Endotoxin at the Maternal–Fetal Interface in a Resource-Constrained Setting: Risk Factors and Associated Birth Outcomes

Emily A. McDonald,^{1,2*} Ronald Stuart,¹ Ayush Joshi,¹ Hannah W. Wu,^{1,2} Remigio M. Olveda,³ Luz P. Acosta,³ Veronica Tallo,³ Palmera I. Baltazar,³ Jeffrey A. Bailey,^{4,5} Jonathan D. Kurtis,^{1,6} and Jennifer F. Friedman^{1,2}

¹Center for International Health Research, Rhode Island Hospital, Providence, Rhode Island; ²Department of Pediatrics, Alpert Medical School of Brown University, Providence, Rhode Island; ³Department of Immunology, Research Institute for Tropical Medicine, Manila, Philippines; ⁴Division of Transfusion Medicine, University of Massachusetts Medical School, Worcester, Massachusetts; ⁵Program in Bioinformatics and Integrative Biology, University of Massachusetts Medical School, Worcester, Massachusetts; ⁶Department of Pathology and Laboratory Medicine, Alpert Medical School of Brown University, Providence, Rhode Island

Abstract. Low- and middle-income countries (LMICs) carry a high burden of infectious diseases associated with impaired gut integrity, leading to microbial translocation. Pregnancies in this setting are at high risk of fetal growth restriction (FGR). We examined the association among specific risk factors for impaired gut integrity (schistosomiasis, hookworm infection, and alcohol consumption), blood endotoxin levels, and FGR. Endotoxins, lipopolysaccharide-binding proteins (LBPs), and cytokines were measured in blood from women at 32 weeks gestation, the maternal–fetal interface (MFI) at delivery, and cord blood at delivery. Resolution of schistosomiasis had no impact on endotoxin levels; however, maternal hookworm infection and alcohol consumption were associated with modest increases in endotoxin at the MFI. Cytokines responses within the maternal peripheral blood and blood from the MFI were positively associated with endotoxins, but many cord blood cytokines were negatively associated with endotoxins. Newborns with FGR also had higher levels of endotoxins at the MFI. Risk factors for microbial translocation may lead to increased levels of endotoxins at the MFI, which may contribute to poor growth in utero.

INTRODUCTION

In low- and middle-income countries (LMICs), perinatal deaths remain one of the top five causes of death, accounting for more than 5% of total deaths in these nations.¹ Low birth weight (LBW), occurring as a result of short gestation or fetal growth restriction (FGR), is of great public health significance as it underlies 60–80% of these deaths.² In LMICs, as compared with higher income nations, FGR contributes more significantly to the burden of LBW than does preterm birth.³ Despite this, significant lacunae remain in our understanding of the etiology of FGR, hampering the design of rational interventions to address this enormous burden.

In LMICs, there also exist specific insults to gut health and integrity that are thought to culminate in the movement of microbial contents across the gut wall, a process termed microbial translocation. Specifically, schistosomiasis is thought to result in microbial translocation as eggs pass from the bloodstream through the gut wall to perpetuate the lifecycle.^{4,5} In our previous studies, we demonstrated that women infected with *Schistosoma japonicum* had increased levels of endotoxin, a highly immunogenic component of the cell wall of gram-negative bacteria that are prevalent in the human gut, in both peripheral blood at 32 weeks gestation and at the maternal–fetal interface (MFI).⁵ In addition, studies from other groups have also demonstrated that hookworm infection is associated with increased endotoxin in the systemic circulation.⁶ Alcohol consumption represents another risk factor for gut permeability, and is more prevalent in some settings, which may lack the social stigma and public health messages regarding the potential harmful effects of consumption during pregnancy.^{7–12}

In this report, we have leveraged blood samples collected during a recently completed randomized control trial of

praziquantel during pregnancy in Leyte, The Philippines. The trial enrolled women infected with *S. japonicum* and examined the impact of treatment of schistosomiasis with praziquantel at 14 ± 2 weeks gestation on birth outcomes. In addition, women in our study reported relatively high rates of alcohol consumption during pregnancy, and the prevalence of hookworm infection in this population was relatively high. The well-characterized samples from this trial allowed us to examine the impact of specific risk factors for microbial translocation on the levels of endotoxin in maternal and fetal blood, as well as the admixture found specifically at the MFI.

We hypothesized that alcohol consumption, persistent schistosomiasis infection in the control group, and/or hookworm infection would result in gut microbial translocation with consequent hematogenous spread of microbial products to the placenta, culminating in elevated endotoxins in the mother and/or newborn. We further hypothesized that increased endotoxin levels would be associated with risk for poor fetal growth, as these microbial products have been demonstrated to elicit pro-inflammatory immune responses at the MFI linked to poor intrauterine growth.^{5,13–17} To examine these hypotheses, we 1) evaluated the relationship between the aforementioned risk factors and endotoxin levels; 2) examined the relationship between endotoxins and cytokines as potential mediators of inflammation; and 3) evaluated the relationship between endotoxin levels at the MFI and poor fetal growth.

METHODS

Ethical considerations and informed consent. This study was approved by the institutional review boards of Rhode Island Hospital and The Research Institute for Tropical Medicine (Philippines). All subjects provided informed consent before enrollment. All methods were performed in accordance with the relevant guidelines and regulations.

Study site and population. Characteristics and enrollment strategies of this study population have been described

* Address correspondence to Emily A. McDonald, Center for International Health Research, Rhode Island Hospital, 55 Claverick St., Providence, RI 02903. E-mail: emily_mcdonald@brown.edu

elsewhere.¹⁸ All women were positive for schistosomiasis and were randomized to praziquantel treatment to resolve infection, or placebo at 14 ± 2 weeks gestation. Of note, treatment did not impact birth weight, risk for LBW, or small for gestational age (SGA).¹⁸ All women in this cohort delivered vaginally.

Sample collection. At enrollment, we collected health related epidemiologic and demographic data, described in our original publication.¹⁹ Most alcohol consumption was in the form of a locally fermented palm wine (tuba), which was found by mass spectrometry to contain 7.3–7.5% alcohol (data not shown). Alcohol consumption was determined by self-report at 12 weeks of gestation through two questions—“Do you currently drink tuba or any alcoholic beverages?” and “If yes, how many glasses do you drink a week?” Answers to the first question regarding alcohol consumption were analyzed as a dichotomous variable (yes/no). Answers to the second question were dichotomized similarly to other studies, with two or less drinks/week classified as occasionally/never and three or more drinks/week classified as routine consumption.²⁰ Geohelminth (*Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*) and schistosome infections were determined at enrollment and again approximately 8 weeks after treatment.

Maternal blood was collected at 32 ± 2 weeks gestation via venous puncture. Cord blood was collected directly from the umbilical cord after delivery of the placenta and cleaning of the outside of the umbilical cord with alcohol wipe(s). Blood from the MFI was collected from the pool of blood resulting immediately after placental wedge biopsy using a sterile scalpel, needle, and syringe. The surface of the placenta was wiped with alcohol pads before incision for wedge biopsy. Blood samples were collected in red top vacutainer tubes and serum obtained by allowing the blood to clot and centrifuging for 15 minutes at 5,000 × g.

Anthropometric measurements. Newborn weight and length were measured within 24 hours of delivery. Size for gestational age was calculated based on the INTERGROWTH-21st healthy reference standard, which provides the percentile for birth weight by gestational age.²¹ Weight-for-height z-scores (WHZ) were calculated using the 2006 World Health Organization Anthro Growth Standards (www.who.int/childgrowth/software/en/). Newborns with a WHZ less than two standard deviations of the norm were considered to have compromised intrauterine growth, specifically in utero wasting.

Endotoxin and LPS-binding protein (LBP) assays. Endotoxin levels were quantified in serum samples from maternal blood, the MFI, and cord blood using an Federal Drug Administration-cleared, chromogenic limulus amebocyte lysate assay (Q1000; Lonza, Basel, Switzerland). Samples were diluted 1:3 and maintained at 37°C throughout the assay. Endotoxin levels were dichotomized based on the upper quartile of endotoxins for this population (i.e., “high” endotoxin levels are all values in the upper 25% of the study population). LPS-binding protein levels were quantitated in maternal and cord blood samples using an enzyme-linked immunosorbent assay-based assay and manufacturer’s instructions (Abcam, Cambridge, MA).

Cytokine assays. Multiplexed cytokine assays were performed on maternal, MFI, and cord serum as described previously.²² We used a multiplexed sandwich antibody-based assay already developed in our laboratory to measure interleukin (IL)-1, IL-6, interferon γ, tumor necrosis factor (TNF) α, IL-4, IL-5, IL-10, IL-13, IL-12, IL-8, IL-2, and chemokine

(C-X-C motif) ligand 9 with a bead-based platform (BioPlex; Bio-Rad, Hercules, CA).

Statistical analysis. Cytokine, LBP, and endotoxin data were log transformed so as to better approach normality. Statistical analyses pertaining to log transformed endotoxin levels were performed using JMP Pro 10 (SAS Institute, Cary, NC). The relationship between endotoxins in each compartment and risk factors, as well as endotoxin and cytokine production and birth outcomes, were examined using multivariate linear regression. Regression models were built by first examining the role of potential confounders in bivariate analyses, with covariates associated with endotoxin levels in any compartment included in multivariate models. Potential confounding and explanatory covariates evaluated included maternal age, socioeconomic status (SES), maternal body mass index, smoking, parity, and placental weight. Given the significant relationship between maternal SES quartile and cord blood endotoxin, SES was included in all regression models. $P < 0.05$ was considered significant. Data are presented as means ± SEM.

RESULTS

The characteristics of the study population are provided in Table 1. Because of randomization, risk factors for microbial translocation (i.e., helminth infection and alcohol

TABLE 1
Characteristics of study population

Maternal characteristics	
<i>Ascaris lumbricoides</i> infected; % (n)	62% (218/353)
<i>Trichuris trichiura</i> infected; % (n)	81% (285/353)
Hookworm infected; % (n)	37% (129/353)
Maternal age; mean (SD)	26.5 (6.42)
Socioeconomic status quartiles (Q1 = highest)	
Q1; % (n)	25% (89/353)
Q2; % (n)	25% (87/353)
Q3; % (n)	37% (132/353)
Q4; % (n)	13% (45/353)
Body mass index; median (IQR)	21.2 (19.6–23.4)
Smoke during pregnancy; % (N)	0% (1/353)
Alcohol consumption during pregnancy; % (N)	76% (270/353)
Routine drinking during pregnancy; % (N)	45% (160/353)
Parity; median (IQR)	3 (2–5)
IL-6 response; % (N)	
Maternal peripheral blood, 32 ± 2 weeks	10% (35/353)
MFI blood, delivery	54% (190/353)
TNFα response; % (N)	
Maternal peripheral blood, 32 ± 2 weeks	3% (12/353)
MFI blood, delivery	19% (68/353)
IL-4 response; % (N)	
Maternal peripheral blood, 32 ± 2 weeks	5% (18/353)
MFI blood, delivery	10% (35/353)
Infant characteristics	
Gestational age (weeks); median (IQR)	38.7 (38.0–39.4)
Birth weight (kg); mean (SD)	2.86 (0.39)
Small for gestational age; % (n)	23% (81/353)
Premature; % (n)	8% (29/353)
IL-6 response; % (N)	94% (235/250)
TNFα response; % (N)	86% (215/250)
IL-4 response; % (N)	83% (207/250)

IQR = interquartile range; MFI = maternal-fetal interface; SD = standard deviation.

consumption) were evenly distributed between women who received placebo and those who received praziquantel. Interestingly, more than 76% of all women reported at least some alcohol consumption at 12 weeks gestation, with 45% of women reporting routine consumption during pregnancy (three or more drinks/week).

Risk factors for microbial translocation and endotoxin levels. Levels of endotoxin in maternal peripheral blood, cord blood, or blood collected following placental biopsy were not associated with treatment of schistosomiasis nor infection with the soil-transmitted helminths, *A. lumbricoides* or *T. trichiura*. Maternal hookworm infection at 12 weeks gestation was positively associated with endotoxin levels in blood collected at the MFI ($P = 0.02$, Figure 1A). Endotoxins in the cord blood of newborns was elevated in pregnancies in which the mother reported any alcohol consumption after controlling for SES ($P = 0.02$, Figure 1B). There was a trend toward higher endotoxin levels at the MFI only among women who reported routine consumption of alcohol while pregnant (≥ 3 drinks/week, $P = 0.071$, data not shown). Conversely, maternal endotoxins in peripheral blood showed a trend downward, with report of any alcohol consumption ($P = 0.074$, data not shown), but no association with the amount of alcohol consumed.

Association with endotoxin and cytokine levels within compartments. The overall cytokine response was strikingly different between compartments, with most cord blood samples displaying a robust cytokine signature, whereas less than 10% of maternal peripheral blood samples displayed cytokine production (Table 1). Based on this divergence between the compartments, we chose to assess the relationship (s) between endotoxin levels and cytokine response by dichotomizing representative cytokines based on those samples displaying any production. With this approach, women who displayed a pro-inflammatory cytokine response based on IL-8 production also had elevated endotoxin levels in peripheral blood ($P = 0.02$, Figure 2A). Similarly, endotoxin levels at the MFI were higher in those women who displayed IL-8 and/or TNF α responses within the MFI as well ($P < 0.0001$, Figure 2B and C). Cord blood endotoxin levels also were elevated in those pregnancies for which the MFI compartment displayed a pro-inflammatory environment based on IL-8 ($P < 0.0001$,

Figure 2D). Endotoxin levels in cord blood were also elevated in those samples producing IL-8; however, a negative association between cord blood TNF α and cord blood endotoxin levels was observed ($P < 0.0001$, Figure 2E and F).

To substantiate our endotoxin data, we also measured LBP levels in maternal peripheral blood, MFI blood, and cord blood. A largely pro-inflammatory response within each compartment was associated with elevated LBP within that compartment, with maternal IL-8 responders displaying higher levels of LBP in maternal blood, and cord blood IL-8 or TNF α responders displaying higher levels of LBP in cord blood ($P < 0.02$, Figure 3).

In addition to pro-inflammatory cytokines, we also measured Th2-related cytokines, such as IL-4. In maternal periphery and MFI compartments, samples with detectable IL-4 also displayed elevated endotoxin levels ($P < 0.03$, Figure 4A and B). By contrast, cord blood samples with detectable IL-4 displayed lower endotoxin levels ($P < 0.0001$, Figure 4C). No association was observed between LBP and IL-4 response in any compartment (data not shown).

Endotoxin levels at the MFI are associated with poor intrauterine growth. Finally, we examined the association between endotoxin levels and birth outcomes. No association between endotoxin levels in maternal or cord blood compartments was found with any adverse birth outcome examined (i.e., SGA, LBW, preterm birth, in utero "wasting"). However, endotoxins measured in the blood collected after placental biopsy were associated with babies born SGA ($P = 0.03$, Figure 5A) or LBW ($P = 0.04$ after controlling for SES and gestational age; data not shown). Endotoxin levels at the MFI were also associated with lower placental weights in these pregnancies ($P < 0.01$; Figure 5B).

DISCUSSION

Herein, we report an association between blood endotoxin levels and cytokine production, as well as potential risk factors for increased endotoxin levels at the MFI and how this impacts birth outcomes. Although treatment of maternal schistosomiasis failed to impact endotoxin levels in this study, those mothers diagnosed with hookworm early in gestation

FIGURE 1. Specific risk factors for microbial translocation are associated with elevated endotoxin levels at the maternal-fetal interface and in cord blood. (A) Maternal hookworm infection determined at 12 weeks gestation is associated with higher endotoxin levels in blood collected following placental biopsy. * $P = 0.02$. (B) Endotoxin levels in cord blood are higher in those pregnancies whose mothers reported any alcohol consumption during gestation. * $P = 0.02$.

FIGURE 2. Endotoxin association with pro-inflammatory cytokine response. (A) Within maternal peripheral blood, endotoxin levels were higher in those mothers who had detectable IL-8 within peripheral blood. $*P = 0.02$. (B and C) In blood collected from the maternal-fetal interface (MFI), endotoxin levels were higher in those mothers who had detectable IL-8 or TNFα also at the MFI. $*P < 0.0001$. (D and E) Within cord blood, endotoxin levels were positively associated with those pregnancies displaying detectable IL-8 at the MFI or within cord blood; however, a (F) negative association with cord blood TNFα response was associated with cord blood endotoxin levels. $*P < 0.0001$.

did display significantly higher endotoxin levels, but only in blood collected at the MFI. Why we would observe this association in this compartment and neither of the other two is an area of ongoing investigation. We also failed to observe an association between maternal hookworm infection and LBP levels in any of the compartments, suggesting that the clearance of LPS specifically at the MFI may be mediating the impact of hookworm infection on endotoxin levels within the placenta. This is substantiated by the robust cytokine association with endotoxins at the MFI (endotoxin level was elevated in samples with measurable levels of 10/11 cytokines assessed), but relative lack of association with LBP and cytokine production in this compartment (3/11 cytokine showed association with elevated levels of LBP at the MFI).

The lack of impact of praziquantel treatment on endotoxin levels is likely because of persistent inflammatory responses to dying eggs trapped in the intestinal wall, allowing continued microbial translocation.²³ Interestingly, we found that maternal consumption of alcohol during pregnancy impacted the levels of endotoxin measured in the cord blood of exposed infants. Alcohol intake has been demonstrated to disrupt gut integrity, culminating in increased systemic endotoxin levels.^{24,25} Many mechanisms have been proposed for this,

including alterations in the type of enteric bacteria, alterations in bacterial metabolism, and disruption of the gut mucosal barrier.⁷⁻¹¹ Importantly, a single oral dose of ethanol has been demonstrated to increase gastroduodenal permeability and systemic endotoxemia, even among individuals without evidence of chronic liver disease.^{10,12,26} Our finding that maternal alcohol exposure is associated with cord blood endotoxin levels could suggest hematogenous spread of microbes from a compromised intestinal mucosa culminating in elevated bacterial products crossing the MFI, clearing from maternal circulation, and entering the fetal circulation. However, the fact that we only observed a trend toward increased endotoxin levels at the MFI, and only in those women with higher alcohol consumption rates, also suggests that this insult is multifactorial.

It is quite likely that inflammation at the placenta is also driving some of the poor growth characteristics in this cohort. This is further substantiated by the divergent associations of pro-inflammatory cytokines with cord endotoxin levels, but a robust positive association between cord blood endotoxin and cytokine production at the MFI. We expected that the association between cytokine production and endotoxin levels observed in the maternal periphery and blood from the MFI

FIGURE 3. LPS-binding protein (LBP) is positively associated with cytokine response within the same compartment. (A) Within maternal peripheral blood, LBP levels were higher in those mothers who had detectable IL-8 within peripheral blood. $^*P = 0.02$. (B and C) Within cord blood, LBP levels were positively associated with those pregnancies displaying detectable IL-8 or TNF α in cord blood. $^*P < 0.02$.

would be recapitulated in cord blood. In fact, we observe a significantly higher amount of samples with detectable cytokine levels compared with either maternal or MFI blood, and lower endotoxin levels associated with many of these cytokine responses in cord blood. These data suggest that 1) endotoxin level is not eliciting the same inflammatory response in fetal circulation, perhaps because of differing acylation states,²⁷ 2) the fetal ability to respond to endotoxins is immature, and/or 3) the fetus is responding differently to a chronic endotoxin insult present through most of gestation. Given that LBP, the protein responsible for driving much of the acute immunological response to LPS, is associated with a pro-inflammatory response in maternal and fetal compartments, the differential association of cytokine production with endotoxins by the fetus is striking. Furthermore, maternal LBP levels were approximately twice as high as those found in the cord blood samples, whereas total endotoxin levels were higher in cord blood compared with maternal peripheral blood, suggesting

that the clearance rates between these two compartments may be divergent.

Significantly increased endotoxin levels at the MFI in SGA infants suggest that the overall abundance of microbial products, particularly because of Gram-negative bacteria, at the MFI is heightened in cases of poor intrauterine growth. The presence of these microbial products may lead to chronic inflammation. In our previous studies, we found that endotoxins were positively correlated with pro-inflammatory cytokines such as TNF α and IL-6 at the MFI.⁵ In rodent models, intraperitoneal injection of endotoxin culminates in increased production of TNF α in maternal serum, the placenta, and amniotic fluid.²⁸ Furthermore, chemical inhibition of TNF α can reverse the LPS-mediated FGR in this model.^{13,28} We also observe a pronounced association of pro-inflammatory cytokines with endotoxin levels at the MFI in this study. Given the known relationship between alcohol exposure and poor intrauterine growth, it is also possible that alcohol exposure

FIGURE 4. Endotoxin association with Th2 cytokine response. (A) Within maternal peripheral blood, endotoxin levels were higher in those mothers who had detectable IL-4 within peripheral blood. $^*P = 0.03$. (B) In blood collected from the maternal-fetal interface (MFI), endotoxin levels were higher in those mothers who had detectable IL-4 also at the MFI. $^*P = 0.007$. (C) Within cord blood, endotoxin levels were negatively associated with IL-4 production within cord blood. $^*P < 0.0001$.

FIGURE 5. Endotoxin levels at the maternal-fetal interface (MFI) are linked to fetal growth. (A) Babies born small for gestational age (SGA) had higher endotoxin levels in blood taken from placental bed biopsy at delivery compared with appropriate for gestational age (AGA) counterparts. * $P < 0.05$. (B) Placental weight is lower in those pregnancies with the highest endotoxin levels at the MFI, after controlling for socioeconomic status and gestational age. * $P < 0.04$.

contributes to poor in utero growth through increased cytokine production at the MFI.²⁹ In addition, both hookworm and schistosome infections are associated with elevated cytokine levels during pregnancy, and the impact of these infections on intrauterine growth may be mediated through cytokine production at the MFI.^{19,30}

There remain limitations to this study. Although care was taken to minimize external contamination of blood collected from the MFI following wedge resection, it remains possible that some differences observed between blood collected at the MFI and the other compartments are a result of contamination. However, introduction of any bacterial products by sampling technique should not differ based on any predictors/outcomes assessed in this study as all midwives used the same procedure. We did not capture the presence of ascending infections in this cohort, which may account for some of the elevated endotoxin levels at the MFI or in cord blood, although the incidence of preterm delivery, the birth outcome most commonly associated with ascending infections, was not associated with endotoxin levels in any compartment. Furthermore, LBP levels in either blood from the MFI or the umbilical cord was not associated with adverse birth outcomes, suggesting the observed association between endotoxin and intrauterine growth was not due to acute infection. In addition, SES was included in all statistical models to control for possible effects of varying maternal hygiene practices. Finally, the use of samples from a randomized, controlled trial mitigates the risk of unequal distribution of both measured and unmeasured potential confounders in this population.

To our knowledge, this study is the first to report an association between maternal alcohol consumption or helminth infection and endotoxin levels in circulation. As we have previously observed in women residing in the same region, a number of pro-inflammatory cytokines were associated with higher endotoxin levels at the MFI. Interestingly, although cord blood endotoxin levels were also associated with pro-inflammatory cytokine responses at the MFI, the association within cord blood was mixed, with some cytokine levels

elevated and others lower in samples with high endotoxin levels. This is an intriguing finding that warrants further investigation. Finally, this is the first study to link endotoxins at the MFI with fetal growth in utero, a finding that may have broad implications for the health of neonates, particularly in an LMIC setting, where unique insults may be addressed to improve pregnancy outcomes.

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Authors' addresses: Emily A. McDonald, Ayush Joshi, Hannah W. Wu, and Jennifer F. Friedman, Center for International Health Research, Rhode Island Hospital, Providence, RI, E-mails: emily_mcdonald@brown.edu, ayush_joshi@brown.edu, haiwai_wu@brown.edu, and jennifer_friedman@brown.edu. Ronald Stuart and Jonathan D. Kurtis, Center for International Health Research, Brown University, Providence, RI, E-mails: ronald_stuart@brown.edu and jonathan_kurtis@brown.edu. Remigio M. Olveda, Luz P. Acosta, Veronica Tallo, and Palmera I. Baltazar, Department of Immunology, Research Institute for Tropical Medicine, Manila, Philippines, E-mails: rolvedamd_ritm_doh@yahoo.com, ipacosta@yahoo.com, veronica.tallo2015@gmail.com, and palmerabaltazar@yahoo.com. Jeffrey A. Bailey, Division of Transfusion Medicine, University of Massachusetts Medical School, Worcester, MA, E-mail: jeffrey.bailey@umassmed.edu.

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