Malaria in pregnancy alters L-arginine biosynthesis in Malawian women and L-arginine supplementation improves birth outcomes in a pre-clinical model

One-sentence summary: This study identified a role for dysregulation of NO biosynthetic pathways in the pathogenesis of MIP, in an experimental mouse model and in a clinical cohort of pregnant women, and supports the evaluation of interventions to enhance L-arginine bioavailability as strategies to improve birth outcomes.

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**Abstract**

Reducing adverse birth outcomes due to malaria in pregnancy (MIP) is a global health priority. However there are few safe and effective interventions. L-arginine is an essential amino acid in pregnancy and an immediate precursor in the biosynthesis of nitric oxide (NO) but there are limited data on the impact of MIP on NO biogenesis. We hypothesized that hypoarginemia contributes to the pathophysiology of MIP and that L-arginine supplementation would improve birth outcomes. In a prospective study of pregnant Malawian women, we show that MIP was associated with lower levels of L-arginine and higher levels of endogenous inhibitors of NO biosynthesis, asymmetric (ADMA) and symmetric dimethylarginine (SDMA), which were associated with adverse birth outcomes. In a model of experimental MIP, L-arginine supplementation in dams improved birth outcomes (e.g. decreased stillbirth and increased birth weight) compared with controls. The mechanism of action was via normalized angiogenic pathways and enhanced placental vascular development as visualized by placental micro-CT imaging. These data define a role for dysregulation of NO biosynthetic pathways in the pathogenesis of MIP and support the evaluation of interventions to enhance L-arginine bioavailability as strategies to improve birth outcomes.

INTRODUCTION

An estimated 125 million women become pregnant in malaria-endemic regions every year, with over 85 million at risk of *Plasmodium falciparum* malaria ([*1-3*](#_ENREF_1)). Pregnant women, in particular first time mothers, are more likely to be infected with falciparum malaria and to experience complications including maternal anemia, pregnancy loss, and low birth weight resulting from small-for-gestational age (SGA) outcomes and/or preterm birth (PTB) ([*4-7*](#_ENREF_4)). Malaria in pregnancy (MIP) leads to the sequestration of malaria-infected red blood cells in the intervillous space of the placenta and the recruitment of mononuclear cells generating a localized immune response at the maternal-fetal interface ([*8*](#_ENREF_8)*,* [*9*](#_ENREF_9)). MIP-induced immune responses in the placenta can disrupt normal angiogenic processes, resulting in placental insufficiency and the inability of the placental to support rapid fetal growth in the third trimester, ultimately leading to SGA, PTB, and low birth weight (LBW) ([*10*](#_ENREF_10)).

Despite the negative impact of MIP on global maternal-child health, there are currently limited intervention strategies to improve maternal and neonatal outcomes. Evidence from preeclampsia and other causes of adverse pregnancy outcomes suggests that interventions to promote placental angiogenesis may improve birth outcomes ([*11-16*](#_ENREF_11)). L-arginine is an essential amino acid in pregnancy and an immediate precursor in the biosynthesis of nitric oxide (NO) via a family of nitric oxide synthase (NOS) enzymes ([*17-20*](#_ENREF_17)). NO plays a central role in endothelial growth and function as a critical regulator of the vascular endothelial growth factor (VEGF) family of proteins (including placental growth factor (PGF), the angiopoietins (Ang1 and Ang2) and their respective soluble receptors (sFlt-1 and sTie-2) ([*21*](#_ENREF_21)). The VEGF family of proteins are essential for proper placental vascularization, vessel growth and remodelling throughout pregnancy ([*12*](#_ENREF_12)*,* [*22*](#_ENREF_22)*,* [*23*](#_ENREF_23)). NO production increases pro-angiogenic VEGF-A and PGF in human trophoblast cultures, while inhibition of NO synthesis results in elevated sFlt-1 and hypertensive responses in pregnant mice ([*21*](#_ENREF_21)*,* [*24*](#_ENREF_24)*,* [*25*](#_ENREF_25)).NO also reduces expression of endothelial adhesion receptors and pro-inflammatory cytokines, which contribute to increased monocyte accumulation and MIP pathogenesis ([*26*](#_ENREF_26)*,* [*27*](#_ENREF_27)).

There is considerable evidence that reduced bioavailable NO contributes to the pathophysiology of severe malaria ([*28-30*](#_ENREF_28)). Malaria-induced hemolysis depletes L-arginine as well as NO, contributing to hypoarginemia and impaired NO synthesis ([*28*](#_ENREF_28)). NO bioavailability is further impaired by the generation of endogenous inhibitors of NO biogenesis, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). ADMA is a competitive inhibitor of NOS, whereas SDMA enhances inflammation and oxidative stress and, at high concentrations, impairs arginine transport into cells ([*31*](#_ENREF_31)*,* [*32*](#_ENREF_32)). A reduced ratio of L-arginine to ADMA (a measure of L-arginine bioavailability) has been reported in children and adults with severe malaria ([*33*](#_ENREF_33)*,* [*34*](#_ENREF_34)). NO may also be depleted by NO-scavenging by cell-free hemoglobin as a result of malaria-induced hemolysis ([*33*](#_ENREF_33)). In severe malaria, reduced NO bioavailability contributes to endothelial dysfunction, and can be reversed in both human infection and experimental models following parenteral L-arginine infusion ([*35*](#_ENREF_35)*,* [*36*](#_ENREF_36)). Pregnancy can also contribute to hypoargininemia, as arginine is continuously metabolized to meet the high NO demands required to support placental vascular growth and remodelling ([*37*](#_ENREF_37)). Moreover, diets deficient in L-arginine are common in low resource and malaria-endemic regions and may further deplete bioavailable L-arginine during pregnancy ([*38*](#_ENREF_38)*,* [*39*](#_ENREF_39)). We hypothesized that maternal circulating levels of L-arginine, ADMA and SDMA would be altered in women with MIP and that L-arginine supplementation during pregnancy would improve pregnancy outcomes (birth weight and viability) in a pre-clinical model of MIP by regulating angiogenesis and promoting placental vascular development.

**RESULTS**

**Malaria in pregnancy at 16-28 weeks of gestation is associated with altered ADMA, SDMA levels and L-arginine:ADMA ratio in a prospective cohort of Malawian women**

We assessed the L-arginine-NO biosynthetic pathway in plasma samples collected from 384 primigravid Malawian women at enrolment (between 16-28 weeks gestation) in a randomized clinical trial ([*40*](#_ENREF_40)). Demographic data of the study population are presented in Table 1. Malaria at enrolment was common in this population with 24% (n=91/379) of women positive by microscopy, and 57% (n=217/381) of women positive by PCR. Submicroscopic infections were common with 48.1% (n=137/285) of smear negative women testing positive by PCR. Smear positive malaria at enrolment was associated with a significant reduction in birth weight compared to women who tested smear negative for malaria (mean (SD), smear positive, 2699g (409) vs. smear negative, 2805g (432), *P =* 0.04). Submicroscopic malaria infections were not associated with changes in birth weight (*P =* 0.73). At delivery, 48% (n=171/356 of women had histological evidence of malaria in the placenta (active or past infection). Placental malaria was associated with adverse birth outcomes with 60.3% of infants born SGA positive for malaria by histology compared to 44.6% of appropriate-for-gestational age (AGA) infants (*P =* 0.01). There was no difference in the proportion of women with histologically-defined placental malaria according to treatment arm (*P =* 0.48), and treatment arm was not associated with adverse birth outcomes (LBW, PTB, SGA, *P >* 0.05 for all) or birth weight (mean (SD), IPTp-DP, 2773g (461) vs. ISTp-DP, 2790g (388), *P =* 0.69).

Women with smear-positive malaria at enrolment had higher levels of SDMA and ADMA than women who were smear negative (Table 2). There was no association between plasma L-arginine levels according to microscopy-defined malaria at enrolment. Women with PCR-defined malaria or submicroscopic malaria had significantly higher levels of SDMA and ADMA, and a lower L-arginine:ADMA ratio than women with PCR-negative malaria (Table 2). These effects remained significant following adjustment for maternal age and gestational age at enrolment where a one unit increase in ADMA or SDMA was associated with a relative risk (95% CI) of PCR-defined malaria of 6.40 (3.72, 11.04) and 3.94 (1.61, 9.66) respectively (Table 2). This effect was strongest in the 48.1% of women with submicroscopic infections where a one unit increase in ADMA or SDMA was associated with a 10.29 (5.04, 21.02) and 8.44 (1.77, 40.29) increased relative risk of submicroscopic malaria respectively (Table 2).

**Association between the L-arginine pathway and maternal nutritional status at enrolment**

As L-arginine is a conditionally essential amino acid obtained through the consumption of dietary protein, we explored whether levels of L-arginine, SDMA, and ADMA were related to maternal nutritional status (maternal body mass index, BMI; or middle-upper arm circumference, MUAC) by regression analysis (Table 3). An increase in L-arginine or the L-arginine:ADMA ratio were positively associated with MUAC following adjustment for maternal age and gestational age at enrolment (*P =* 0.005 and *P =* 0.003 respectively, Table 3), suggesting higher L-arginine levels are associated with improved nutritional status. There was a strong negative relationship between ADMA and SDMA and maternal hemoglobin following adjustment for maternal age and gestational age at enrolment (*P <*0.0001 and *P =* 0.001 respectively, Table 3).

**Increased ADMA at enrolment is associated with adverse birth outcomes, including an increased relative risk (RR) of delivering a small-for-gestational age (SGA) infant**

We investigated the association between the L-arginine pathway and adverse birth outcomes. A total of 167 (43.5%) women had an adverse birth outcome consisting of PTB or SGA. Adverse birth outcomes (PTB or SGA as a composite outcome) were associated with increased ADMA at enrolment compared to normal birth outcome (mean (SD): term/AGA, 0.43umol/L (0.07); adverse birth outcome, 0.46 umol/L (0.09), *P* = 0.007 (Student’s t-test). Using log binomial regression, ADMA was associated with an increased relative risk of having a SGA infant (adjusted RR, 95% CI: 21.2, 2.27-197.9, *P* = 0.007) following adjustment for maternal age, gestational age at enrolment, body mass index, socioeconomic status, smear positive malaria at enrolment, and treatment group (Table 1).

**Malaria at enrolment is associated with lower levels of L-arginine across pregnancy and higher levels of ADMA**

In order to evaluate the kinetics of the L-arginine pathway across pregnancy, we quantified longitudinal levels of L-arginine, ADMA and SDMA in the plasma of 94/384 women included in this study who had between 2 and 5 samples collected prior to delivery (mean of 3.3 visits, n=603 samples tested). We observed an increase in plasma levels of ADMA (Figure 1A), and SDMA (Figure 1B) over pregnancy (*P =* 0.01 and *P <*0.0001 respectively, linear regression of biomarker levels by gestational age). There was no change in levels of L-arginine (Figure 1C) or L-arginine:ADMA (Figure 1D) over pregnancy, (*P =* 0>0.05 for both outcomes).

At enrolment 61.3% (n=57/93) of these women were positive for malaria by PCR, 23.1% (n=21/91) by microscopy, 53.6% (n=37/69) of infections were submicroscopic (PCR positive and microscopy negative), and 51.1% (n=45/88) had histologically defined placental malaria at delivery (Table 1). At enrolment, ADMA levels were higher than SDMA levels, but SDMA increased more than ADMA over gestation and SDMA levels surpassed those of ADMA by 36 weeks of gestation. Using linear mixed effects modelling, malaria by microscopy at enrolment was associated with significantly higher levels of SDMA over pregnancy (χ2(1)=4.38, *P <* 0.04); however, there were no differences in levels of ADMA (χ2(1)=0.17, *P >*0.65) or L-arginine (χ2(1)=0.00, *P* >0.95) over pregnancy based on malaria status at enrolment (Figure 2A-C). Whereas, PCR-defined malaria at enrolment was associated with higher ADMA over gestation (χ2(1)=7.70, *P* = 0.006), and lower L-arginine (χ2(1)=4.64, *P =* 0.031) (Figure 2D-F).

As increased ADMA at enrolment was associated with increased relative risk of SGA, we explored this relationship further comparing ADMA levels over pregnancy (Figure 3). We used linear mixed effects modelling to evaluate the relationship between the ADMA concentrations and the SGA outcome, adjusting for gestational age, maternal age, BMI, malaria status, socioeconomic status and the interaction between treatment arm and gestational age. Those destined to have SGA births had significantly higher levels of ADMA than those destined to have AGA births (χ2(2)=8.76, *P <*0.02). The groups converged over time as demonstrated by the addition of the interaction term between SGA and gestational age (χ2(1)=4.62, *P <*0.04; Supplementary Table 1). Thus, increased ADMA early in pregnancy (reflecting reduced NO biosynthesis) is associated with SGA, but the effect diminishes over gestation as those with lower baseline levels of ADMA, show increases later in pregnancy.

**Dietary L-arginine** **supplementation improves fetal weight and fetal viability in an experimental model of malaria in pregnancy**

Based on the findings of altered pathways of L-arginine and NO biosynthesis in humans, we next explored mechanism and interventions in an experimental malaria in pregnancy (EMIP) mouse model. EMIP recapitulates several features of human MIP including placental parasite accumulation, damage to the syncytiotrophoblast, a low birth weight phenotype (Figure 4A, *P*  < 0.001) and reduced fetal viability (a surrogate for stillbirth) assessed at gestational day 19 (Figure 4B, *P* < 0.001) ([*10*](#_ENREF_10)*,* [*41*](#_ENREF_41)). We used the EMIP model to examine the impact of dietary L-arginine on birth outcomes. L-arginine supplementation to dams did not influence maternal peripheral parasite densities (gestational day 19) or litter size (Supplementary Table2). L-arginine supplementation did not alter birth weight or fetal viability in offspring from uninfected, control litters (Figure 4A,B). However, in malaria-infected dams L-arginine supplementation increased fetal weight (Figure 4A, *P* < 0.05 and Supplementary Table 3) and increased the number of viable pups per litter (Figure 4B, *P* < 0.05, Supplementary Table 4). No differences were observed in placental weight between treatment groups (Supplementary Table 3). EMIP was associated with an increased relative risk of 24.62-fold of delivering a non-viable pup (95% confidence interval: 12.90, 46.98, *P* < 0.001). L-arginine supplementation during EMIP was associated with a 2.5-fold decrease in the relative risk of delivering a non-viable pup (reduced from 24.62 to 9.65 (5.32, 17.51), *P* < 0.05, Supplementary Table 4).

**Experimental malaria in pregnancy (EMIP) decreases circulating L-arginine and dietary supplementation with L-arginine reduces inhibitors of NO synthesis, ADMA and SDMA**

We performed mass spectrometry on serum collected at gestational day 19, in order to quantify circulating levels of L-arginine, ADMA and SDMA in malaria-infected and uninfected pregnant dams. At gestational day 19 (day 6 of infection) levels of L-arginine were significantly reduced in malaria-infected dams (Figure 5A, *P* < 0.001). Malaria-infected dams receiving L-arginine supplementation showed reduced serum ADMA (Figure 5B, *P* < 0.01) and SDMA (Figure 5C, *P* < 0.05) and a trend towards increased L-arginine/ADMA ratio (Figure 5D, *P* < 0.10) compared with the control group.

**L-arginine** **supplementation in EMIP alters the expression of inflammatory and angiogenic mediators in the placenta**

We hypothesized that L-arginine supplementation increases fetal weight and viability by reducing malaria-induced inflammation in the placenta and by promoting the placental vascular development and remodelling required for healthy pregnancy outcomes. Therefore we examined expression of inflammatory and angiogenic factors in placental tissue from viable pups collected at gestational day 19 (Figure 6). EMIP resulted in increased placental expression of pro-inflammatory C5a receptor (C5aR; Figure 6B, *P* < 0.001) and ICAM-1 (Figure 6C, *P* < 0.001) and pro-angiogenic Ang2 (Figure 6F, *P* < 0.01). L-arginine supplementation did not alter gene expression in placental tissue from uninfected dams compared to control uninfected dams. In malaria-infected dams supplemented with L-arginine, we observed reduced gene expression of inflammation-related proteins C5 (Figure 6A, *P* <0.001) and ICAM-1 (Figure 6C, *P* < 0.01). L-arginine supplementation in malaria-infected dams also resulted in changes to angiogenic mediators with an upregulation of Tie-2 (Figure 6D, *P* < 0.01), and Ang1 (Figure 6E, *P* < 0.05) and a down regulation in Ang2 (Figure 6F, *P* < 0.05). In addition, there was reduced expression of the pro-angiogenic factor VEGF-A (Figure 6G, *P* < 0.01), as well as its negative regulator Flt-1 (Figure 6H, *P* < 0.05) in placental tissue from malaria-infected dams receiving L-arginine supplementation compared to malaria-infected control dams. Overall, L-arginine supplementation during EMIP resulted in a more balanced angiogenic response favouring vessel remodelling in the placenta.

**L-arginine** **supplementation during EMIP leads to an increase in placental vascular development and remodelling in malaria-infected dams**

To examine whether changes observed in placental tissue expression of angiogenic factors associated with L-arginine supplementation were related to functional changes in placental vascular development we performed micro-CT imaging of placentas collected prior to the onset of the low birth weight and stillbirth phenotypes ([*10*](#_ENREF_10)). We have previously reported malaria-induced changes in placental vascular development in association with enhanced C5a-C5aR signalling ([*10*](#_ENREF_10)) and hypothesized that L-arginine supplementation, similar to C5aR blockade, would increase placental vascularization and improve birth outcomes. In uninfected litters supplemented with L-arginine, we did not observe differences in placental vascularization compared to uninfected control litters (Figure 7). By contrast, malaria-infected dams receiving L-arginine had an increased total number of placental vessel segments compared with L-arginine-treated uninfected controls (*P* = 0.02, Figure 7A,B). Placentas from L-arginine-treated malaria-infected dams showed higher number of vessel segments in vessels with diameter <50μm compared with placentas from vehicle-control treated malaria-infected litters (*P* < 0.001, Figure 7C).

**Dietary L-arginine supplementation increases fetal weight and viability in the context of an L-arginine** **deficient diet**

Pregnant women in malaria-endemic areas are particularly vulnerable to hypoarginemia due to diets that are relatively deficient in L-arginine, since staple food stuffs (e.g. maize, plantains, yams and cassava) are low in dietary L-arginine ([*42*](#_ENREF_42)). Therefore we modelled this scenario by placing dams on an L-arginine-deficient diet and hypothesized that this would increase the impact of L-arginine supplementation on birth outcomes in EMIP. Compared with controls receiving regular chow, offspring of uninfected dams on the L-arginine deficient chow were lower birth weight (Figure 8A, *P* < 0.01) and supplementation with L-arginine reversed the low birth weight phenotype (Figure 8A, *P* > 0.05). Litters born to malaria-infected dams on the deficient chow that were receiving L-arginine supplementation had increased birth weight (Figure 8A, *P* < 0.01) and fetal viability (Figure 8B, *P* < 0.05) compared with infected control litters on the deficient chow.

**DISCUSSION**

MIP is a leading global cause of maternal morbidity and adverse pregnancy outcomes. The WHO recommends the use of intermittent preventative treatment and insecticide-treated nets for the prevention of MIP; however escalating drug and insecticide resistance threaten this approach ([*1*](#_ENREF_1)*,* [*40*](#_ENREF_40)). Importantly we also lack effective and safe interventions, especially in early pregnancy, to prevent or reduce malaria-associated placental pathology that directly contributes to poor birth outcomes. Here we investigated the L-arginine-NO biosynthetic pathway in the pathogenesis of MIP and provide several lines of evidence supporting this axis as a potential therapeutic target. First, in a prospective study of pregnant women in Malawi we report MIP-related decreases in circulating levels of L-arginine, and increases in inhibitors of NO biosynthesis, ADMA and SDMA, and their association with poor birth outcomes. In an experimental model of MIP we corroborated the human data that alterations in NO biogenesis were associated with adverse birth outcomes. We then used this pre-clinical model to explore mechanism and interventions and show that L-arginine dietary supplementation improved fetal weight and markedly reduced stillbirth. The effect of supplementation on fetal weight was enhanced when dams were placed on an L-arginine deficient diet simulating diets prevalent in low resource settings. The mechanism of L-arginine action was via reduced expression of placental inflammatory factors, normalized expression of angiogenic mediators and a corresponding increase in placental vascular development as evidenced by micro-CT imaging.

NO regulates essential mediators of placental vasculogenesis and angiogenesis, including the VEGF-A and the angiopoietin-Tie2 pathways, and is critical to implantation, trophoblast invasion, placental and embryo development ([*17*](#_ENREF_17)*,* [*21*](#_ENREF_21)*,* [*43*](#_ENREF_43)*,* [*44*](#_ENREF_44)). NO has been shown to increase expression of Ang1 in endothelial cells and NO-production is necessary for VEGF-A-mediated angiogenesis ([*43*](#_ENREF_43)*,* [*45*](#_ENREF_45)). Pathological pregnancy outcomes including preeclampsia, fetal growth restriction and resulting SGA, have been linked to L-arginine deficiency, reduced NO bioavailability and oxidative stress ([*17*](#_ENREF_17)*,* [*46*](#_ENREF_46)*,* [*47*](#_ENREF_47)). In this prospective study of pregnant Malawian women we demonstrate that MIP impacts NO biogenesis by increasing levels of endogenous inhibitors, ADMA and SDMA, and decreasing L-arginine resulting in decreased L-arginine bioavailability (i.e. a reduced L-arginine:ADMA ratio) and conditions that enhance inflammation while impairing L-arginine appearance and intracellular influx ([*30-32*](#_ENREF_30)*,* [*37*](#_ENREF_37)*,* [*46*](#_ENREF_46)). The impact of malaria on the L-arginine pathway was most evident in PCR-detectable infections at enrolment (16-28 weeks of pregnancy), and affected over half of the women enrolled in this study. These changes occurred relatively early in gestation and could contribute to sustained changes in NO bioavailability over pregnancy. Consistent with this hypothesis, increased ADMA between weeks 16-28 of pregnancy was associated with impaired fetal growth and this change was evident across pregnancy. Our results support a mechanistic role for altered L-arginine-NO biosynthesis and related placental insufficiency in malaria-induced SGA outcomes. However, other pathways may also contribute including those that regulate the nutrient transport across the placenta ([*48*](#_ENREF_48)).

Collectively our results suggest that targeting NO biosynthesis in MIP may be an effective intervention to improve birth outcome. In support of this hypothesis, dietary L-arginine supplementation in the EMIP model normalized angiogenic and inflammatory pathways, and enhanced placental vascular development. We observed reduced levels of circulating L-arginine in both treated and untreated malaria-infected dams. While L-arginine supplementation did not increase L-arginine in plasma it was associated with reduced ADMA and SDMA levels compared to malaria-infected untreated dams. Plasma samples were collected via cardiac puncture at gestational day 19 when dams are ill due to malaria infection and drink less water, and therefore may ingest less L-arginine. As L-arginine supplementation reduced circulating inhibitors of NO biosynthesis, ADMA and SDMA, nitric oxide bioavailability may have increased even in the absence of increased L-arginine levels. Our findings are supported by previous studies reporting reduced levels of ADMA in association with L-arginine supplementation ([*49*](#_ENREF_49)*,* [*50*](#_ENREF_50)). While the mechanism by which L-arginine reduces ADMA and SDMA levels is unknown, we speculate that L-arginine supplementation may decrease oxidative stress, conditions under which these endogenous inhibitors are generated ([*49*](#_ENREF_49)*,* [*51*](#_ENREF_51)).

Previous mechanistic studies in pre-clinical models have shown that MIP alters placental vascular development and results in increased placental arterial vascular resistance and adverse birth outcomes including low birth weight offspring and stillbirth ([*10*](#_ENREF_10)). Collectively those findings support the hypothesis that MIP dysregulates placental angiogenesis and vascular remodelling, resulting in placental insufficiency and poor birth outcomes. In this study we confirm and extend those observations, and implicate MIP-induced changes in L-arginine-NO biosynthesis as a putative mediator of the altered angiogenesis observed. Of translational relevance these changes can be corrected, at least in part, by L-arginine supplementation of malaria-infected dams. L-arginine treatment was associated with reduced placental expression of factors that destabilize blood vessels (e.g. C5a, Ang2, VEGF-A) as well as inflammatory cell adhesion molecules (e.g. ICAM-1). Increased levels of these inflammatory factors and mediators of endothelial dysfunction have previously been linked with adverse birth outcomes in other conditions in pregnancy ([*10*](#_ENREF_10)*,* [*16*](#_ENREF_16)*,* [*52*](#_ENREF_52)*,* [*53*](#_ENREF_53)). Expression of Ang2, Tie-2 and VEGF-A is increased in hypoxic conditions, which may also occur during malaria infection in pregnancy ([*54*](#_ENREF_54)*,* [*55*](#_ENREF_55)). We posit that enhanced Tie-2 expression we observed in L-arginine-supplemented dams promotes microvascular stability in the context of malaria-induced inflammation and vascular injury ([*56*](#_ENREF_56)). We observed increased VEGF-A expression in the malaria-infected non-supplemented dams that was reduced with L-arginine supplementation. Taken together these results are consistent with the hypothesis that L-arginine supplementation improves birth outcomes by reducing the expression of pro-inflammatory factors and by normalizing angiogenic processes and promoting placental function and fetal growth.

 In order to link the observed L-arginine related changes in inflammatory and angiogenic factors to a functional vascular correlate, we used micro-CT to visualize the impact of dietary supplementation on placental vascular structure and development. Consistent with our earlier studies, malaria-infection was associated with altered vascular branching in the smaller vessels ([*10*](#_ENREF_10)). Abnormal placental vascular development has previously been linked to poor birth outcomes including fetal growth restriction and preeclampsia ([*57*](#_ENREF_57)*,* [*58*](#_ENREF_58)). In this study, L-arginine supplementation in malaria-infected dams was associated with an increase in the total number of vessel segments, especially in small diameter vessels (<50μm). These small terminal capillaries are the primary sites of vascular remodelling later in pregnancy ([*58*](#_ENREF_58)) and therefore represent a biologically relevant site of action for the L-arginine-NO pathway. Collectively the results suggest that L-arginine supplementation contributes to increased fetal weight and viability via expansion of the vascular network of the placenta, allowing for increased blood volume and surface area for nutrient exchange. In previous pre-clinical studies, MIP was associated with elevated arterial resistance and poor birth outcomes that were reversed following disruption of C5a signalling and we report similar results here with L-arginine supplementation. However L-arginine dietary supplementation represents a more feasible, safe, inexpensive and acceptable intervention strategy for pregnancy compared to biologics for C5 blockade ([*59*](#_ENREF_59)).

Altered angiogenesis may represent a common pathway of injury leading to adverse birth outcomes associated with multiple pathological conditions in pregnancy, including preeclampsia, and L-arginine supplementation during pregnancy may improve birth outcomes in high risk women ([*16*](#_ENREF_16)*,* [*17*](#_ENREF_17)*,* [*46*](#_ENREF_46)). Several lines of evidence support this hypothesis. In many malaria-endemic regions malaria-induced reductions in L-arginine levels may be further compounded by the lack of dietary L-arginine intake ([*60*](#_ENREF_60)). Most regions with high rates of poor birth outcomes also have high rates of malnutrition, due in part to low daily protein intake and therefore low L-arginine intake ([*39*](#_ENREF_39)*,* [*61*](#_ENREF_61)). Low dietary intake of L-arginine has been linked to an increased risk of preterm birth in Tanzanian women ([*61*](#_ENREF_61)). Moreover a previous randomized trial used medical food to supplement L-arginine in the diet ([*62*](#_ENREF_62)) and reported reduced incidence of pre-eclampsia in a high-risk cohort of women receiving L-arginine supplementation. In the present study the beneficial impact of L-arginine supplementation was most marked in animals on an L-arginine deficient diet suggesting that L-arginine supplementation may be most efficacious in women in low resource settings who are most vulnerable to malaria-associated adverse birth outcomes.

While the mouse model can provide important mechanistic insights into the pathophysiology of malaria infection in pregnancy, it also has limitations. The model replicates important components of *P. falciparum* malaria-infection in pregnancy, including the induction of an inflammatory response in the placenta, shared placental vascular development and placental pathology, and associated adverse birth outcomes including intrauterine growth restriction and decreased fetal viability. However, there are also differences including higher parasitaemia levels in the mouse model, which are not observed in multigravid clinical cohorts, and the lack of VAR2CSA-mediated blinding of parasitized erythrocytes in the placenta. Notably the mouse model used in this study most closely models infection in non-immune primigravid women where importantly, higher parasite burdens and the greatest risk of adverse birth outcomes are observed. Moreover, while adhesion in the placenta is not mediated by the same receptors as *P. falciparum*, binding and accumulation of *Plasmodium berghei*-parasitized erythrocytes in the placenta is observed ([*41*](#_ENREF_41)).

In summary we provide evidence supporting a role for L-arginine-NO biosynthesis in the pathophysiology of malaria infection in pregnancy. In a prospective study of women with MIP, alterations in this pathway were associated with adverse birth outcomes. We demonstrate that similar changes occur in a pre-clinical model of MIP and use this model to demonstrate that strategies to enhance L-arginine bioavailability improves birth outcomes, at least in part by reducing placental inflammation, regulating angiogenesis and enhancing placental vascular development. We propose that novel interventions aimed at promoting regulated angiogenesis in the placenta may improve birth outcomes and reduce the global burden of malaria in pregnancy.

MATERIALS AND METHODS

**Clinical cohort**

Samples were collected as part of a multi-site, open-label, two-arm, randomized superiority trial in southern Malawi (Pan African Clinical Trials Registry PACTR20110300280319, ISRCTN Registry ISRCTN69800930), which took place between 2011 and 2013 as described ([*40*](#_ENREF_40)). Briefly, eligibility criteria included: HIV-negative women with an estimated gestational age between 16 and 28 weeks gestation by ultrasound, LMP or both; hemoglobin >7g/dL at baseline; a willingness to deliver in hospital; and had not received a dose of sulfadoxine pyrimethamine (SP) in pregnancy. Women were randomized to receive over the 2nd and 3rd trimester of pregnancy either: i) three or four doses of sulfadoxine/pyrimethamine (intermittent preventive treatment in pregnancy IPTp-SP); or ii) screening with malaria rapid diagnostic tests (First Response Malaria pLDH/HRP-2 Combo Test, Premier Medical Corporation Ltd, USA), and treatment of RDT positive women with a standard 3-day course of dihyrdoartemisinin-piperaquine (intermittent screening and treatment in pregnancy, ISTp-DP; Euartesim, Sigma Tau, Italy, 40mg/320mg tablets). We randomly selected 384 primigravidae for assessment of L-arginine, SDMA, and ADMA provided they met the following inclusion criteria: live birth with known birth weight and singleton delivery. Of the 384 women included, 379 had an enrollment sample tested and 94 had multiple samples tested over pregnancy for longitudinal assessment of L-arginine, SDMA, and ADMA.

**Ethics**

Written informed consent was obtained for all study participants. This study was reviewed and approved by the Liverpool School of Tropical Medicine, the Malawian National Health Science Research Committee, and the University Health Network Research Ethics Committee.

**Sample size calculation**

Our primary endpoint for the human cohort was the association between the arginine pathway and adverse birth outcomes in primigravidae. Using pilot data from the booking visit, we estimated a sample size of 323 women assuming a mean difference in ADMA of 8ng/mL and a standard deviation of 19 with 20% of women expected to have an adverse birth outcome (β=0.80, α=0.05). In the event the data were not normally distributed, we adjusted our sample size upwards by 15% to generate a final minimum sample size of 372 women.

**Assessment of** **L-arginine, ADMA, and SDMA**

EDTA plasma samples were tested for L-arginine, asymmetric dimethyl arginine (ADMA) or symmetric dimethyl arginine (SDMA) using high-pressure liquid chromatography electrospray tandem mass spectrometry as described below. The coefficients of variation for arginine testing were 5.2% for L-arginine, 2.0% for SDMA, and 1.4% for ADMA. Levels of L-arginine, ADMA, and SDMA are quantified as ng/mL and the ratios are expressed as L-arginine/ADMA, L-arginine/SDMA and ADMA/SDMA ([*63*](#_ENREF_63)*,* [*64*](#_ENREF_64)).

**Experimental Malaria in Pregnancy (EMIP) murine model**

The EMIP model used in this study is a validated murine model of MIP, which replicates key pathogenic factors of human MIP ([*41*](#_ENREF_41)). Female wild-type BALB/c mice between 6-8 weeks of age were mated with male wild-type BALB/c mice (8-9 weeks of age, obtained from Jackson Laboratories (Bar Harbor, ME). Naturally mated pregnant mice were infected on G13 with 106 *Plasmodium berghei* ANKA (PbA)-infected erythrocytes in RPMI media via injection into the lateral tail vein. Control pregnant females were injected on G13 with RPMI media alone. Thin blood smears were taken daily and stained with Giemsa stain (Protocol Hema3 Stain Set, Sigma, Oakville, ON) to monitor parasitemia. All experimental protocols were approved by the University Health Network Animal Care Committee and performed in accordance with current institutional regulations.

**Dietary L-arginine** **Supplementation**

On the day of pairing, mice were randomly assigned to one of the following treatment groups: (i) vehicle control - regular drinking water; or (ii) 1.2% L-arginine in drinking water (L-arginine monohydrochloride A6969, Sigma Aldrich, Oakville, Ontario). Mice received L-arginine-supplemented drinking water (or vehicle control) beginning before pregnancy and a minimum of 13 days before malaria-infection (depending on what day they became pregnant after pairing). A dose of 1.2% was selected as it represents approximately twice the daily intake of L-arginine in regular chow (50 mg/day assuming daily intake of 3-5 grams of chow with 1% L-arginine), based on the assumption that mice drink 5-6 mL of water per day (60 mg/day intake via supplemented water). There was no difference in the daily intake of dams receiving the vehicle-control and L-arginine–supplemented water at a dose of 1.2% L-arginine. All mice received treatment via ad libitum access to bottled drinking water throughout pregnancy. All supplementation treatments were given in autoclaved water and water bottles.

**L-arginine Deficient Chow**

Dams that received L-arginine deficient chow were placed on a diet of exclusively deficient chow (Harlan Laboratories, Macison, WI) beginning at gestational day 9 (confirmation of pregnancy) until tissue collection. Dams were kept on their regular chow diet until this time (G9) to minimize disruptions to their environment (i.e., change in diet) during pairing and early pregnancy. On gestational day 9 mice were randomly assigned receive (i) vehicle control - regular drinking water, or (ii) 1.2% L-arginine in drinking water (L-arginine monohydrochloride A6969, Sigma Aldrich, Oakville, Ontario). All mice received treatment via ad libitum access to bottled drinking water throughout pregnancy. All supplementation treatments were given in autoclaved water and water bottles.

**Tissue Collection**

The EMIP model followed the same protocol outlined above. Dams were sacrificed at G19, yolk sacs were dissected from uteri, fetuses were removed and weighed, and placentas were snap frozen and stored at -80 °C until analyzed. Fetal viability was determined by assessing pedal withdrawal reflex. Non-viable fetuses (i.e., lacking the pedal withdrawal reflex) were considered stillbirths. All fetuses were weighed at this time. RNA extraction was performed on snap-frozen fetal placenta tissue collected at G19. Serum from mice was collected from cardiac punch was stored at -80°C prior to analysis.

**Placenta Transcript Analysis**

Only placentas collected from viable fetuses were used in the transcript analysis. Tissue was homogenized in TRIzol (0.5mL/100mg tissue; Invitrogen, Burlington, ON) according to the manufacturer’s protocol and RNA was extracted. Extracted RNA (2 μg per sample) was then treated with DNase I (Ambion, Streetsville, ON) and reverse transcribed to cDNA with SuperScript III (Invitrogen, Burlington, ON) in the presence of oligo (dT)18 primers (for primer sequences refer to Supplementary Table 5) (Fermentas, Burlington, ON). Residual RNA was degraded with RNase H (Invitrogen, Burlington, ON). Sample cDNA was amplified in triplicate with SYBR Green master mix (Roche, Laval, QC) in the presence of 1 μM both forward and reverse primers in a Light Cycler 480 (Roche, Laval, QC). Transcript number was calculated based on Ct compared to the standard curve of mouse genomic DNA included on each plate by Light Cycler 480 software (Roche, Laval, QC), and normalized to geometric average of the housekeeping genes GAPDH and HRPT expression levels. Primer sequences (5’–3’) are listed in Supplementary Table 5.

**High Pressure Liquid Chromatography (HPLC)-Electrospray Tandem Mass Spectrometry**

Levels of L-arginine, ADMA and SDMA were assayed by mass spectrometry as described ([*65*](#_ENREF_65)). Briefly, the chromatographic conditions included a 125 x 3 mm Nucleuosil 100-5 silica column with a 4 x 2 mm silica filter insert. Mobile phase A consisted of 1 L of water mixed with 0.25 mL of TFA and 10 mL of propionic acid. Mobile phase B consisted of 1 L of acetonitrile mixed with 0.25 mL of trifluoroacetic acid and 10 mL of propionic acid. Isocratic elution with one part mobile phase A and nine parts mobile phase B was delivered at a flow rate of 0.5 mL/min at a temperature of 30°C. Samples were prepared with 60 µl of serum, 20 µl of the respective internal standard. Samples (10 µl) were injected automatically and the electrospray ion source run time duration was 3-6.5 minutes under the following conditions: 32 (arbitrary units); auxiliary gas, 20 (arbitrary units); needle voltage, +4.5 kV; and capillary temperature, 300°C.

**Placental Micro-CT Scans**

Detailed methods for preparing the fetoplacental vasculature for micro-CT imaging have been described previously ([*66*](#_ENREF_66)). Briefly, uteri were extracted from dams at gestational day 18 and anesthetized via hypothermia (immersion in ice-cold PBS). Each individual fetus is then extracted from the uterus while maintaining the vascular connection to the placenta. The embryo is briefly resuscitated via immersion in warm PBS to resume blood circulation. Embryos that could not be resuscitated are not perfused and were removed from the study. A catheter is then inserted into the umbilical artery and the fetus is perfused with saline (with heparin, 100units/mL) followed by radio-opaque silicone rubber contrast agent (Microfil; Flow Technology, Carver, MA). Following perfusion specimens are post-fixed with 10% Formalin and imaged using micro-computed tomography (micro-CT). Specimens were scanned at 7.1μm resolution for 1 hour using a Bruker SkyScan1172 high resolution Micro-CT scanner. 996 views were acquitted via 180-degree rotation with an X-ray source at 54 kVp and 185 uA. Three-dimensional micro-CT data were reconstructed using SkyScan NRecon software. The structure of the vasculature was identified automatically using a segmentation algorithm as described in detail previously ([*67*](#_ENREF_67)). The leaves of the vascular tree were pruned to 35 μm to improve data consistency. Analysis was performed on wild type (unexposed (n = 7) and malaria exposed (n = 8)) offspring of control (non-supplemented dams and unexposed (n = 7) and malaria exposed (n = 7)) offspring of L-arginine supplemented dams. Each group contains a minimum of 3 dams per group and 1-3 specimens per litter.

**Statistical Analyses**

Statistical analysis was performed using STATA v14 (StataCorp. College Station, TX), R v3.2.1 (R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism v6 (GraphPad Software Inc., La Jolla, CA). Student’s t-test, one-way ANOVA (non-parametric Kruskal-Wallis, *P* < 0.05) and post-test, independent samples t-test, Chi Squared and relative risk were used to examine statistical significance between experimental groups. Analysis of the cumulative distribution of vessel diameters for each placenta was fit with a natural spline with eight degrees of freedom. A two-way ANOVA was conducted to determine whether there was an effect of treatment group on the spline parameters. There was a significant interaction between spline coefficient and group (*P* <0.001) and therefore a post hoc analysis was performed to compare pairs of treatment groups. Post-tests on all groups were conducted using Dunn’s multiple comparison test (*P* < 0.05). For the human study, relative risk was calculated using a log binomial model including all variables with a *P* <0.20 by bivariate analysis. In addition, treatment arm, maternal age and malaria status at enrollment (by microscopy) were included in the model.To compare the association between markers of NO biosynthesis and nutritional status (maternal BMI, mid-upper-arm circumference, hemoglobin), linear regression was used adjusting for maternal age and gestational age at enrolment. For longitudinal analysis, we used linear mixed effects modelling with the lme4 ([*68*](#_ENREF_68)) package in R ([*69*](#_ENREF_69)) to evaluate the relationship between longitudinal ADMA concentrations and the SGA outcome. We first constructed a null model with six fixed effects: the linear effect of gestational age, maternal age, enrolment BMI, enrolment malaria status, socio-economic status, and the interaction between gestational age and treatment arm. This interaction term adjusted for the possibility that ADMA’s rate of change was affected by either treatment. Using likelihood ratio tests, we then assessed whether adding SGA as a fixed effect significantly improved the model fit, followed by adding the interaction between SGA and gestational age (Supplementary Table 1). For random effects, all models included a by-participant intercept and by-participant slope for the effect of gestational age. Biomarker levels were transformed using the natural logarithm to stabilize their variance. No apparent deviation from homoscedasticity or normality was apparent on the residual plots. Similarly, but without adjusting for other covariates, LME models were used to assess the relationship between malaria status at enrolment (by microscopy and PCR) and gestational changes in ADMA, SDMA, and L-arginine concentrations.

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**Figure Legends**

**Figure 1:** Longitudinal assessment of the L-arginine pathway over pregnancy.Levels of L-arginine, ADMA and SDMA measured by mass spectrometry in n = 603 plasma samples from the study cohort of pregnant women (n = 94) beginning at 16 weeks gestation. Longitudinal assessment of changes over pregnancy in: (**A**) ADMA (*P* = 0.01), (**B**) SDMA (*P* <0.0001), and (**C**) L-arginine (*P* >0.05), (**D**) L-arginine:ADMA (*P* >0.05) from 16 weeks of gestation to deliveryby linear regression.

**Figure 2.** Malaria at enrolment is associated with altered NO-biosynthesis over pregnancy.

Individual data points colored according to malaria status by microscopy for levels of (**A**) L-arginine (*P* >0.95), (**B**) ADMA (*P* >0.65), and (**C**) SDMA (*P* <0.04) or malaria status by PCR for (**D**) L-arginine (*P* = 0.031), (**E**) ADMA (*P* = 0.006), and (**F**) SDMA (*P* = 0.57). The overlaid regression lines are from linear mixed effects models fitted for a subject with average values (conditional on fixed effects only).

**Figure 3.** Elevated ADMA in pregnancy is associated with small-for-gestational age (SGA) birth outcomes. Those destined to have SGA births had higher levels of ADMA than those destined to have AGA births (χ2(2)=8.76, *P* <0.02). Individual data points colored according to SGA or appropriate-for-gestational age (AGA) status with overlaid regression lines from linear mixed effects models, fitted for a subject with average values (conditional on fixed effects only).

**Figure 4:** Dietary L-arginine supplementation improves fetal outcomes in experimental malaria in pregnancy. **(A)** Fetal weight (g) of uninfected vehicle control treated litters (n = 22), uninfected L-arginine supplemented litters (n = 37), malaria (*Plasmodium berghei* ANKA, PbA) infected vehicle control treated litters (n = 26), and malaria (PbA) infected L-arginine supplemented litters (n = 36). Box plots depict median, interquartile range with whiskers depicting maximum and minimum values. **(B)** Percent viable pups per litter of uninfected vehicle control treated litters (n = 22), uninfected L-arginine supplemented litters (n = 37), malaria (PbA) infected vehicle control treated litters (n = 26), and malaria (PbA) infected L-arginine supplemented litters (n = 36). Figures depict mean ± standard deviation. Results of independent samples t-test (fetal weight) and chi-squared test (viability), \*\* *P* < 0.001, \* *P* < 0.05.

**Figure 5:** L-arginine, ADMA and SDMA levels measured by mass spectrometry in maternal serum. **(A)** Malaria infection is associated with decreased L-arginine serum levels.Malaria-infected dams receiving L-arginine supplementation show reduced serum levels of **(B)** ADMA and **(C)** SDMA. Box plots depict median, interquartile range with whiskers depicting maximum and minimum values. **(D)** Malaria infection is associated with a trend towards a decreased L-arginine /ADMA ratio. Malaria-infected vehicle control treated (Veh) (n = 18) and malaria infected (PbA) L-arginine treated (L-arg) dams (n = 17) had a reduced (*P* < 0.10) ratio of L-arginine to ADMA compared with uninfected (UI) vehicle control (Veh) (n = 14) and uninfected L-arginine treated (L-arg) dams (n = 16). Figures depict mean ± standard deviation. Results of one-way ANOVA and post-test, \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001.

**Figure 6:** Expression of inflammatory and angiogenic mediators in placental tissue by RTPCR. **(A)** C5, **(B)** C5a receptor, **(C)** ICAM-1, **(D)** Tie-2, **(E)** Ang1, **(F)** Ang2, **(G)** VEGF-A and **(H)** Flt-1 (VEGF receptor) in uninfected (UI) vehicle control (Veh) treated dams (n = 12), uninfected (UI) L-arginine treated (L-arg) dams (n = 12), malaria infected (*Plasmodium berghei* ANKA, PbA) vehicle control treated (Veh) dams (n = 12) and malaria infected (PbA) L-arginine treated (L-arg) dams (n = 12). Box plots depict median, interquartile range with whiskers depicting maximum and minimum values. Results of one-way ANOVA and post-test, \**P* < 0.05, \*\**P* < 0.01, \*\*\* *P* < 0.001

**Figure 7:** L-arginine supplementation increases the number of small vessels in placentas from malaria-infected litters. Representative micro-CT images of fetoplacental arterial vasculature at gestational day 18 in placentas from malaria-infected **(A)** vehicle control treated and **(B)** L-arginine-treated mice colour-coded by vessel diameter. **(C)** Cumulative distribution of vessel diameters in placentas from uninfected vehicle control-treated (n = 7), uninfected L-arginine -treated (n = 7), malaria-infected vehicle control-treated (n = 8) and malaria infected L-arginine -treated (n = 7) litters. Cumulative vessel segments depicts median and SEM of vessels larger than the threshold diameter with results of two-way ANOVA and post-hoc test, \**P* < 0.001.

**Figure 8:** Dietary L-arginine supplementation improves birth outcomes in malaria-infected dams receiving L-arginine deficient chow. **(A)** Fetal weight (g) of uninfected vehicle (Veh) control treated litters on regular chow (n = 22), uninfected L-arginine (L-arg) supplemented litters on regular chow (n = 36), uninfected vehicle (Veh) control treated litters (n = 10), uninfected L-arginine (L-arg) supplemented litters (n = 10), malaria (*Plasmodium berghei* ANKA, PbA) infected vehicle control treated litters (n = 12), and malaria (PbA) infected L-arginine supplemented litters (n = 11) on L-arginine deficient chow. Box plots depict median, interquartile range with whiskers depicting maximum and minimum values, and **(B)** Percent viable pups per litter of uninfected vehicle control (n = 22) on regular chow, uninfected L-arginine supplemented litters (n = 36) on regular chow, uninfected vehicle control treated litters (n = 10), uninfected L-arginine supplemented litters (n = 10), malaria (PbA) infected vehicle control treated litters (n = 12), and malaria (PbA) infected L-arginine supplemented litters (n = 11) on L-arginine deficient chow. Figure depicts mean ± standard deviation. Results of one-way ANOVA and post-test, \*\* *P* < 0.01, \**P* < 0.05.

**Supplementary Table 1:** Linear mixed effects modeling of longitudinal changes in ADMA levels and small-for-gestational age.

**Supplementary Table 2:** Dam peripheral parasitaemia (G19) and litter size from all cohorts. Values are presented as mean ± SD. Results of one-way ANOVA and post-test.

**Supplementary Table 3:** Impact of dietary L-arginine supplementation on fetal and placental weight. Values are presented as mean ± SD. \**P* =0.012 in an independent sample t-test.

**Supplementary Table 4**: Impact of dietary L-arginine supplementation on fetal viability. Values are expressed as percent of viable pups per study and total number (n) of pups per group. Results of one-way ANOVA and post-test, \**P* < 0.05.

**Supplementary Table 5:** RTPCR Primer Sequences (5’ – 3’)

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