and *de novo* synthesis (Fig. 1A and B). *De novo* synthesis requires enteral absorption of glutamine, enterocyte conversion of glutamine to citrulline, and the subsequent conversion of citrulline to arginine in the kidney. While this *de novo* synthesis comprises a minority of the total supply, it can be an important factor under stress conditions, such as severe infection. These low concentrations of amino acid precursors for arginine biosynthesis, particularly glutamine, may be highly relevant to malaria pathogenesis. Glutamine has a recognized role in maintaining the intestinal barrier function (21–23) and has a potential prognostic utility in critical illnesses (24, 25). As the precursor to antioxidant cofactors, such as glutathione and NAD, low plasma glutamine levels might reduce the ability to resist the oxidative stress of malaria (26–29) and other disease states. While one observational study found glutamine levels to be in the normal range among Gabonese children with severe malaria (30), low blood glutamine levels have been described in Ghanaian children with severe malaria (31) and Malawian children with cerebral malaria (32). Similar to the findings of these two studies, we observed that the plasma glutamine concentration not only was signifi-

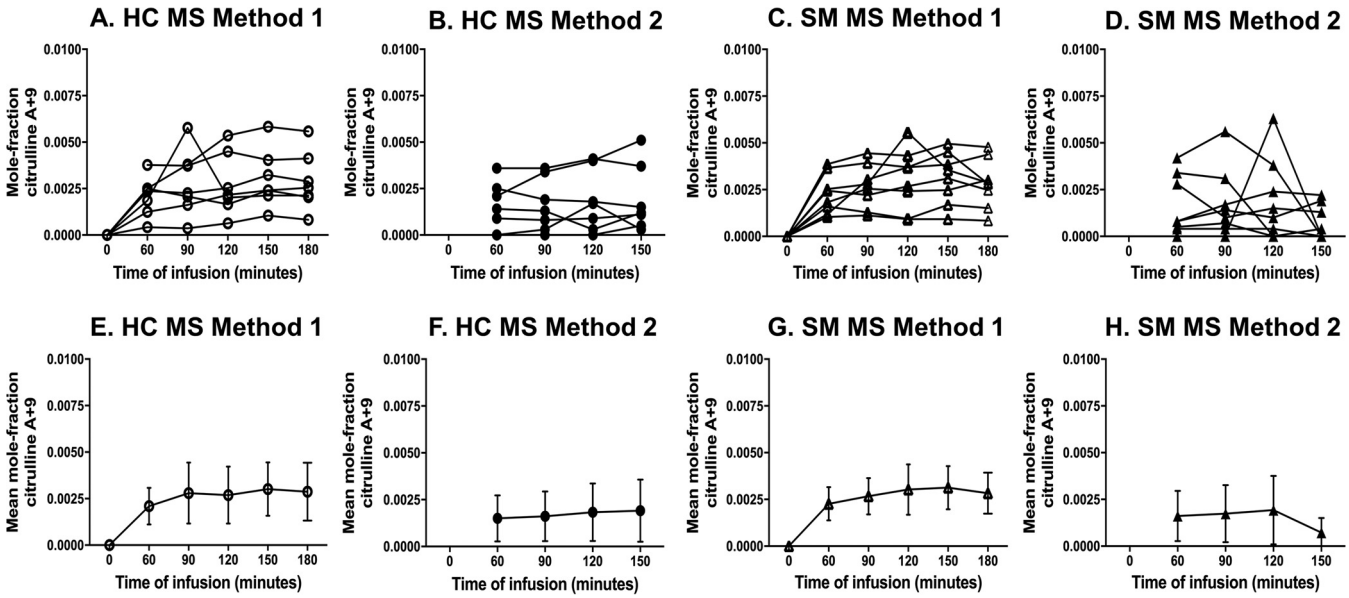

FIG 8 Plasma mole fractions of citrulline A+9 during heavy isotope-labeled arginine infusion among healthy children (HC) and children with severe malaria (SM). Citrulline (A+9) represents heavy isotope-labeled arginine (A + 10) that has been metabolized via the NOS pathway, producing citrulline (A+9) and NO (Fig. 1C). (A to D) Mole fraction of citrulline A+9 for each of the 7 HC and for each of the 8 SM measured at time intervals during arginine A+10 infusion. (A) Mole fraction measurements by MS method 1 for each HC before the priming dose and at 30-min intervals through 180 min. (B) Mole fraction measurements by MS method 2 for each HC at time points of 60 to 150 min. (C) Mole fraction measurements by MS method 1 for each SM before the priming dose and at 30-min intervals through 180 min. (D) Mole fraction measurements by MS method 2 for each SM at time points of 60 to 150 min. (E to H) Mean mole fraction for the respective clinical group (aggregate average for the 7 HC and aggregative average for the 8 SM) at each time point (vertical lines with whiskers represent the 95% confidence interval). (E) Values for HC measured by MS method 1; (F) values for HC measured by MS method 2; (G) values for SM measured by MS method 1; (H) values for SM measured by MS method 2.

cantly lower in CM than in Tanzanian UM and HC but also was lower than established reference ranges for children (33, 34). As highlighted in the first study of plasma glutamine in severe malaria (31), a low glutamine level is noteworthy in the context of human malaria, in which an impaired intestinal barrier function has been demonstrated (35, 36) and is a key predisposing factor for malaria-associated nontyphoidal *Salmonella* bacteremia (37–39). A low glutamine level likely exacerbates the impaired barrier function arising from heavy parasite infiltration of the intestinal vasculature (40, 41). Heavy parasite sequestration in the intestinal microvasculature could also affect amino acid absorption and amino acid synthesis by enterocytes.

Potential importance of small intestine in arginine metabolism. Intestinal dysfunction is especially relevant to our study, since the small intestine is the primary source for arginine *de novo* synthesis. Enterocyte synthesis of citrulline from glutamine and proline accounts for 80 to 90% of systemic citrulline, the immediate precursor for arginine *de novo* synthesis (42). We observed very low levels of plasma citrulline in CM and SM, and a low plasma citrulline level is a general marker for intestinal dysfunction (43). Citrulline synthesis can be impaired by the accumulation of lactate, which inhibits proline oxidase, an enzyme required for enteric citrulline synthesis (Fig. 1A) (44, 45). Hyperlactatemia is a known complication with prognostic significance in severe malaria (46). Alkaitis et al. (17) reported low levels of plasma citrulline and ornithine in falciparum malaria and concluded that the low citrulline level is likely due to the decreased appearance from enteral sources and that the low ornithine level is due to decreased arginine appearance from nonarginine sources, such as glutamate and proline. By juxtaposing the deficiencies of plasma glutamine and citrulline, our results implicate the small intestine as a new focus for the study of malaria pathogenesis. Low plasma glutamine, glutamate, and proline levels are consistent with the conclusions of Alkaitis et al. regarding the causes of low citrulline and ornithine levels in malaria (17), and they suggest a link to the low NO bioavailability in falciparum malaria. Interventions to modulate the intestinal pathology in malaria, such as enteral glutamine

supplementation, might reduce the endothelial pathology in malaria by enhancing NO production (47).

Arginine flux studies in health and disease. Low plasma levels of glutamine, citrulline, and the other arginine precursor amino acids (glutamate, proline, and ornithine) suggest an impaired ability to compensate for hypoargininemia via *de novo* arginine synthesis. We did not measure the fluxes of these individual precursor amino acids, so we cannot definitively identify the metabolic aberration that results in hypoargininemia, but our findings of low arginine precursor amino acid levels at presentation coincide with the findings of arginine flux studies in a murine malaria model that suggest that hypoargininemia in malaria results from a low influx of arginine (17). Using this experimental model, researchers also found decreased arginine use by NOS but no overall change in arginine flux among the mice infected with *Plasmodium berghei* ANKA.

Our isotope infusion protocol was based on studies in adult human volunteers that showed a consistent arginine flux of approximately 70 μmol per hour per kilogram of body weight (48, 49). Similar to those studies, we found that by 60 min after prime/constant-rate infusion of labeled arginine, mole fractions were constant in both the HC and SM study groups during the subsequent 2 h. The median arginine flux values for HC and SM were higher than the published values noted in healthy adults but lower than the values (125 μmol/hour/kg) observed in neonates (50). Ethnic and dietary differences might also contribute to the higher values that we observed in Tanzanian children than in North American adults.

A previous study of arginine metabolism in 47 Gabonese children with uncomplicated malaria employed labeled arginine infusion (51). The investigators observed a mean arginine flux of 84 μmol/h/kg, but they did not study a healthy control group for comparison. Another study of 10 children with bacterial sepsis found mean $\pm$ standard deviation (SD) arginine flux rates of 67 $\pm$ 21 to 72 $\pm$ 17 μmol/h/kg for the two different heavy isotope-labeled arginine molecules infused (52). In two controlled studies of arginine flux in adults with sepsis or septic shock, arginine flux measurements were either slightly lower (53) or not significantly increased (54) compared to those in healthy controls. A third controlled study of adults with sepsis found no difference in overall arginine production but found decreased *de novo* arginine synthesis and decreased NO production (55). Thus, our finding that arginine flux is not significantly increased in SM compared to HC is consistent with controlled comparisons among adults with sepsis.

Our observations from these two malaria cohorts—that arginine flux is not significantly increased in patients with severe malaria and that the plasma concentrations of amino acid precursors for arginine synthesis are low in patients with cerebral malaria—suggest that hypoargininemia in human malaria likely results from an insufficient biosynthetic supply of arginine. An impaired arginine supply via protein catabolism could also contribute to a low-arginine state in the context of relatively normal arginine flux. Though previous data suggested that increased plasma and cellular arginase activity (16) could be responsible for hypoargininemia, *in vitro* measurements of enzyme activity may not capture dynamic alterations of *in vivo* metabolic flow. Our observation that arginine flux is not increased in SM has implications for considering adjunctive therapies aimed at mitigating the microvascular dysfunction associated with low NO in SM. Since we did not observe increased metabolic turnover of arginine in SM and since there are multiple barriers to NOS-dependent NO production in SM (10, 15, 16, 18, 26, 56–59), NOS-independent adjunctive therapies (e.g., administration of an NO donor, such as sodium nitrite) might better increase the NO level than adjunctive therapies which aim to increase the level of the plasma arginine substrate for NO synthesis.

Limitations. (i) Our small sample size for the infusion study may have limited our power to detect any difference in arginine flux between HC and SM. Nonetheless, despite the small sample size, we observed significantly lower plasma arginine levels in

SM than in HC, yet our infusion study showed no indication that this difference in plasma arginine levels was due to the increased metabolic flux of arginine. (ii) The lack of elevated plasma lactate levels in SM and the preponderance of hyperparasitemia as the sole enrollment criterion suggest that the severe falciparum malaria cases were not very severe. A group of SM with more severe disease may have revealed differences in arginine flux from those in HC. (iii) Measuring flux closer to the time of diagnosis of severe falciparum malaria in the district hospital rather than 2 h later in our Clinical Research Unit at Hubert Kairuki Memorial University (HKMU) Hospital might have demonstrated increased arginine flux in SM. However, concerns about clinical severity or the timing of sampling are unlikely biases since hypoargininemia is observed even in patients with uncomplicated malaria (14, 17). (iv) We were unable to measure *de novo* NO synthesis rates since we did not use labeled citrulline to measure citrulline flux. Similarly, without citrulline flux and ornithine flux measurements, our measurements of the labeled products of arginine A+10 (Fig. 6, 7, and 8) cannot be used to infer increased or decreased metabolism through the arginase pathway in SM compared to HC.

Conclusions. We found no statistically significant increase in arginine flux in SM compared to HC, but the levels of plasma arginine and the amino acid precursors for *de novo* arginine biosynthesis were lower in children with malaria. The metabolic causes of low plasma arginine levels remain incompletely understood. In the context of two hallmarks of severe malaria—intestinal dysfunction and endothelial dysfunction associated with low NO levels—our finding of low plasma glutamine levels suggests a novel mechanistic link in malaria pathogenesis. Further studies that measure the production of arginine, citrulline, and NO will help determine the mechanisms of hypoargininemia and hypoglutaminemia and their relationship to NO bioinsufficiency in severe malaria. The results of these studies may lead to novel adjunctive treatments for falciparum malaria.

MATERIALS AND METHODS

Ethics. These studies were approved by the ethics committees at the Hubert Kairuki Memorial University (HKMU) Hospital and the United Republic of Tanzania National Institute for Medical Research and the institutional review boards of the Duke University Hospital System, the University of Utah, and the Durham VA Medical Center. Written informed consent was obtained from the parents or guardians of all participating children. We followed United States Department of Health and Human Services guidelines for human subjects research.

Study site. The observational study of arginine amino acid precursors and the infusion study of arginine flux were both conducted at the HKMU Hospital in Dar es Salaam, Tanzania. Eligible children were recruited from the inpatient wards and outpatient clinics of the HKMU Hospital, the Amana District Hospital, the Mwanayamala District Hospital, and the Temeke District Hospital in Dar es Salaam. We enrolled subjects into the observational study of arginine amino acid precursors from November 2007 through January 2012 as part of a study on the pathogenesis of cerebral malaria. For the arginine flux study using a heavy isotope tracer, we conducted enrollment from August 2013 through June 2014.

Arginine amino acid precursor observational study. (i) Participants and clinical investigations. All children were 6 months to 6 years of age. Enrollment criteria for healthy children (HC), children with uncomplicated falciparum malaria (UM), and children with cerebral falciparum malaria (CM) were as previously described (26). Briefly, healthy children were enrolled from the outpatient well-child clinics at the Amana District Hospital and the Mwanayamala District Hospital and were eligible if they met the following criteria: they had no signs or symptoms of active illness, no febrile illness within the previous 2 weeks, no history or evidence of an active inflammatory condition, and a negative result by a *Plasmodium falciparum* rapid diagnostic test (Paracheck-Pf; Omega Diagnostics). All children with severe malaria in this study had the cerebral malaria phenotype, as defined by the World Health Organization (WHO) case definition in place at the time of the study: any level of *P. falciparum* parasitemia on peripheral blood smear, unarousable coma (Blantyre coma score $\leq$ 2) that was not attributable to hypoglycemia (blood glucose level $<$ 40 mg/dl) and that persisted for more than 60 min after any convulsion, and no other identifiable cause of coma (60). Inclusion criteria for uncomplicated falciparum malaria were as follows: a clinical syndrome consistent with malaria and a documented fever (temperature $\geq$ 38°C) or history of fever within 48 h from the time of enrollment, *P. falciparum* parasitemia of $>$10,000 parasites/μl on Giemsa-stained blood film plus a positive result by a *P. falciparum* rapid diagnostic test (Paracheck-Pf; Omega Diagnostics), no other cause of fever identified, and no WHO warning signs suggestive of severe disease (60). These warning signs were the following: inability to suckle, eat, or drink; excessive vomiting; abnormal respiratory rate or respiratory distress, as evidenced by accessory muscle use, suprasternal retractions, or intercostal retractions; a recent history of convulsions; altered mental status; and an inability to sit unaided. Children were excluded from the UM or CM

group if there was any evidence of mixed infection with other *Plasmodium* spp. or evidence of mixed infection with bacterial pathogens (septicemia; urinary tract infection; or bacterial meningitis, established by cerebrospinal fluid [CSF] culture or CSF cell count).

Routine laboratory measurements included complete blood count (model Act 10; Beckman-Coulter) and creatinine and blood glucose levels (Abbott iStat). Giemsa-stained thick and thin peripheral blood smears were read by a trained study laboratory technologist.

(ii) Amino acid measurements. Whole blood was collected into heparin-containing tubes at enrollment and spun immediately, and the supernatant plasma was stored in cryovials at −80°C. The plasma concentrations of glutamine, glutamate, proline, ornithine, citrulline, and arginine were quantified at a reference laboratory (ARUP Laboratories) by ion-exchange chromatography (33) and compared to established pediatric reference ranges (33, 34).

Arginine flux infusion study. (i) Participants and clinical investigations. Children aged 4 to 10 years were eligible to participate in the labeled arginine infusion study. Enrollment criteria for children with severe falciparum malaria (SM) were defined as those with a *P. falciparum* parasitemia of >2,500 parasites/μl, no other identifiable cause of illness, and one or more of the following modified World Health Organization criteria for severe falciparum malaria: unarousable coma (Blantyre coma score $\leq$ 2), severe respiratory distress, metabolic acidosis (venous bicarbonate concentration $<$ 15 mmol/liter or venous lactate concentration $>$ 5 mmol/liter), severe anemia (hemoglobin concentration $<$ 5 g/dl), or hyperparasitemia (>5% of red blood cells) (60, 61). SM received antimalarial therapy and other supportive care per standard Tanzanian national protocols. Parenteral treatment with either quinine or an artemisinin derivative was initiated as soon as the diagnosis of malaria was suspected, and there was no delay in treatment or care due to study procedures. Enrollment criteria for healthy children (HC) were the following: they were subjectively well with no current illness, they had no history of fever in the preceding 14 days and no parasitemia by microscopy, and they were not taking any regular medication.

For all participants, routine laboratory measurements included a complete blood count (model Act 10; Beckman-Coulter) and determination of plasma bicarbonate, creatinine, glucose, and lactate levels (Abbott iStat or Siemens Dimension Vista 1500 when Abbott iStat was unavailable).

Power calculations for sample size estimates were as follows: the anticipated observed difference in arginine flux was estimated based upon prior measurements of mean $\pm$ SD plasma arginine values in Tanzanian children with severe malaria (45 $\pm$ 15 μM) and in healthy controls (100 $\pm$ 32 μM) (14). We set the criteria for significance (α) at 0.05. In order to have a power (β) of 95% to yield a statistically significant result between healthy control children and children with severe malaria, the proposed sample size was a minimum of 6 participants in each group. Our protocol called for *a priori* enrollment of at least 2 additional children per group to account for potential dropout.

(ii) Tracer infusion protocol. HC observed an overnight fast, and we recorded the time since the last meal for both HC and SM. Peripheral intravenous catheters (20 or 22 gauge) were placed under aseptic technique in both upper extremities. Using an 11 Elite precision pump (Harvard Apparatus) for all infusions, we first infused a priming dose (6.45 μmol/kg) of L-arginine uniformly labeled with $^{13}C_6$ and $^{15}N_4$ (Tracer Technologies) over 3 min, followed by a constant infusion of 6.45 μmol/h/kg over 3 h. We also refer to L-arginine uniformly labeled with $^{13}C_6$ and $^{15}N_4$ as the tracer or arginine (A+10).

Venous blood was sampled from the upper-extremity venous catheter that was not receiving the tracer infusion (i.e., the extremity contralateral to the extremity receiving the infusion). Venous blood was drawn at the baseline, prior to initiation of the infusion, and then at 60, 90, 120, 150, and 180 min. Fifteen minutes before the scheduled venous sampling, the site for blood sampling was warmed to 55 to 60°C using an electric heating pad. At the designated time point, we cleared the line by withdrawing 1 ml of blood from the catheter and discarded it. After this, we collected 1.5 to 2 ml of blood in 4-ml lithium heparin tubes and placed the tubes into a 4°C container at the bedside. These blood samples were processed immediately by centrifuging them at 1,200 $\times$ *g* (4°C) for 15 min, removing the plasma, and storing the samples at −80°C. Plasma samples were shipped from the HKMU Hospital to Duke University using liquid nitrogen cryopreservation shippers.

(iii) Amino acid measurements and kinetics calculations. We measured plasma amino acid levels with a Hitachi L-8800 amino acid analyzer. For measurements of isotope abundance, we employed two independent mass spectrometry (MS) methods. MS method 1 was conducted *a priori*. MS method 2 employed a more sensitive instrument for isotope abundance measurements. The second method was conducted *post hoc* in order to cross validate our measurements with MS method 1, thereby confirming the analytical validity of our results.

(iv) MS method 1. We measured isotope abundance by ultraperformance liquid chromatography (UPLC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) in the positive ion mode using a Waters Xevo TQ MS system equipped with an Acquity UPLC system (62). Arginine, citrulline, and ornithine were extracted from plasma samples using a cation Oasis MCX 30-mg exchange cartridge (Waters), followed by methanolysis using 3 M HCl in methanol at 65°C for 60 min. We dried these under nitrogen before reconstitution in 200 μl of matrix buffer B (methyl cyanide [MeCN], H_2O [95:5, vol/vol] containing 2 mM ammonium acetate [NH_4Ac] and 0.1% formic acid). We injected reconstituted samples (5 μl) into the UPLC-ESI-MS/MS system. Separation was done using a Waters BEH Amide UPLC column (particle size, 1.7 μm; 2.1 by 100 mm) with gradient elution using the following buffers: MeCN-H_2O (5:95, vol/vol) containing 2 mM NH_4Ac and 0.1% formic acid (buffer A) and buffer B.

Labeled arginine, citrulline, and ornithine and their isotope abundances were analyzed by selected ion monitoring (SIM) with a mass resolution of 0.5 atomic mass unit. In general, the unlabeled molecular ion is expressed as A, and *n* heavy isotope-labeled molecular ion is expressed as A+*n* (in which the heavy isotope is incorporated at the *n* position). A tracer-to-tracee ratio (TTR), also described as isotope

enrichment, is the abundance of an *n*-labeled tracer with molecular ion A+*n* relative to the abundance of the target tracee metabolite with molecular ion A and its isotope molecular ions in natural abundance: A+1, A+2, A+3, etc. Usually, A+4 and heavier isotope-labeled molecular ions are negligible in natural abundance. The abundances of the following citrulline and ornithine isotopes were measured: citrulline (A+9), a product of NOS; citrulline (A+7), a product of ornithine transcarbamoylase; and ornithine (A+7), a product of arginase (45, 63) (see Fig. 1C for a schematic of the heavy isotope atoms on the labeled molecules of arginine, citrulline, and ornithine). The isotope signals of citrulline corresponding to A (*m/z* 190), A+1 (*m/z* 191), A+7 (*m/z* 197), and A+9 (*m/z* 199) were eluted at retention time 2.22 min during the UPLC profile; A was the most abundant isotope signal of the protonated derivative (*m/z* 190). In the same injection, the isotope signals of arginine corresponding to A+1 (*m/z* 190), A+2 (*m/z* 191), and A+10 (*m/z* 199) were collected at retention time 2.82 min. The isotope signals of ornithine corresponding to A (*m/z* 147), A+1 (*m/z* 148), and A+7 (m/z154) were analyzed by SIM at retention time 2.88 min during the UPLC gradient using the same inlet method but in a separate injection. Isotope measurements were performed by MS method 1 on samples collected at time points of 0, 60, 90, 120, 150, and 180 min during the heavy isotope-labeled arginine infusion.

(v) MS method 2. Isotope abundance was also measured using a ZipChip capillary electrophoresis (CE) system with an HS chip (908 Devices, Inc.) coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific). These methods were as follows: 20 μl plasma was transferred into a labeled Eppendorf tube. To this tube we added 140 μl methanol at room temperature, followed by the addition of 40 μl 0.5 M ammonium acetate. The sample was shaken at 1,000 relative centrifugal force (rcf) on a ThermoMixer (Eppendorf) at room temperature for 10 min. Samples were cooled to dry ice temperature and then spun at 20,000 rcf at 4°C for 10 min, before aliquoting the supernatant into glass autosampler vials.

Isotope incorporation data were measured for arginine, citrulline, and ornithine in the following manner: the ZipChip CE system was operated using an autosampler to deliver 10 μl of sample, and injection of 8 nl was performed using pressure. The capillary electrophoresis (CE) separation was performed at 1,000 V/cm with pressure assist at 2 min for a total of 4 min. The mass spectrometry analysis for arginine (isotopes A and A+10) and citrulline (isotopes A, A+7, and A+9) was performed in positive ion mode with a selected ion monitoring (SIM) scan method of 170 to 190 *m/z* and 2e5 ions as the automatic gain control (AGC) target with a maximum ion accumulation time of 60 ms at a 240,000 resolution, with data collected in the centroid mode. Ornithine (isotopes A and A+7) was measured using a separate CE injection under the same conditions, but with Q Exactive HF settings of 70 to 500 *m/z*, 3e6 ions as the AGC target, and 120,000 resolution. Accurate mass extraction with a tolerance of 2 ppm was performed using Skyline (v4.1) software. Isotopic incorporation of the nonnative stable isotopes was calculated as a percentage of the total, considering the contribution of native isotopes A and A+1 for arginine, citrulline, and ornithine. Isotope measurements by MS method 2 were performed on samples collected at time points of 60, 90, 120, and 150 min during the heavy isotope-labeled arginine infusion. Measurements at time points of 0 and 180 min were omitted because we aimed to use MS method 2 to cross validate our measurements at steady state. Accordingly, time point 0 min was extraneous and heavy isotope-labeled arginine measurements by MS method 1 had shown a drop from those at steady state at time point 180 min, and therefore, a measurement at time point 180 min was not included in the MS method 2 cross validation of steady-state measurements.

(vi) Isotope enrichment and arginine flux calculations. Arginine A+10 enrichment (the arginine A+10 tracer-to-tracee ratio [$TTR_{Arg\ A+10}$]) was calculated according to equation 1:

$$TTR_{Arg\ A+10} = \frac{100 \times [A+10]}{[A+1]} \Big/ 1{,}184.41 \quad (1)$$

where [A+10] and [A+1] are the absolute peak intensities of isotope molecular ions A+10 and A+1, respectively, and 1,184.41 is the theoretical sum of the peak intensities of A, A+1, A+2, and A+3 in natural abundance normalized to the A+1 peak intensity (arbitrarily given a value of 100). The peak intensities of A+4 and A+5, etc., are considered negligible and were not included in the calculation.

Citrulline A+9 enrichment (the citrulline A+9 tracer-to-tracee ratio [$TRR_{Cit\ A+9}$]) was also calculated according to equation 2:

$$TTR_{Cit\ A+9} = \frac{100 \times [A+9]}{[A]} \Big/ \text{sum (percent BPI)} \quad (2)$$

where [A+9] and [A] are the absolute peak intensities of isotope molecular ions A+9 and A, respectively, and sum (percent BPI) is the theoretical sum of the percent base peak intensity (BPI) in natural abundance. In a mass spectrum, the peak with the highest intensity is referred to as the base peak, its intensity is arbitrarily set at a value of 100, and the intensities of other peaks are set as a percentage of the base peak intensity (BPI). Here, unlabeled citrulline molecular ion A is the most abundant ion and has the highest intensity, and its BPI is 100%. The percent BPI of A+1, A+2, and A+3 are the peak intensities of A+1, A+2, and A+3 normalized to A (arbitrarily set at a value of 100), respectively. In the isotope model of the protonated citrulline molecular ion, the sum (percent BPI) is 108.8 in natural abundance. Enrichment of citrulline A+7 and ornithine A+7 was also calculated as the tracer-to-tracee ratio in a manner analogous to that used in equation 2 above. At steady state, the arginine flux rate (i.e., the rate of appearance or disappearance of arginine) was equal to the isotope infusion rate divided by isotope enrichment.

Data analysis. For both the observational study of arginine amino acid precursor concentrations and the arginine flux study, statistical analyses were performed with Stata (v14) software (StataCorp). Variables with a normal distribution are presented as means with 95% confidence intervals, Student's *t* test was used to assess differences between groups, and analysis of variance (ANOVA) with the Bonferroni correction was used for comparisons across more than 2 groups (HC, UM, and CM for the observational study). For variables that did not have a normal distribution, results are presented as the median with the interquartile range (IQR), the Wilcoxon rank-sum test was used to assess differences between groups, and the Kruskal-Wallis test was used for comparisons across more than two groups (HC, UM, and CM for the observational study). The chi-square test was used to compare proportions between groups. A two-sided *P* value of ≤0.05 was the threshold for statistical significance.

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D.L.G., B.K.L., J.B.W., N.M.A., T.W.Y., and E.D.M. conceived and designed the study. S.M.F., J.P.M., A.R.K., E.D.M., J.B.W., and M.P.R. performed the study. H.Z., S.Y., D.S.M., and J.W.T. performed the laboratory analyses. M.P.R., T.W.Y., N.M.A., D.L.G., J.B.W., H.Z., S.Y., D.S.M., and J.W.T. analyzed the data. M.P.R., H.Z., S.M.F., J.P.M., A.R.K., S.Y., D.S.M., J.W.T., E.D.M., N.M.A., T.W.Y., B.K.L., D.L.G., and J.B.W. wrote and edited the manuscript.

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We declare no conflicts of interest.

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