EBioMedicine 73 (2021) 103683

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.elsevier.com/locate/ebiom

# The Angiopoietin-Tie2 axis contributes to placental vascular disruption and adverse birth outcomes in malaria in pregnancy

Vanessa Tran[1,2,*], Andrea M. Weckman[1,2,*], Valerie M. Crowley[1], Lindsay S. Cahill[3], Kathleen Zhong[1], Ana Cabrera[4], Robyn E. Elphinstone[1,2], Victoria Pearce[1], Mwayiwawo Madanitsa[6], Linda Kalilani-Phiri[5], Victor Mwapasa[5], Carole Khairallah[7], Andrea L. Conroy[8], Feiko O. ter Kuile[7], John G. Sled[3], Kevin C. Kain[1,2,9,#]

[1] SAR Laboratories, Sandra Rotman Centre for Global Health, University Health Network-Toronto General Hospital Research Institute, Toronto, Canada
[2] Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Canada
[3] Mouse Imaging Centre, Hospital for Sick Children, Toronto, Canada
[4] Department of Pathology and Laboratory Medicine, Schulich School of Medicine & Dentistry, Western University, London, Canada
[5] College of Medicine, University of Malawi, Blantyre, Malawi
[6] Department of Clinical Sciences, Academy of Medical Sciences, Malawi University of Science and Technology, Thyolo, Malawi
[7] Department of Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
[8] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, United States
[9] Tropical Disease Unit, Division of Infectious Diseases, Department of Medicine, University of Toronto, Toronto, Canada

ARTICLE INFO

*Article history:*
Received 26 July 2021
Revised 28 September 2021
Accepted 25 October 2021

*Keywords:*
Malaria
pregnancy
angiopoietin-Tie2
placental vasculature
angiopoietin-1

ABSTRACT

*Background:* Malaria during pregnancy is a major contributor to the global burden of adverse birth outcomes including fetal growth restriction, preterm birth, and fetal loss. Recent evidence supports a role for angiogenic dysregulation and perturbations to placental vascular development in the pathobiology of malaria in pregnancy. The Angiopoietin-Tie2 axis is critical for placental vascularization and remodeling. We hypothesized that disruption of this pathway would contribute to malaria-induced adverse birth outcomes.

*Methods:* Using samples from a previously conducted prospective cohort study of pregnant women in Malawi, we measured circulating levels of angiopoietin-1 (Angpt-1) and Angpt-2 by Luminex (n=1392). We used a preclinical model of malaria in pregnancy (*Plasmodium berghei* ANKA [PbA] in pregnant BALB/c mice), genetic disruption of Angpt-1 (*Angpt1*$^{+/-}$ mice), and micro-CT analysis of placental vasculature to test the hypothesis that disruptions to the Angpt-Tie2 axis by malaria during pregnancy would result in aberrant placental vasculature and adverse birth outcomes.

*Findings:* Decreased circulating levels of Angpt-1 and an increased ratio of Angpt-2/Angpt-1 across pregnancy were associated with malaria in pregnancy. In the preclinical model, PbA infection recapitulated disruptions to the Angiopoietin-Tie2 axis resulting in reduced fetal growth and viability. Malaria decreased placental Angpt-1 and Tie2 expression and acted synergistically with reduced Angpt-1 in heterozygous dams (*Angpt1*$^{+/-}$), to worsen birth outcomes by impeding vascular remodeling required for placental function.

*Interpretation:* Collectively, these data support a mechanistic role for the Angpt-Tie2 axis in malaria in pregnancy, including a potential protective role for Angpt-1 in mitigating infection-associated adverse birth outcomes.

*Funding:* This work was supported by the Canadian Institutes of Health Research (CIHR), Canada Research Chair, and Toronto General Research Institute Postdoctoral Fellowship Award. The parent trial was supported by the European & Developing Countries Clinical Trials Partnership and the Malaria in Pregnancy Consortium, which was funded by the Bill & Melinda Gates Foundation. The funders had no role in design, analysis, or reporting of these studies.



https://doi.org/10.1016/j.ebiom.2021.103683

Research in context

**Evidence before this study**

Despite progress in preventing and treating *Plasmodium falciparum* malaria in pregnancy, the global burden of malaria-related adverse birth outcomes (e.g., low birth weight, preterm birth, and foetal loss) remains high. Existing evidence implicates disruptions to placental vascular development as a key mediator of the pathobiology of malaria-associated adverse birth outcomes. The angiopoietin signalling axis has been implicated in the pathogenesis of severe malaria in non-pregnant individuals. Furthermore, there is evidence in cross-sectional studies for systemic and placental dysregulation of Angpt-1 and Angpt-2 by *P. falciparum* infection during pregnancy. However, a mechanistic role for the angiopoietin signalling family in the pathogenesis of malaria in pregnancy has not been established.

**Added value of this study**

Here, we explore a putative mechanistic role for the Angpt-Tie2 signalling axis in the pathogenesis of malaria in pregnancy. We used longitudinal clinical data from a large cohort of pregnant women to extend existing cross-sectional data and characterize the longitudinal dynamics of Angpt-1 and Angpt-2 across pregnancies complicated by malaria. We used a preclinical model of malaria in pregnancy, combined with genetic reduction of Angpt-1 (*Angpt1*$^{+/-}$), and micro-CT imaging of placental vasculature to show that Angpt-1 may play a putative protective role in the pathogenesis of malaria in pregnancy via compensatory placental vascular remodelling.

**Implications of all the available evidence**

Our data provide preliminary evidence for a mechanistic link between malaria-associated disruptions to the Angpt-Tie2 axis, aberrant placental vascular development and remodelling, and adverse birth outcomes. Previous cross-sectional studies in *Plasmodium*-infected pregnancies showed disruptions to the angiopoietins at delivery, at which point intervention is futile. Here, we combined clinical and preclinical data to provide a more comprehensive picture of the disruption of Angpt-1 and Angpt-2 homeostasis across gestation and its subsequent impact on placental vascular development and birth outcomes.

## 1. Introduction

Despite progress in preventing and treating *Plasmodium falciparum* malaria in pregnancy, the global burden of malaria-related adverse birth outcomes (e.g., low birth weight, preterm birth, and foetal loss) remains high [1,2]. Advances in our understanding of malaria-associated adverse birth outcomes have implicated early dysregulation of inflammatory and angiogenic pathways, and consequent disruptions to placental vascular development, as key mediators of its pathobiology [2–6].

Developing a healthy placental vascular system is essential to sustain foetal growth and support a successful pregnancy. Placental vascular development is a complex process that requires tight regulation and crosstalk between inflammatory and angiogenic signalling pathways [7,8]. There is considerable evidence supporting the hypothesis of a hierarchical relationship between maternal infection and immune activation, dysregulation of angiogenic pathways and placental development, and adverse birth outcomes [reviewed in [9]]. The angiopoietin protein family has critical roles in placental and embryonic development including regulation of angiogenesis, trophoblast function, and spiral artery remodelling [10,11]. Angiopoietin-1 (Angpt-1) and its antagonist angiopoietin-2 (Angpt-2), both signal through the Tie2 receptor and their respective placental expressions are spatially and temporally coordinated across pregnancy [10,12-14]. Angpt-1 stabilizes vasculature and promotes vessel maturation, while Angpt-2 destabilizes vasculature to promote plasticity and vascular remodelling [15]. Regulation of the angiopoietins requires a fine balance, and disruptions of Angpt-1, Angpt-2, and the Angpt-2/Angpt-1 ratio have been linked to pathological pregnancies and adverse birth outcomes [3,5,10,16-18].

Clinical and preclinical studies of paediatric and adult *P. falciparum* infection support a mechanistic role for Angpt-1 and Angpt-2 in the pathogenesis of severe malaria in non-pregnant individuals [19–23]. Moreover, there is evidence in cross-sectional studies for systemic and placental dysregulation of Angpt-1 and Angpt-2 by *P. falciparum* infection during pregnancy [3,5,24,25]. We previously reported that disruptions to C5a-C5a receptor signalling in preclinical and clinical malaria in pregnancy led to foetal growth restriction via aberrant placental vascular development [3]. In that prospective study of pregnant women in Malawi, structural equation modelling was applied to statistically demonstrate that decreases in circulating Angpt-1 were downstream of C5a in its mediation of adverse birth outcomes [3]. However, a mechanistic role for the angiopoietin signalling family in the pathogenesis of malaria in pregnancy has not been established.

Here we show that the Angpt-Tie2 axis plays a role in the pathogenesis of malaria in pregnancy. In a longitudinal cohort study, we extend existing cross-sectional data by showing that Angpt-1 is decreased and the Angpt-2/Angpt-1 ratio is increased across pregnancies complicated by *Plasmodium falciparum* infection. Using an experimental model of malaria in pregnancy, we show that Angpt-1 plays a potential protective role in the pathogenesis of antenatal malaria via compensatory placental vascular remodelling. Collectively, our data suggest a mechanistic role for Angpt-1 in the pathogenesis of malaria and malaria-associated placental vascular development and adverse birth outcomes.

## 2. Methods

### 2.1. Clinical cohort study design and analyte data

This study cohort was nested within a three-site, two-arm, randomized superiority trial of malaria prevention during pregnancy in Malawi [26]. Between 2011 and 2013, HIV-negative pregnant women were enrolled in the parent trial and randomized to receive either intermittent preventative treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP), or intermittent screening and treatment in pregnancy with dihydroartemisinin-piperaquine (ISTp-DP). Women enrolled in the parent trial attended 3-4 antenatal visits in the second and third trimester, scheduled every 4-6 weeks. Women were tested for peripheral malaria at each scheduled (3-4) and unscheduled (up to 8 [26]) visit including delivery, by real-time polymerase chain reaction (PCR). At delivery, the presence of placental malaria (assessed by placental histopathology and/or by PCR in placental blood), as well as birth weight and gestational age were recorded. Gestational age was determined by ultrasound as described [26]. Comprehensive information on parent trial inclusion/exclusion criteria, malaria testing, and treatment courses has been published [26].

[#] Corresponding author: Dr. Kevin C. Kain, University Health Network, Toronto General Hospital, MaRS Centre, TMDT, 10th Floor 10-351, Toronto, Ontario M5G 1L7, (+1) 416 581 7704

*E-mail address:* kevin.kain@uhn.ca (K.C. Kain).

[*] Co-first authors

A maternal plasma sample was collected at each scheduled antenatal visit. Of the 1873 participants enrolled in the parent trial, women were included in this nested study of placental pathobiology if they met the inclusion criteria for plasma analyte testing as described [2], including enrolment in the parent trial <24 weeks gestation and an available plasma sample (kept frozen at -80°C) [n=1628]. In addition, participants without a known birth outcome or placental malaria status were excluded from the current analysis to reach the final cohort size of n=1392. Women enrolled in the parent trial that were excluded from our final cohort had similar demographic characteristics to our cohort including treatment group allocation, socioeconomic status, rates of placental malaria, rates of adverse live birth outcomes, and primigravidity (all $p>0.05$; Wilcoxon rank sum (continuous) or chi-square test (categorical)). Women excluded from our cohort had a higher median age (22 vs. 21; Wilcoxon rank sum, $p=0.006$), and higher rates of foetal loss (5.1% vs. 1.3%; chi-square test, $p<0.0001$).

In this study, the primary malaria outcome was placental malaria. Placental malaria was defined by placental histology (including past and active infections) and/or infection in the placental blood by PCR. Placental malaria was chosen based on the study's focus on placental vascular development. Adverse birth outcomes were defined as: adverse live birth outcomes, which included low birth weight (<2500g), small-for-gestational age [27], and preterm birth (<37 weeks gestation); or foetal loss, which included miscarriage and stillbirth [26]. Multianalyte Luminex Human Discovery Assay (R&D Systems, Minneapolis, MN; custom plate LXSAHM-01) was used to measure angiopoietin-1 (Angpt-1; 1:2 dilution) and angiopoietin-2 (Angpt-2; 1:2 dilution) in maternal plasma at up to three gestational windows per woman that corresponded with antenatal visits (13-23 weeks gestation, 28-33 weeks gestation, and 34-36 weeks gestation) [2]. All samples were processed according to manufacturers' instructions. The methods of assessment were the same across groups, and malaria status and birth outcomes were unknown to the individuals performing the analyte assays.

### 2.2. Statistical analyses of clinical data

Statistical analyses of clinical data were performed using R version 4.0.3 (R Core Team, 2020). Relative risk (with 95% confidence intervals) of live adverse birth outcomes or foetal loss according to maternal placental malaria status was calculated using log-binomial regression. Models were adjusted for factors (chosen *a priori*) known to be associated with adverse birth outcomes, including gestational age at enrolment, maternal age, socioeconomic status (calculated as described in [26]), and parent trial treatment group. In this cohort, gravidity was strongly correlated with maternal age (Spearman's rho = 0.84; $p<0.0001$), and so it was not included as a covariate in log-binomial regression or linear mixed-effects models to avoid problems with multicollinearity. However, as primigravids are more susceptible to placental malaria and experience more severe birth outcomes in the context of *P. falciparum* infection [1], analyses were stratified by gravidity (primigravidae and multigravidas) to investigate its impact as an effect modifier. Angiopoietin analyte data were log-transformed for longitudinal analysis. To assess the impact of placental malaria on longitudinal angiopoietin concentrations, as well as differences in longitudinal angiopoietin concentrations in pregnancies resulting in adverse birth outcomes, we used linear mixed-effects modelling (LME) with the lme4 package in R [28]. We modelled repeated measures angiopoietin data as a continuous dependent variable. For each analyte we built a null model, which included maternal age, socioeconomic status, and a restricted cubic spline [29] of gestational age as fixed effects, and a by-participant intercept as a random effect. Parent trial treatment group (IPTp-SP vs. ISTp-DP) was not associated with demographic variables, malaria status, or analyte data (Supplementary Table 1); however, treatment group was included as a fixed effect in all models to account for the parent trial design. The effect of placental malaria on angiopoietin concentrations across pregnancy was analysed by adding placental malaria status as a fixed effect in a second model. A third model included an interaction term between the spline of gestational age and placental malaria status to account for variation in the effect of placental malaria status on angiopoietin concentration according to gestational age. Models were compared for best fit using a likelihood ratio test. Longitudinal analysis of angiopoietins across pregnancy according to birth outcome was conducted in the same way; however, placental malaria was included as a fixed effect in all models and birth outcome was added to the null model as the variable of interest.

### 2.3. Experimental model of malaria in pregnancy

To examine the role of Angpt-1 in the pathology of malaria in pregnancy, we used a well-validated experimental model of malaria in pregnancy [3,4,30]. For experiments measuring Angpt-1 and Angpt-2 dynamics across pregnancy, wildtype BALB/c female and male mice (7-8 weeks old; purchased from Jackson Laboratories [stock no: 000651] were paired (triad breeding) for timed-mating. Mice purchased from a commercial supplier were allowed to acclimatize for one week before commencement of experiments. For genetic experiments, *Angpt1*$^{+/-}$ mice were bred starting from *Angpt1*$^{del}$ on a 129Sv/J mixed background obtained from our collaborator Dr. Sue Quaggin (Samuel Lunenfeld Research Institute, Toronto, ON). Targeted mutations were transferred to the BALB/c background by backcrossing to wild type BALB/c mice for 10 generations. *Angpt1*$^{+/-}$ and wildtype female littermate controls were paired (triad breeding) with wildtype BALB/c males purchased from Jackson Laboratories (8-15 weeks old; [stock no: 000651] for timed-mating. When mating was confirmed (by presence of vaginal plug), female mice were weighed and separated into cages of 3-4 potentially pregnant females. Individual mice were identified using a standard ear-punch system. On gestational (G) day 13, naturally mated pregnant dams were infected with *Plasmodium berghei* ANKA (MR4 [ATCC/MR4] Malaria Research & Reference Reagent Resource Center, cat#MRA-311) thawed from cryopreservation and passaged through a male BALB/c mouse. Dams were injected intravenously via the lateral tail vein with either $10^6$ parasitized erythrocytes (infected group) prepared in Gibco Roswell Park Memorial Institute (RPMI) medium (ThermoFisher), or the same volume of RPMI alone (uninfected control group). After infection, mice were monitored daily and euthanized if they showed overt signs of distress (hypothermia, loss of >20% body weight, ruffled coat, inactivity). Experiments were planned to achieve a sample size of 5 biological replicates (pregnant dams) per group, with a minimum of 2 experimental replicates. Final sample size was determined by success of timed pregnancy (assessed twice before randomization: by weight gain ≥1.5g on G9 and weight gain ≥2.5g on G13). Mice that reached the pre-established weight gain cut-offs were included in the study and randomized to exposure group (i.e., infected vs. uninfected) on G13, by alternating assignation. To reduce confounding, dams within a single cage were split between infected and uninfected exposure groups and all cages were located together within the animal housing facility. On the day of sacrifice, mice were excluded from the cohort if it was discovered by dissection that they were not pregnant.

Pregnant dams were sacrificed with carbon dioxide inhalation (2.4L/min/20% filling; using an 1l.9L container). As the infected dams are visibly ill, laboratory personnel were not blinded at this stage. To reduce bias, all sacrifices for an experiment were performed sequentially, within the same timeframe. Maternal whole blood (~500-1000 $\mu$L) was collected by cardiac puncture with

heparin, spun down to isolate plasma, and plasma was stored at -80°C for future analysis. Foetuses were dissected from uteri and yolk sacs and weighed. Foetal viability was assessed by visual examination and pedal withdrawal reflex. Placentas were snap-frozen in liquid nitrogen and stored at -80°C. For assessment of maternal data (i.e., peripheral plasma angiopoietin levels), each dam was considered an experimental unit. For assessment of foetal and placental data (i.e., foetal weight and viability, placental qRT-PCR, placental vasculature), each foetus and/or placenta was an experimental unit.

### 2.4. Placental transcript analysis

Levels of Angpt-1, Angpt-2, and Tie2 were determined by quantitative real-time PCR (qRT-PCR) as described previously [5]. Briefly, RNA was extracted from snap-frozen mouse placentas after homogenization in TRIzol (Invitrogen, cat#15596018) as per manufacturer's instructions. RNA was treated with Dexoyribonuclease I (RNase-free) (Thermo Scientific, cat#EN0521) and reverse transcribed to complimentary DNA (cDNA) with iScript cDNA Synthesis Kit (Bio-Rad, cat#1708891) with oligo $(dT)_{18}$ primers. cDNA was amplified and quantified in triplicate by qRT-PCR with LightCycler® 480 SYBR Green I Master (Roche Diagnostics, cat#04707516001) and 1$\mu$M of both forward and reverse primers using a Roche Light Cycler® 480 (Roche, Serial #20377). Samples were run in triplicate. Transcript expression levels were calculated compared to a standard curve of mouse genomic DNA included on each plate and normalized to average GAPDH expression levels. See supplementary methods for primer sequences and thermocycling conditions. Laboratory personnel were blinded to experimental groups and outcomes.

### 2.5. Mouse maternal plasma analyte measurements

Circulating mouse maternal plasma levels of Angpt-1 (1:20 dilution, R&D Systems, cat #DY923) and Angpt-2 (1:160 dilution, R&D Systems, cat#MANG20) were measured by enzyme-linked immunosorbent assay (ELISA) from samples stored at -80°C. Samples were processed according to manufacturer's protocols. Laboratory personnel were blinded to experimental groups and outcomes.

### 2.6. Fetoplacental perfusions and micro-computed tomography

Placentas were perfused via umbilical artery cannulation as described previously [3,31]. Briefly, uteri were collected from pregnant mice on G18, dipped in iodine and placed in cold Dulbecco's Modified Eagle's Medium (DMEM) to anesthetize the foetuses. Once removed from the uterus with fetoplacental vascular connection intact, foetuses were individually warmed with saline to reinitiate heartbeat and placental blood flow. Placentas were perfused with 2% xylocaine and 100 IU/mL heparin in saline followed by radio-opaque silicone rubber contrast agent (Microfil ®; Flow Technology, Carver, MA) until capillaries were visibly perfused. Umbilical vessels were clamped to maintain pressure and left to set for one hour. Placentas were fixed in 10% phosphate-buffered formalin for 24 hours and mounted in 1% agar for imaging by micro-computed tomography (micro-CT) using a Bruker Skyscan 1272 scanner (Bruker Skyscan, Antwerp, Belgium). Three-dimensional data sets were acquired with each specimen rotated 360° and a resolution of 7.1 $\mu$m. The placental vasculature was automatically segmented using a previously described algorithm that identified vessel segments and bifurcations [32]. Detection of vessels with a diameter smaller than 35 $\mu$m was unreliable, and therefore the terminal segments of the vascular tree were pruned to 35 $\mu$m to improve data consistency. Resistance of the foeto-placental arterial vascular network was calculated using a combination of standard formulas for resistances in parallel and in series as described previously [31]. Haemodynamic calculations assumed Poiseuille's law for flow of fluid through a pipe-like structure; equal pressure at each terminal vessel; and a correction factor modelling blood viscosity changes in small vessels derived using adult blood [33]. Laboratory personnel were blinded to experimental groups and outcome.

### 2.7. Statistical analyses of experimental studies

Statistical analyses of experimental studies were performed using GraphPad Prism v9 (GraphPad Software, LLC). For experimental malaria in pregnancy time course data, each time point represents separate groups of mice (from one experimental cohort to keep conditions consistent) at the different gestational ages because specimen collection for analysis required maternal sacrifice. Therefore, repeated measures analysis was not appropriate and time course data were analysed using two-way ANOVA with Bonferroni's multiple comparisons test to compare groups at each gestational age. Assumptions of two-way ANOVAs were checked by visual inspection of residuals (for heteroscedasticity) and Shapiro-Wilk's test for normality of residuals. When these assumptions were not met, data were log-transformed. If assumptions were still not satisfied, time course data were analysed using multiple Mann-Whitney tests with Holm-Sidak correction for multiple comparisons. Maternal plasma and placental qRT-PCR data at G19 were analysed using one-way ANOVA with Tukey's multiple comparisons test. ANOVA assumptions were assessed using Brown-Forsythe and Bartlett's tests for homogeneity of variance and Shapiro-Wilk's test for normality of residuals. When these assumptions were not met, data were log-transformed. If assumptions were still not met, these data were analysed using Kruskall-Wallis non-parametric ANOVA with Dunn's test for multiple comparisons. As ANOVA results can be sensitive to outliers, outliers were identified using the ROUT method with a conservative threshold (Q = 0.1%) to avoid false detection. No outliers (n=2 across all experiments) were removed because sensitivity analysis showed they did not influence the results. Differences in foetal viability were assessed using Fisher's exact test. The cumulative distributions of vessel diameters for the placentas were fit with a natural spline with eight degrees of freedom. A two-way ANOVA was used to determine whether there was an effect of treatment group on the spline parameters, with a Dunn's multiple comparison post hoc test.

### 2.8. Ethics

The parent clinical trial was registered with the Pan-African Clinical Trials Registry [PACTR201103000280319] and the ISRCTN Registry [ISRCTN69800930]. Ethical approval for clinical work was obtained from the Liverpool School of Tropical Medicine [Protocol (10.74)], the Malawian National Health Science Research Committee [NHSRC 916], and University Health Network Research Ethics Board (Toronto) [14-7313 Biomarkers in Pregnancy]. Written informed consent was acquired from all study participants. All experimental protocols involving animals were prepared and approved by the University Health Network Animal Care Committee [UHN ACC] (Animal Use Protocol (AUP) #3568) before study initiation. All work was done in compliance with animal husbandry standard operating procedures set out by UHN ACC.

Data availability: Detailed AUP procedures, including housing and husbandry, ethical animal care, and humane endpoints can be provided upon request. All human and animal data that support the findings of this study are available upon request from the corresponding author, Kevin C. Kain.

Role of funding source: The funders had no role in design, analysis, or reporting of these studies.

**Table 1**
Population characteristics by placental malaria status of pregnant women enrolled in a prospective cohort in Malawi.

| | Entire Cohort | Placental Malaria Negative[a] Median [IQR] or n (%) | Placental Malaria Positive[a] | *p*-value[b] |
|---|---|---|---|---|
| Baseline Characteristics | n=1392 | n=892/1392 (64.1) | n=500/1392 (35.9) | |
| Age (years) | 21 [18, 25] | 23 [20, 27] | 19 [17, 22] | <0.0001 |
| Primigravid | 484 (34.8) | 219 (24.6) | 265 (53.0) | <0.0001 |
| Gestational age at enrolment (weeks) | 20.0 [18.3, 21.7] | 20.1 [18.2, 22.0] | 20.3 [18.6, 22.3] | 0.154 |
| Socioeconomic status[c] | -1.02 [-1.49, 0.43] | -0.90 [-1.45, 0.90] | -1.19 [-1.52, -0.06] | <0.0001 |
| Treatment arm (IPTp-SP) | 688 (49.4) | 454 (50.9) | 234 (46.8) | 0.158 |
| Birth Outcomes[d] | | | | |
| Adverse live birth outcomes | 404 (29.5) | 232 (26.3) | 172 (35.3) | <0.001 |
| Fetal loss | 18 (1.3) | 7 (0.8) | 11 (2.2) | 0.046 |

[a]Placental malaria was defined by placental histology (includes past and active infection) and/or infection in the placental blood by PCR at delivery. [b]Comparing placental malaria negative and positive. [c]3 women were missing data for socioeconomic status (n=1389). [d]Adverse live birth outcomes include low birth weight, small-for-gestational age, or preterm birth; foetal loss includes miscarriage or stillbirth. 24 women were missing data for adverse live birth outcomes (n=1368); however, all women had data for foetal loss (n=1392). Abbreviations: Intermittent preventative treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP); interquartile range (IQR).

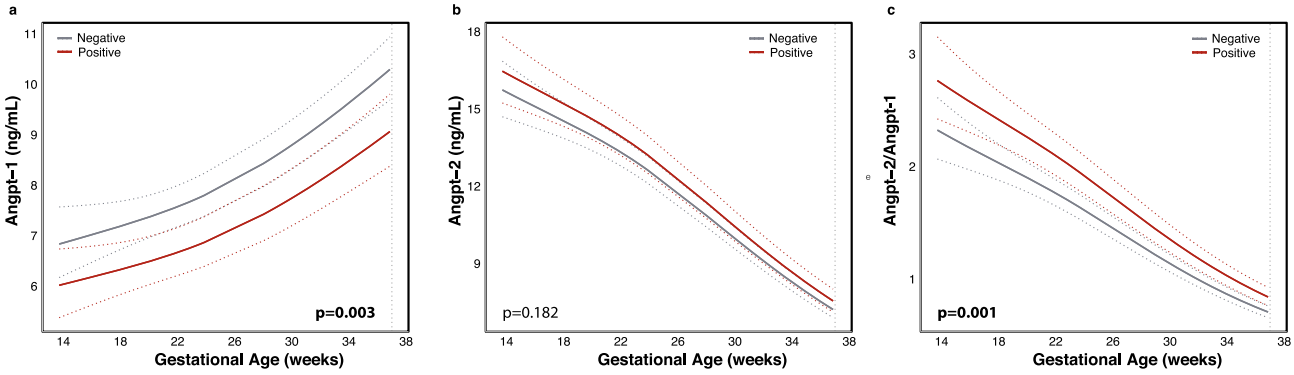


**Figure 1.** Placental malaria is associated with dysregulation of the maternal angiopoietin axis across pregnancy. (a to c) Longitudinal changes in maternal concentrations of (a) angiopoietin-1 (Angpt-1), (b) angiopoietin-2 (Angpt-2), and (c) the Angpt-2/Angpt-1 ratio across pregnancy by placental malaria status (negative or positive) in whole cohort (n=1389 subjects, n=2975 observations). Three women were not included in the analysis because of missing covariate data (socioeconomic status). Longitudinal regression lines depict linear mixed effects model-predicted, back-transformed values and 95% confidence intervals of analytes across pregnancy stratified by placental malaria status, while holding other fixed effects constant [57]. P-value overlaid on graphs determined by likelihood ratio test comparing the model with placental malaria status to the null model (model without). All models adjusted for (fixed effects) a restricted cubic spline of gestational age, maternal age and socioeconomic status at enrolment, parent trial treatment group, and a by-participant intercept as a random effect. Vertical dotted lines represent term (37 weeks gestation).

## 3. Results

### *3.1. Placental malaria is associated with adverse birth outcomes and altered Angpt-2/Angpt-1 concentrations across pregnancy*

We examined the relationships between placental malaria, birth outcomes, and circulating levels of Angpt-1 and Angpt-2 across pregnancy in a cohort of pregnant women in Malawi (n=1392). There was evidence for placental malaria in one third of the cohort (n=500, 35.9%). In 404 pregnancies (29.0%) there were adverse live birth outcomes, and in 18 (1.3%), foetal loss (Table 1).

After adjusting for demographic variables, placental malaria was associated with an increased relative risk of adverse live birth outcomes (aRR [95% CI]: 1.28 [1.07, 1.52], log-binomial regression p=0.007) and foetal loss (aRR [95% CI]: 3.20 [1.21, 8.98], log-binomial regression p=0.021) (Figure S1). Women with evidence of placental malaria also had decreased circulating Angpt-1 across pregnancy (adjusted LME model: $\beta$ [95% CI]: -0.13 [-0.21, -0.04], p=0.003) and an increased ratio of Angpt-2/Angpt-1 (adjusted LME model: $\beta$ [95% CI]: 0.17 [0.07, 0.28], p=0.001), compared to women with no evidence of placental malaria (Figure 1). Women with evidence of peripheral malaria by PCR at any point across pregnancy had a similar profile of decreased Angpt-1 (adjusted LME p=0.049; Figure S2a) and an increased Angpt-2/Angpt-1 ratio (adjusted LME p=0.024; Figure S2c) across pregnancy. We stratified the analysis to assess gravidity (primigravidae and multigravidas) as a potential effect modifier of the relationship between placental malaria and angiopoietins. In primigravidae, women with placental malaria had decreased Angpt-1 (adjusted LME, p=0.043), increased Angpt-2 (adjusted LME, p=0.040), and an increased ratio of Angpt-2/Angpt-1 (adjusted LME, p=0.006) across pregnancy (Figure 2, a-c). Multigravidity was associated with decreased Angpt-1 (adjusted LME, p=0.016) in women who had evidence of placental malaria; however, there were no differences in Angpt-2 or the Angpt-2/Angpt-1 ratio (Figure 2, d-f).

The dynamics of Angpt-1 (adjusted LME, p=0.014; Figure S3a), Angpt-2 (adjusted LME, p<0.001; Figure S3b), and the Angpt-2/Angpt-1 ratio (adjusted LME, p<0.001; Figure S3c) were also altered across pregnancies that resulted in adverse live birth outcomes, after controlling for placental malaria. These analyses revealed an interaction between gestational age and adverse birth outcomes, indicating that the association between the angiopoietins and adverse live birth outcomes varied according to gestational age (Figure S3a-c).

### *3.2. Experimental malaria in pregnancy dysregulates circulating maternal angiopoietins and mRNA expression of angiopoietins and Tie2 in the placenta*

We used a well characterized experimental model of malaria in pregnancy to investigate a potential mechanistic role for angiopoietin signalling in mediating adverse birth outcomes. This model most closely resembles infections in primigravidae. The preclini-

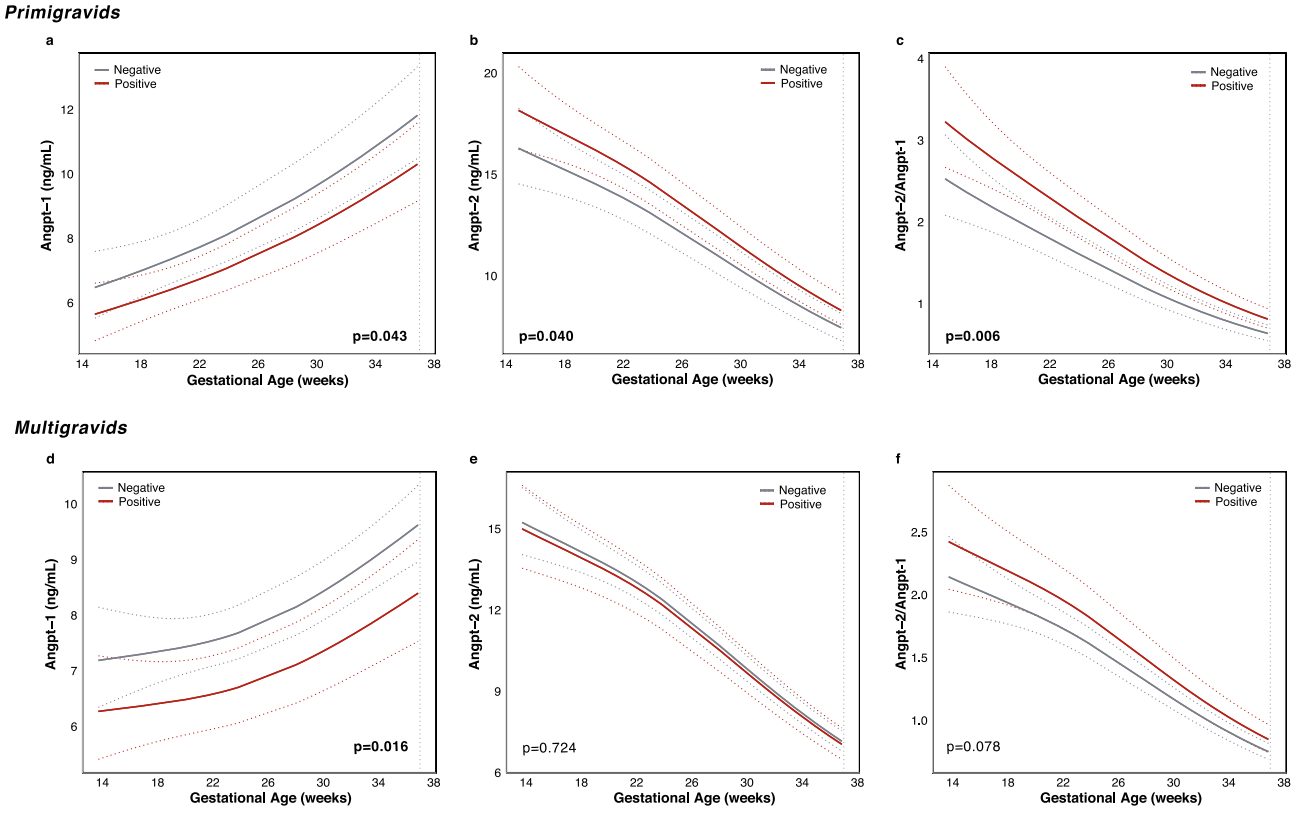


**Figure 2.** Placental malaria is differentially associated with dysregulation of the maternal angiopoietin axis across pregnancy in primigravid pregnancies and multigravid pregnancies. (a to c) Longitudinal changes in maternal concentrations of angiopoietin-1 (Angpt-1), angiopoietin-2 (Angpt-2), and the Angpt-2/Angpt-1 ratio across pregnancy in by placental malaria status (negative or positive) in primigravid (n=481 subjects; n=1006 observations) and (d to f) multigravid pregnancies (n=908 subjects; n=1969 observations). Three women were not included in the analysis because of missing covariate data (socioeconomic status). Longitudinal regression lines depict linear mixed effects model-predicted, back-transformed values and 95% confidence intervals of analytes across pregnancy stratified by placental malaria status, while holding other fixed effects constant [57]. P-value overlaid on graphs determined by likelihood ratio test comparing the model with placental malaria status to the null model (model without). All models adjusted for (fixed effects) a restricted cubic spline of gestational age, maternal age and socioeconomic status at enrolment, parent trial treatment group, and a by-participant intercept as a random effect. Vertical dotted lines represent term (37 weeks gestation).

cal model of malaria emulated the pathology of human malaria in pregnancy, including reduced foetal weight (multiple Mann-Whitney with Holm Sidak correction, $p<0.0001$; Figure 3a) and viability (Fisher's exact test, $p<0.0001$; 3b). An assessment of experimental malaria pathology over the course of pregnancy showed a reduction in foetal weight in *P. berghei*-infected pregnant mice starting at G18 (multiple Mann-Whitney with Holm Sidak correction, $p<0.001$; Figure 3a). This coincided with a reduction in maternal plasma Angpt-1 and an increase in maternal plasma Angpt-2 and the Angpt-2/Angpt-1 ratio (two-way ANOVA with Bonferroni's multiple comparisons, $p<0.01$; Figure 3c, 3e, and 3g), which recapitulates the longitudinal clinical findings in primigravidae. Both foetal growth restriction and increased levels of Angpt-2 were preceded by a significant reduction in circulating Angpt-1 starting at G16 in infected dams (two-way ANOVA with Bonferroni's multiple comparisons; $p<0.05$; Figure 3c).

In placental tissue, Angpt-1 mRNA was significantly reduced (two-way ANOVA with Bonferroni's multiple comparisons, $p<0.01$; Figure 3d) and the Angpt-2/Angpt-1 ratio was increased (two-way ANOVA with Bonferroni's multiple comparisons, $p<0.05$; Figure 3h) in *P. berghei*-infected dams compared to uninfected dams at G18. Placental tissue expression of the Angpt-1 receptor Tie2, was also significantly reduced in *P. berghei*-infected dams compared to uninfected dams (one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$; Figure 4g). Ang-2 mRNA expression in placental tissue was similar between the two groups regardless of gestational timing (Figure 3f). These experimental findings support the human clinical data indicating disruption of systemic angiopoietins by malaria during pregnancy and further indicate that angiopoietin signalling pathways at the placenta may also be disrupted. Given the temporal relationship between the reduction in circulating levels of Angpt-1, increased Angpt-2, and subsequent foetal growth restriction in the preclinical model, we further investigated the role of Angpt-1 by genetic disruption.

### 3.3. *PbA-infection of* Angpt1$^{+/-}$ *heterozygous dams worsens foetal weight and viability*

Total knockout of *Angpt1* is embryonic lethal [11]; therefore, we used a heterozygous *Angpt1*$^{+/-}$ mouse line, which has reduced Angpt-1 expression but maintains normal fecundity and birth outcomes in the absence of an infectious challenge (Figure 4d and Figure 5). We tested the hypothesis that reduced maternal Angpt-1 in the context of *P. berghei* infection would further exacerbate adverse birth outcomes. Levels of maternal plasma Angpt-1 (Kruskall-Wallis with Dunn's multiple comparisons, n.s.; Figure 4a) and Angpt-1 mRNA (one way ANOVA with Tukey's multiple comparisons, $p=0.046$; Figure 4d) in the placenta were decreased by ~50% in uninfected *Angpt1*$^{+/-}$ heterozygous dams compared to wildtype, while levels of Angpt-2 remained similar (Figure 4b and 4e). *P. berghei* infection resulted in decreased circulating Angpt-1 concentrations and increased the Angpt-2/Angpt-1 ratio compared to uninfected dams, irrespective of genotype (Kruskall-Wallis with Dunn's multiple comparisons, $p<0.01$, Figure 4a; and one-way ANOVA with Tukey's multiple comparisons, $p<0.001$, Figure 4c). While wildtype-infected mice showed a significant in-

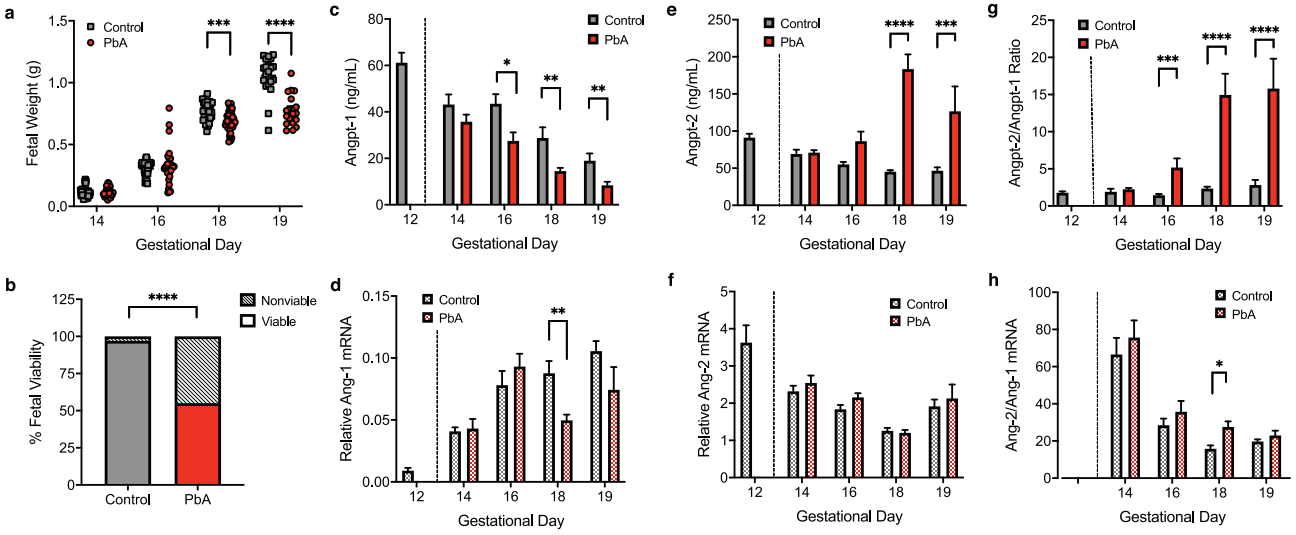


**Figure 3.** Experimental malaria in pregnancy recapitulates human birth phenotypes and dysregulation of angiopoietins across pregnancy. (a) Fetal weight (in grams) across pregnancy in dams injected with RPMI vehicle control or *Plasmodium berghei* (PbA) (***p<0.001, ****p<0.0001, multiple Mann-Whitney tests with Holm-Sidak correction for multiple comparisons; n=17-40 fetuses per group per gestational day (n=5-10 pregnant mice per group per gestational day); n=2 experimental replicates). (b) Fetal viability (as percentage of total fetuses) at gestational day 19 in dams injected with vehicle control or PbA (****p<0.0001, Fisher's exact test; n=62-71 per group; n=3 experimental replicates). (c, e, g) Maternal plasma concentrations of Angpt-1 (c) and Angpt-2 (e) across pregnancy measured by enzyme-linked immunosorbent assay (ELISA) in dams injected with vehicle control or PbA. Ratio of Angpt-2/Angpt-1 presented in (g). Dotted lines represent exposure group injection on gestational day 13. Bars depict mean and SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Bonferroni's multiple comparisons test; n=5-9 dams per group per gestational day; n=2 experimental replicates). (d, f, h) Maternal expression of Angpt-1 (d) and Angpt-2 (f) mRNA in the placenta measured by qRT-PCR in dams injected with vehicle control or PbA. Ratio of Angpt-2/Angpt-1 presented in (h). Dotted lines represent exposure group injection on gestational day 13. Bars depict mean and SEM (**p<0.01, two-way ANOVA with Bonferroni's multiple comparisons test; n=6-20 placentas per group per gestational day; n=2 experimental replicates).

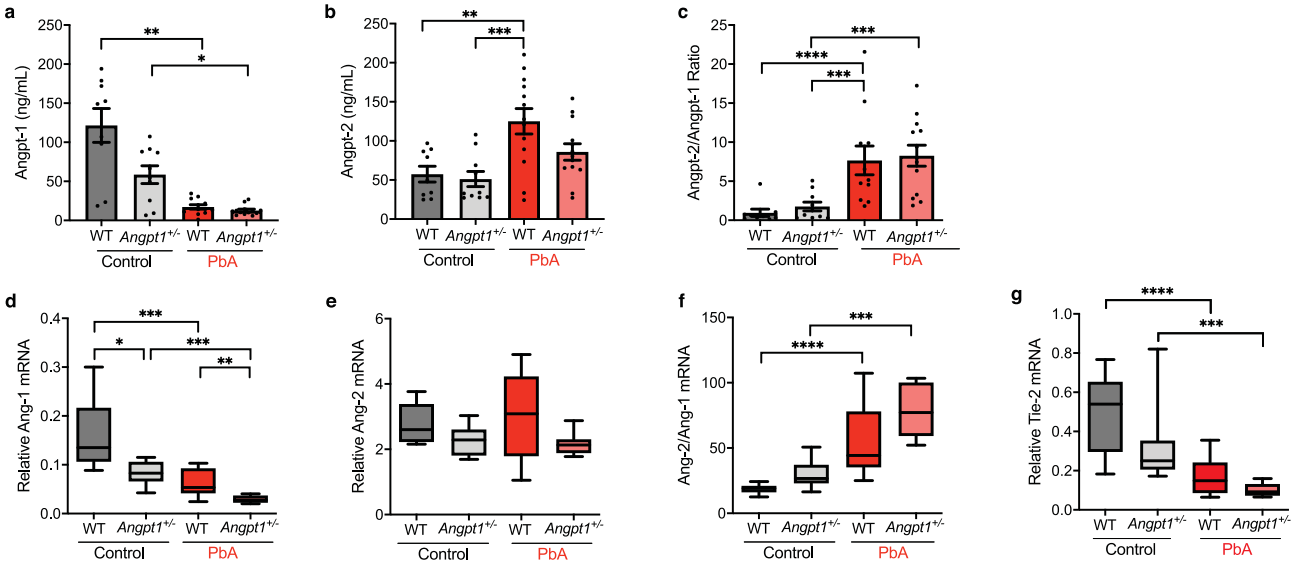


**Figure 4.** Circulating protein concentrations and placental mRNA expression of angiopoietins are altered with *P. berghei* ANKA infection of wild type dams and further exacerbated in infected *Angpt1*$^{+/-}$ heterozygous dams. (a to c) Maternal plasma levels of Angpt-1 (a) and Angpt-2 (b) measured by enzyme-linked immunosorbent assay (ELISA) at gestational day 19 in wildtype or *Angpt1*$^{+/-}$ heterozygous dams injected with vehicle control or *Plasmodium berghei* ANKA (PbA). Ratio of Angpt-2/Angpt-1 is presented in (c). One Angpt-2/Angpt-1 ratio value was included in the analysis (see Methods for handling of outliers) but excluded from the visual depiction for reasons of scale (value = 144). Bars represent mean and SEM. (*p<0.05, **p<0.01, ****p<0.0001, Kruskal-Wallis test with Dunn's multiple comparisons or one-way ANOVA with Tukey's multiple comparisons test; n=9-13 dams per group; n=3 experimental replicates). (d to g) Maternal expression of Angpt-1 (d), Angpt-2 (e), and Tie2 (g) mRNA in the placenta measured by qRT-PCR at gestational day 19 in wildtype or *Angpt1*$^{+/-}$ dams injected with vehicle control or PbA. Ratio of Angpt-2/Angpt-1 presented in (f). Boxplots depict median and interquartile range with min/max whiskers. (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons; n=6-12 placentas per group (n=3-7 pregnant mice) from a representative experiment).

crease in maternal plasma Angpt-2, infected *Angpt1*$^{+/-}$ dams did not (Figure 4b). In the placenta, the ratio of Angpt-2/Angpt-1 mRNA expression trended towards highest in *P. berghei*-infected *Angpt1*$^{+/-}$ dams (one-way ANOVA with Tukey's multiple comparisons, p=0.052; Figure 4f). This imbalance appeared to result from a significant decrease in placental expression of Angpt-1 mRNA in infected *Angpt1*$^{+/-}$ dams (one-way ANOVA with Tukey's multiple comparisons, p<0.01; Figure 4d). Tie2 mRNA expression in the placenta was significantly reduced in response to *P. berghei*-infection, regardless of maternal genotype (one-way ANOVA with Tukey's multiple comparisons, p<0.001; Figure 4g).

Phenotypically, greater dysregulation of the angiopoietin axis in the context of *P. berghei* infection was associated with worsened birth outcomes. *P. berghei* infection in the heterozygous dams

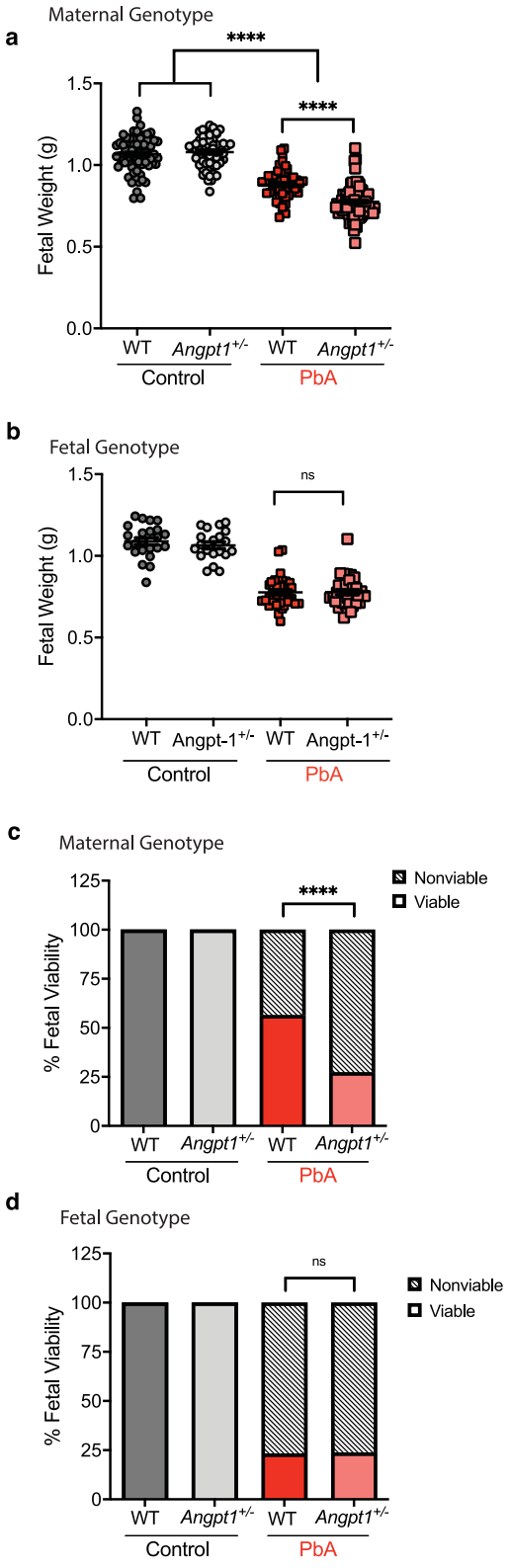


**Figure 5.** *P. berghei* infection exacerbates adverse birth outcomes in *Angpt1*$^{+/-}$ heterozygous dams. (a) Fetal weight (in grams) at gestational day 19 from wildtype or *Angpt1*$^{+/-}$ dams injected with RPMI vehicle control or *Plasmodium berghei* ANKA (PbA) (****$p<0.0001$, two-way ANOVA with Tukey's multiple comparisons test; n=56-86 fetuses per group (n=10-13 pregnant mice); from n=3 experimental replicates). (b) Fetal weight (in grams) at gestational day 19 by fetal genotype (from *Angpt1*$^{+/-}$ maternal background) and exposure group (not significant (ns), two-way ANOVA with Tukey's multiple comparisons test; n=20-37 fetuses per group (n=6-10 pregnant mice); n=2 experimental replicates). (c) Fetal viability (as percentage of total fetuses) at gestational day 19 by maternal genotype (wildtype or *Angpt1*$^{+/-}$) and exposure group (***$p<0.001$, Fisher's exact test; n=62-88 fetuses per group (n=10-14 pregnant mice); from n=3 experimental replicates). (d) Fetal viability (as percentage of total fetuses) at gestational day 19 by fetal genotype (from *Angpt1*$^{+/-}$ maternal background) and exposure group (not significant (ns), Fisher's exact test; n=30-42 fetuses per group (n=6-10 pregnant mice); n=2 experimental replicates).

(*Angpt1*$^{+/-}$) led to decreased foetal growth and viability (two-way ANOVA with Tukey's Multiple comparisons test, $p<0.0001$, Figure 5a; and Fisher's exact test, $p<0.0001$, Figure 5c). This effect was driven by the maternal genotype as there was no difference in weight or viability between wildtype and *Angpt1*$^{+/-}$ foetuses in the context of *P. berghei* infection (Figure 5b and 5d).

### *3.4. Maternal Angpt-1 is involved in placental vascular development and function in experimental malaria in pregnancy*

We have previously shown that compensatory placental vascular remodelling occurs in the context of placental dysfunction (e.g., increased arterial resistance) during *P. berghei* infection [3,4]. Several therapeutic approaches that have improved birth outcomes in experimental malaria in pregnancy, including C5a-C5aR signalling blockade and L-arginine supplementation, have done so by further increasing vascular development to compensate for malaria-mediated placental dysfunction [3,4]. We hypothesized that the observed deficiency in circulating Angpt-1 was worsening birth outcomes by inhibiting compensatory placental vascular remodelling in the opposite manner to C5a-C5aR blockade or L-arginine. To test this hypothesis, we used micro-CT to image placental vasculature in wildtype and *Angpt1*$^{+/-}$ infected and uninfected pregnant mice (Figure 6c, Figure S4). As reported [3,4], wildtype *P. berghei*-infected dams had an increased total number of placental vessel segments, driven by an increase in small vessel segments (35-75 $\mu$m) compared to uninfected mice (two-way ANOVA with Dunn's multiple comparison post-hoc test, $p<0.001$, Figure 6a; and Figures S5a and S5b). The compensatory increase in placental vasculature characteristic of *P. berghei* infection was abrogated in *Angpt1*$^{+/-}$ dams (Figure 6a and Figure S5A and S5B). Regardless of infection, *Angpt1*$^{+/-}$ placentas had fewer large vessel segments (>200 $\mu$m) (Figure S5d), a phenotype consistent with previous research showing that Angpt-1 promotes large vessel size [15,34]. A functional readout of placental vasculature showed that infection elevated placental arterial resistance (two-way ANOVA with Tukey's multiple comparisons, $p<0.01$; Figure 6b). Genetic Angpt-1 deficiency exacerbated the effect of infection, with the highest arterial resistance observed in infected *Angpt1*$^{+/-}$ mice; however, the increase did not reach significance (Figure 6b). Collectively, our preclinical data support a hypothesis whereby experimental malaria in pregnancy induces a reduction in maternal Angpt-1 resulting in disrupted placental vascular remodelling required for optimal foetal growth and survival.

## 4. Discussion

Dysregulation of the angiopoietin-Tie2 axis contributes to the pathogenesis of severe infections and host response to infection, including sepsis, severe bacterial infections, and non-pregnancy severe malaria [35]. Several cross-sectional studies have shown dysregulation of Angpt-1 and Angpt-2 at delivery in women with antenatal malaria [3,5,24,25]. Here, we provide the first mechanistic evidence that implicates this axis in the pathogenesis of adverse birth outcomes associated with malaria during pregnancy. We show that Angpt-1 and the Angpt-2/Angpt-1 ratio are perturbed across pregnancy in women with evidence of placental malaria, as well as in pregnancies resulting in adverse live birth outcomes. We confirm these observations in a preclinical model of malaria in pregnancy and provide direct experimental evidence for a synergistic impact of *P. berghei* infection and reduced circulating Angpt-1 levels on foetal weight and viability. Finally, we show that Angpt-1 may play a protective role in placental adaptations to malaria in pregnancy, and that when it is depleted, compensatory vascular remodelling mechanisms are impaired and adverse birth outcomes increase.

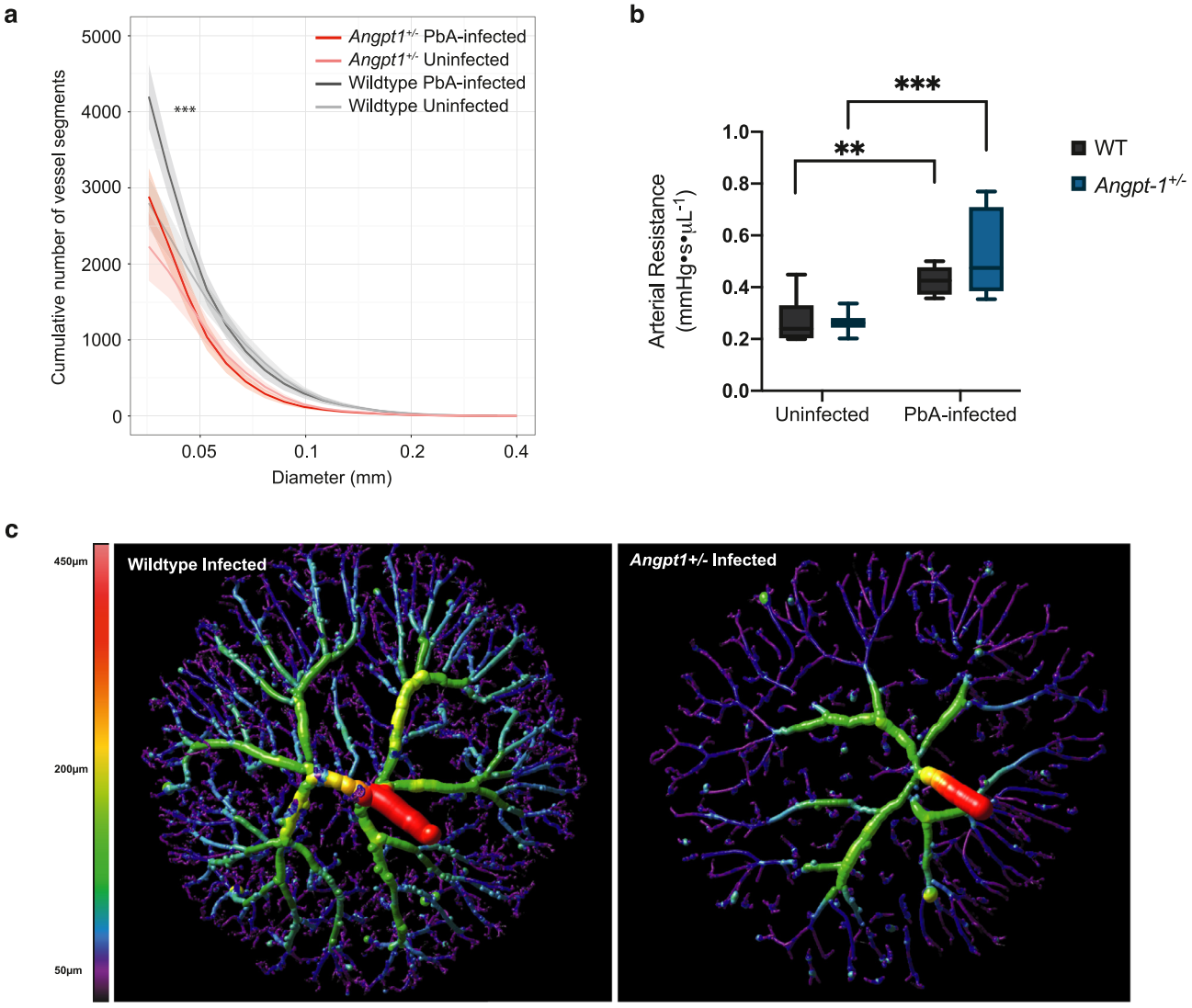


**Figure 6.** Angpt-1 is required for *P. berghei* mediated compensatory placental vascular remodeling. (a) Cumulative distribution of placental vessels by vessel diameter from wildtype (gray lines) or *Angpt1*$^{+/-}$ (red lines) *Plasmodium berghei* ANKA (PbA) infected or uninfected dams. Lines represent mean and SEM of vessels greater than the threshold diameter (>35µm) (***p<0.001; two-way ANOVA of spline parameters with Dunn's multiple comparisons post-hoc test; n=7-8 placentas per group (n=3-5 pregnant mice); n=2 experimental replicates). (b) Arterial resistance in placentas from uninfected and PbA-infected, wildtype or *Angpt1*$^{+/-}$, dams. Boxplots represent median and interquartile range with min/max whiskers. (**p<0.01, ***p<0.001; two-way ANOVA with Tukey's multiple comparisons test; n=6-7 placentas per group (n=3-4 pregnant mice); n=2 experimental replicates). (c) Representative micro-CT imaging of placentas at gestational day 18 from wildtype and *Angpt1*$^{+/-}$ PbA-infected dams, color-coded by vessel diameter. Visual comparison to uninfected controls can be found in Figure S4.

Angpt-1, Angpt-2, and Tie2 are expressed in the placenta early in development and are necessary for embryonic, or in the case of Angpt-2, early postnatal survival [11,14,36,37]. On endothelial cells, Angpt-1 and Angpt-2 act primarily as agonist and antagonist, respectively, to their receptor Tie2 [38]. Increasing concentrations of Angpt-2 block the activity of Angpt-1, highlighting the importance of the Angpt-2/Angpt-1 ratio in the angiopoietin-Tie2 signalling axis [38]. Biologically, Angpt-1 stabilizes newly formed vessels by promoting interactions between endothelial cells and the surrounding extracellular matrix and supporting cells [11]. Angpt-2 can block this activity, leading to either increased vascular remodelling and growth or vascular regression depending on the presence or absence of other factors including vascular endothelial growth factor (VEGF) [38,39]. In a healthy pregnancy, placental expression of Angpt-1 increases as vasculature becomes more established across pregnancy, while Angpt-2 and Tie2 decrease [12]. Perturbations to the longitudinal dynamics of the angiopoietin signalling axis are associated with adverse pregnancy outcomes, including preeclampsia and foetal growth restriction [12,17,40,41]. Our clinical data showed decreased Angpt-1 and an increased Angpt-2/Angpt-1 ratio across pregnancy in women with evidence of placental malaria. In primigravid pregnancies, Angpt-2 was also significantly elevated across gestation in women with evidence of placental malaria. In pregnancies resulting in adverse live birth outcomes, the Angpt-2/Angpt-1 ratio was significantly lower in the late third trimester compared to pregnancies with normal birth outcomes. This pattern has been seen in other pathologic pregnancies, including pregnancies complicated by preeclampsia [18]. Given that there is a higher rate of adverse birth outcomes in pregnancies complicated with malaria compared to those that are not, we hypothesize that reducing Angpt-2 and increasing Angpt-1 may be a late compensation mechanism in pathologic pregnancies, to stabilize placental vasculature and mitigate adverse birth outcomes. However, in the context of malaria in pregnancy our clinical and preclinical data indicate that a high Angpt-2/Angpt-1 ratio

is maintained, preventing compensation, and resulting in increased adverse birth outcomes compared to non-malarial pregnancies.

Our preclinical experiments mimic our human cohort data in terms of decreased circulating Angpt-1, increased Angpt-2 (in primigravid pregnancies), and an increased peripheral plasma Angpt-2/Angpt-1 ratio in response to malaria in pregnancy. While the dysregulation of circulating Angpt-1 and the Angpt-2/Angpt-1 ratio were mirrored by their placental tissue mRNA expression, the dynamics of Angpt-2 alone were not. Circulating Angpt-2 was increased in response to *P. berghei* infection, but placental mRNA expression remained unchanged. This discrepancy is supported by data from non-malarial pathologic pregnancies, including foetal growth restriction and preeclampsia, which describe differences between circulating maternal angiopoietins and placental mRNA levels [18]. In the context of experimental malaria in pregnancy, the mismatch between circulating levels versus placental Angpt-2 transcript levels may not be due to placental mRNA per se but could be due to systemic endothelial cell activation caused by malaria and associated inflammation in the maternal circulation, as well as release of preformed Angpt-2 from Weibel-Palade bodies in the endothelium of the placental vasculature [35]. Placental expression of Tie2 was also significantly reduced in our *P. berghei*-infected dams. Decreased expression of Tie2 would be expected to reduce Tie2 signalling and compromise placental endothelial function, even in the presence of Angpt-1 [42]. A critical role for Angpt-1 over Angpt-2 in this mechanism is supported by data from preclinical models of severe malaria, which show that despite high levels of circulating Angpt-2, its inhibition did not improve outcomes [19]. Conversely, genetic and pharmacological strategies established Angpt-1 as necessary for survival and maintaining endothelial integrity at the blood-brain barrier [19].

Aberrant placental vascular development and remodelling, resulting in placental dysfunction and insufficiency, has emerged as a key mediator of adverse birth outcomes in the context of maternal infections including malaria [3,4,6,9]. Inflammatory and angiogenic pathways, and the interactions between them, have essential physiologic roles in healthy placental and foetal development, and their combined dysregulation in pathologic pregnancies and malaria in pregnancy is well-documented [2,3,43-47]. Evidence from malaria in pregnancy, as well as other infections and pathologies, suggests a relationship between complement dysregulation (e.g., excessive C5a) and the angiopoietin family [3,43,48]. In pregnant women with *P. falciparum* infection, structural equation modelling provided a statistical hierarchical relationship between C5a and the angiopoietins, suggesting C5a was an upstream initiator, reducing Angpt-1 levels to ultimately mediate foetal growth restriction [3]. Future studies should directly investigate the putative hierarchical relationship between C5a and Angpt-1 in malaria in pregnancy to further elucidate targetable molecular pathways in the pathogenesis of adverse birth outcomes.

Collectively, our clinical and preclinical data, along with pre-existing data, support the idea that the angiopoietin-Tie2 axis could be a modifiable pathway to improve birth outcomes in malaria in pregnancy [49]. There is precedent from other severe malaria outcomes that are mediated through the angiopoietin-Tie2 pathway, where therapeutic administration of recombinant Angpt-1 improved survival and the integrity of the blood brain barrier in a preclinical model of cerebral malaria [19]. However, the high cost of pharmaceutical recombinant proteins and monoclonal antibodies [50] impedes their use in low-resource settings, where the burden of malaria is highest. Furthermore, without also rectifying malaria-induced reductions in Tie2 expression in the placenta, administering recombinant Angpt-1 may not be effective [42]. As an alternative, we have shown that dietary supplementation with L-arginine, a precursor to nitric oxide, increases placental expression of both Angpt-1 and Tie2 in malaria in pregnancy [4]. In the context of experimental malaria in pregnancy, L-arginine supplementation improved foetal weight and viability and did so by a compensatory increase in placental vasculature [4]. L-arginine is an inexpensive intervention with a known safety profile in pregnancy, and evidence for its capacity to modulate several pathways relevant to the pathogenesis of adverse birth outcomes in malaria in pregnancy, including the angiopoietin-Tie2 pathway [4,49,51].

### 4.1. Caveats and Limitations

This study presents a novel longitudinal assessment of angiopoietin dynamics across pregnancies complicated by *Plasmodium falciparum* infection. Our findings are strengthened by the combination of human data and preclinical mechanistic data to support a clinically relevant hypothesis. Dysregulation of the angiopoietins across gestation was common to both placental malaria and adverse birth outcomes in the human cohort, yet these data are associative and do not establish causality. Using a preclinical mouse model of malaria in pregnancy enables more mechanistic investigations that would be challenging to conduct for ethical and logistical reasons in human populations. Although there are differences between the mouse and the human placenta, there are also structural and molecular similarities, especially in the context of vascular development and function, including high concordance in proteomic and transcriptomic profiles [52–54]. Importantly, Angpt-1 and Angpt-2 sequences are almost identical between mice and humans [30,55], and there is strong precedent for their analogous roles in humans and mice in both physiological processes and disease pathogenesis [19,27,56]. However, there are limitations in the translation from human pregnancy to an experimental model of malaria in pregnancy. The experimental model uses malaria-naïve, nulliparous mice. This most closely simulates infections in nonimmune primigravidae (for whom the human data most closely matched the preclinical data), and results in higher parasitaemia and more severe acute disease than would be expected in human pregnancies. This could in part explain discrepancies in angiopoietin-Tie2 profiles between mouse and human data (i.e., greater effect of malaria on Angpt-2 in the mouse model). Furthermore, our data revealed a complex relationship between systemic angiopoietins and placental Angpt-Tie2 signalling, and their relative importance during malaria in pregnancy. In the preclinical genetic model of malaria, systemic levels of Angpt-1 and -2 did not explain the synergistic effect of *Angpt1*$^{+/-}$ genotype and PbA infection on adverse birth outcomes (worst outcomes in PbA-infected *Angpt1*$^{+/-}$ mice). Differences in placental Angpt-1 mRNA expression and placental vasculature in infected *Angpt1*$^{+/-}$ dams better mirrored the exacerbated birth outcomes; however, the precise mechanistic pathway requires further investigation to fully elucidate. While our clinical data would be strengthened by a matching assessment of human placental Tie2 and Angpt-2/Angpt-1 expression, collecting placental samples in a large cohort of women in a low-income setting would be difficult. The preclinical model allowed us to more feasibly establish a link between malaria during pregnancy, systemic, and placental dysregulation of the angiopoietins, and provided further advantages including the ability to conduct micro-CT analysis of the full placental vascular tree, which would not have been possible in the clinical study.

Despite the limitations with directly comparing human and animal studies, the data presented here provide preliminary evidence for a mechanistic link between malaria-associated changes in the angiopoietin-Tie2 axis, alterations in placental vascular development and remodelling, and adverse birth outcomes. Previous cross-sectional studies in *P. falciparum*-infected pregnancies showed disruptions to the angiopoietins at delivery, at which point intervention is futile. Here, we combined clinical and preclinical data to provide a more comprehensive picture of the disruption of Angpt-1

and Angpt-2 homeostasis across gestation and its subsequent impact on placental vascular development and birth outcomes.

## 5. Contributors

AMW, VT, JGS, KCK conceived and designed preclinical experiments. AMW, VT, VC, LSC, KZ, AC, REE performed preclinical experiments and clinical analyte measurement. AMW, VT, VC, LSC, VP analysed the data. AMW, VT, VC, REE, LSC verified the underlying data. AMW, VT, KCK wrote the original manuscript. MM, ALC, LKP, CK, VM, FOK designed, funded, and ran the parent clinical trial from which the clinical data was derived. All authors reviewed and edited the final version of the manuscript.

## Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

## Acknowledgements

This study would not have been possible without all the mothers who enrolled in the trial, and the excellent field team in Malawi. This work was supported by the Canadian Institutes of Health Research (CIHR) Foundation Grant (FDN-148439) [KCK], Canada Research Chair [KCK], Toronto General Research Institute Postdoctoral Fellowship Award [VT], and CIHR Doctoral Award (GSD-157907) [AMW]. The parent trial was supported by the European & Developing Countries Clinical Trials Partnership (Award Number IP.2007.31080.003) [FOtK] and the Malaria in Pregnancy Consortium, which was funded by the Bill & Melinda Gates Foundation to the Liverpool School of Tropical Medicine (Award Number 46099) [FOtK]. The funders had no role in design, analysis, or reporting of these studies.

## Data Sharing Statement

All human and animal data that support the findings of this study are available upon request from the corresponding author, Kevin C. Kain.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103683.

## References

[1] Rogerson SJ, Desai M, Mayor A, Sicuri E, Taylor SM, van Eijk AM. Burden, pathology, and costs of malaria in pregnancy: new developments for an old problem. Lancet Infect Dis 2018;18(4):e107–3118.
[2] Elphinstone RE, Weckman AM, McDonald CR, Tran V, Zhong K, Madanitsa M, et al. Early malaria infection, dysregulation of angiogenesis, metabolism and inflammation across pregnancy, and risk of preterm birth in Malawi: A cohort study. PLoS Med 2019;16(10):e1002914.
[3] Conroy AL, Silver KL, Zhong K, Rennie M, Ward P, Sarma JV, et al. Complement activation and the resulting placental vascular insufficiency drives fetal growth restriction associated with placental malaria. Cell Host Microbe 2013;13(2):215–26.
[4] McDonald CR, Cahill LS, Gamble JL, Elphinstone R, Gazdzinski LM, Zhong KJY, et al. Malaria in pregnancy alters l-arginine bioavailability and placental vascular development. Sci Transl Med 2018;10(431):eaan6007.
[5] Silver KL, Zhong K, Leke RG, Taylor DW, Kain KC. Dysregulation of angiopoietins is associated with placental malaria and low birth weight. PLoS One 2010;5(3):e9481.
[6] Moeller SL, Nyengaard JR, Larsen LG, Nielsen K, Bygbjerg IC, Msemo OA, et al. Malaria in early pregnancy and the development of the placental vasculature. J Infect Dis 2019;220(9):1425–34.
[7] Fiedler U, Augustin HG. Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol 2006;27(12):552–8.
[8] Naldini A, Carraro F. Role of inflammatory mediators in angiogenesis. Curr. Drug Targets Inflamm Allergy. 2005;4(1):3–8.
[9] Weckman AM, Ngai M, Wright J, McDonald CR, Kain KC. The impact of infection in pregnancy on placental vascular development and adverse birth outcomes. Front Microbiol 2019;10:1924.
[10] Dunk C, Shams M, Nijjar S, Rhaman M, Qiu Y, Bussolati B, et al. Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development. Am J Pathol 2000;156:2185–99.
[11] Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996;87:1171–80.
[12] Geva E, Ginzinger DG, Zaloudek CJ, Moore DH, Byrne A, Jaffe RB. Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2. J Clin Endocrinol Metab 2002;87:4213–24.
[13] De A, Maulik D, Lankachandra K, Mundy DC, Ye SQ, Gerkovich MM. Fetoplacental regional variations in the expression of angiopoietin-1, angiopoietin-2, and Tie2 in normal-term and near-term pregnancies. J Matern Fetal Neonatal Med 2016;29(21):3421–8.
[14] Seval Y, Sati L, Celik-Ozenci C, Taskin O, Demir R. The distribution of angiopoietin-1, angiopoietin-2 and their receptors tie-1 and tie-2 in the very early human placenta. Placenta 2008;29:809–15.
[15] Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000;407:242–8.
[16] Kappou D, Sifakis S, Androutsopoulos V, Konstantinidou A, Spandidos DA, Papantoniou N. Placental mRNA expression of angiopoietins (Ang)-1, Ang-2 and their receptor Tie-2 is altered in pregnancies complicated by preeclampsia. Placenta 2014;35:718–23.
[17] Leinonen E, Wathén KA, Alfthan H, Ylikorkala O, Andersson S, Stenman UH, et al. Maternal serum angiopoietin-1 and -2 and tie-2 in early pregnancy ending in preeclampsia or intrauterine growth retardation. J Clin Endocrinol Metab 2010;95:126–33.
[18] Umapathy A, Chamley LW, James JL. Reconciling the distinct roles of angiogenic/anti-angiogenic factors in the placenta and maternal circulation of normal and pathological pregnancies. Angiogenesis 2020;23:105–17.
[19] Higgins SJ, Purcell LA, Silver KL, Tran V, Crowley V, Hawkes M, et al. Dysregulation of angiopoietin-1 plays a mechanistic role in the pathogenesis of cerebral malaria. Science Transl Med 2016;8 358ra128358ra128.
[20] Yeo TW, Lampah DA, Gitawati R, Tjitra E, Kenangalem E, Piera K, et al. Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe falciparum malaria. Proc Natl Acad Sci USA 2008;105(44):17097–102.
[21] Lovegrove FE, Tangpukdee N, Opoka RO, Lafferty EI, Rajwans N, Hawkes M, et al. Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children. PLoS ONE 2009;4:e4912.
[22] de Jong GM, Slager JJ, Verbon A, van Hellemond JJ, van Genderen PJ. Systematic review of the role of angiopoietin-1 and angiopoietin-2 in *Plasmodium* species infections: biomarkers or therapeutic targets? Malar. J. 2016;15:581.
[23] Petersen JE, Mkumbaye SI, Vaaben AV, Manjurano A, Lyimo E, Kavishe RA, et al. Plasma Ang2 and ADAM17 levels are elevated during clinical malaria; Ang2 level correlates with severity and expression of EPCR-binding PfEMP1. Sci Rep 2016;6:35950. doi:10.1038/srep35950.
[24] Ataíde R, Murillo O, Dombrowski JG, Souza RM, Lima FA, Lima GF, et al. Malaria in pregnancy interacts with and alters the angiogenic profiles of the placenta. PLoS Negl Trop Dis 2015;9:e0003824.
[25] Singh PP, Bhandari S, Sharma RK, Singh N, Bharti PK. Association of angiopoietin dysregulation in placental malaria with adverse birth outcomes. Disease Markers 2020;2020:6163487.
[26] Madanitsa M, Kalilani L, Mwapasa V, van Eijk AM, Khairallah C, Ali D, et al. Scheduled Intermittent Screening with Rapid Diagnostic Tests and Treatment with Dihydroartemisinin-Piperaquine versus Intermittent Preventive Therapy with Sulfadoxine-Pyrimethamine for Malaria in Pregnancy in Malawi: An Open-Label Randomized Controlled Trial. PLoS Med 2016;13(9):e1002124.
[27] Schmiegelow C, Scheike T, Oesterholt M, Minja D, Pehrson C, Magistrado P, et al. Development of a fetal weight chart using serial trans-abdominal ultrasound in an East African population: a longitudinal observational study. PLoS One 2012;7(9):e44773.
[28] Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw 2015;67:1–48.
[29] Harrell Jr. FE. rms: Regression Modeling Strategies. R Package Version 5.1–3.1 [software]. 2019. https://CRAN.R-project.org/package=rms. [cited 2019 Jun 4]
[30] Neres R, Marinho CR, Gonçalves LA, Catarino MB. Penha-Gonçalves C. Pregnancy outcome and placenta pathology in Plasmodium berghei ANKA infected mice reproduce the pathogenesis of severe malaria in pregnant women. PLoS One 2008;3(2):e1608.
[31] Rennie MY, Detmar J, Whiteley KJ, Yang J, Jurisicova A, Adamson SL, et al. Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons. Am J Physiol Heart Circ Physiol 2011;300:H675–84.
[32] Rennie MY, Detmar J, Whiteley KJ, Jurisicova A, Adamson SL, Sled JG. Expansion of the fetoplacental vasculature in late gestation is strain dependent in mice. Am J Physiol Heart Circ Physiol 2012;302:H1261–73.
[33] Pries AR, Secomb TW. Rheology of the microcirculation. Clin Hemorheol Microcirc 2003;29:143–8.
[34] Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH,

et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282(5388):468–71.
[35] Leligdowicz A, Richard-Greenblatt M, Wright J, Crowley VM, Kain KC. Endothelial activation: The Ang/Tie axis in sepsis. Front Immunol 2018;9:838.
[36] Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron–Maguire M, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376:70–4.
[37] Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, et al. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Dev Cell 2002;3:411–23.
[38] Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277(5322):55–60.
[39] Lobov IB, Brooks PC, Lang RA. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci USA 2002;99(17):11205–10.
[40] Zhang EG, Smith SK, Baker PN, DS Charnock-Jones, et al. The regulation and localization of angiopoietin-1, -2, and their receptor Tie2 in normal and pathologic human placentae. Mol Med 2001;7(9):624–35.
[41] Bolin M, Wiberg-Itzel E, Wikström AK, Goop M, Larsson A, Olovsson M, et al. Angiopoietin-1/angiopoietin-2 ratio for prediction of preeclampsia. Am J Hypertens 2009;2(8):891–5.
[42] Ghosh CC, David S, Zhang R, Berghelli A, Milam K, Higgins SJ, et al. Gene control of tyrosine kinase TIE2 and vascular manifestations of infections. Proc Natl Acad Sci USA 2016;113(9):2472–7.
[43] f G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. J. Exp. Med. 2006;203:2165–75.
[44] McDonald CR, Darling AM, Conroy AL, Tran V, Cabrera A, et al. Inflammatory and angiogenic factors at mid-pregnancy are associated with spontaneous preterm birth in a cohort of Tanzanian women. PLoS One 2015;10:e0134619.
[45] Darling AM, McDonald CR, Conroy AL, Hayford KT, Liles WC, et al. Angiogenic and inflammatory biomarkers in midpregnancy and small-for-gestational-age outcomes in Tanzania. Am J Obstet Gynecol 2014;211:509 .e1–509.e8.
[46] Umbers AJ, Aitken EH, Rogerson SJ. Malaria in pregnancy: small babies, big problem. Trends Parasitol 2011;27(4):168–75.
[47] Conroy A, Serghides L, Finney C, Owino SO, Kumar S, et al. C5a enhances dysregulated inflammatory and angiogenic responses to malaria in vitro: potential implications for placental malaria. PLoS One 2009;4:1–10.
[48] Gao S, Emin M, Thoma T, Pastellas K, Castagna F, Shah R, et al. Complement promotes endothelial von Willebrand factor and angiopoietin-2 release in obstructive sleep apnea. Sleep 2021;44(4):zsaa286.
[49] Ngai M, Weckman AM, Erice C, McDonald CR, Cahill LS, Sled JG, et al. Malaria in pregnancy and adverse birth outcomes: New mechanisms and therapeutic opportunities. Trends Parasitol 2020;36(2):127–37.
[50] Puetz J, Wurm FM. Recombinant proteins for industrial versus pharmaceutical purposes: a review of process and pricing. Processes 2019;7:476.
[51] Weckman AM, McDonald CR, Baxter JB, Fawzi WW, Conroy AL, Kain KC. Perspective: L-arginine and L-citrulline supplementation in pregnancy: a potential strategy to improve birth outcomes in low-resource settings. Adv Nutr 2019;10(5):765–77.
[52] Cox B, Kotlyar M, Evangelou AI, Ignatchenko V, Ignatchenko A, Whiteley K, et al. Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology. Mol Syst Biol 2009;5:279.
[53] Rossant J, Cross JC. Placental development: lessons from mouse mutants. Nat. Rev. Genet. 2021;2(7):538–48 200.
[54] Hemberger M, Hanna CW, Dean W. Mechanisms of early placental development in mouse and humans. Nat Rev Genet 2020;21(1):27–43.
[55] Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996;87(7):1161–9.
[56] Saharinen P, Eklund L, Alitalo K. Therapeutic targeting of the angiopoietin-TIE pathway. Nat Rev Drug Discov 2017;16(9):635–61.
[57] Lüdecke D. ggeffects: tidy data frames of marginal effects from regression models. J. Open Source Softw. 2018;3(26):772.