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Meredith A. Kelleher, PhD, Ji Yeon Lee, MD PhD, Victoria Roberts, PhD, Christopher M. Novak, MD, Ahmet A. Baschat, MD, Terry K. Morgan, MD PhD, Miles J. Novy, MD, Juha P. Rasanen, MD PhD, Antonio E. Frias, MD, Irina Burd, MD PhD

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Maternal Azithromycin therapy for *Ureaplasma parvum* intra-amniotic infection improves fetal hemodynamics in a non-human primate model

Meredith A. KELLEHER PhD[1], Ji Yeon LEE MD PhD[2], Victoria ROBERTS PhD[1], Christopher M. NOVAK MD[2], Ahmet A. BASCHAT MD[3], Terry K. MORGAN MD PhD[4], Miles J. NOVY MD[1], Juha P. RASANEN MD PhD[4,5], Antonio E. FRIAS MD[1,4], Irina BURD, MD PhD[2,6]

[1] Division of Reproductive & Development Sciences, Oregon National Primate Research Center, Beaverton, OR, USA
[2] Integrated Research Center for Fetal Medicine, Department of Gynecology and Obstetrics, Johns Hopkins University, School of Medicine, Baltimore, MD, USA
[3] Johns Hopkins Center for Fetal Therapy Department of Gynecology and Obstetrics, Johns Hopkins University, School of Medicine, Baltimore, MD, USA
[4] Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, OR, USA
[5] Department of Obstetrics and Gynecology, Helsinki University Hospital and University of Helsinki, Helsinki, Finland
[6] Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
Corresponding Author: Meredith A. Kelleher, PhD
Division of Reproductive & Developmental Sciences
Oregon National Primate Research Center
ONPRC 1121, MC L584
505 NW 185th Ave, Beaverton, OR 97006.
Tel: 503-346-5417
Fax: 503-346-5585
Email: kellehem@ohsu.edu

Condensation: Antimicrobial therapy improves umbilical artery pulsatility index and cardiac output in a non-human primate model of intra-amniotic *Ureaplasma* infection.

Short Title: Antimicrobial therapy for intra-amniotic *Ureaplasma* infection improves fetal hemodynamics.

AJOG At a Glance

A. The study utilized Doppler ultrasonography in a non-human primate model of preterm labor to determine if intrauterine infection and inflammation was associated with altered fetal

hemodynamic and cardiovascular function and whether these effects would be ameliorated *in utero* by maternal antibiotics.

B. Intrauterine *Ureaplasma* infection alters the fetal hemodynamic profile, with potential compromise of cardiovascular function, which was mitigated by maternal antibiotic.

C. Our study provides additional new evidence that Doppler ultrasonography is a useful method to evaluate fetal cardiovascular status in the context of intrauterine infection and preterm labor and, in this setting, to assess the efficacy and safety of therapeutic interventions.

Work was performed at the Oregon National Primate Research Center, Beaverton, OR, USA.

Conflict of Interest The authors report no conflict of interest.

Portions of this study have been presented at meetings of the Society of Maternal Fetal Medicine and the American Society of Reproductive Immunology.

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Abbreviations

pPROM - preterm premature rupture of membranes

FIRS - fetal inflammatory response syndrome

IAI - intra-amniotic infection

AZI - azithromycin

PI - pulsatility index

UA - umbilical artery

RPA - right pulmonary artery

MCA - middle cerebral artery

CPR - cerebro-placental ratio

CO - cardiac output

RCO/LCO - ratio of right ventricular cardiac output to left ventricular cardiac output

VTI - velocity time integrals

HR - heart rate

AF - amniotic fluid

NHP - non-human primate

E/A Ratio - marker of ventricular function

Tei Index - myocardial performance index

SF - shortening fraction, measure of systolic performance

Keywords: Doppler ultrasound, chorioamnionitis, Azithromycin, preterm birth

ABSTRACT

Background: *Ureaplasma parvum* infection is a prevalent cause of intrauterine infection that is associated with preterm birth, preterm premature rupture of membranes, the fetal inflammatory response syndrome and adverse postnatal sequelae. Elucidation of diagnostic and treatment strategies for infection-associated preterm labor may improve perinatal and long-term outcomes for these cases.

Objective: This study assesses the effect of intra-amniotic *Ureaplasma* infection on fetal hemodynamic and cardiac function and the impact of maternal antibiotic treatment on these outcomes.

Study Design: Chronically catheterized pregnant rhesus monkeys were assigned to control (n=6), intra-amniotic inoculation with *Ureaplasma parvum* (10^7 CFU/ml, IAI, n=15); and intra-amniotic infection plus Azithromycin treatment (12.5 mg/kg BID I.V., IAI+AZI, n=8) groups. At ~135days gestation (term=165 days), pulsed and color Doppler ultrasonography was utilized to obtain measurements of fetal hemodynamic**s** (pulsatility index of umbilical artery, ductus venosus, descending aorta, ductus arteriosus, aortic isthmus, right pulmonary artery, middle cerebral artery and cerebro-placental ratio**,** and left and right ventricular cardiac outputs) and cardiac function (E/A ratio, Tei index). These indices were stratified by amniotic fluid pro-inflammatory mediator levels and cardiac histology.

Results: Umbilical and fetal pulmonary artery vascular impedances were significantly increased in IAI animals ($p<0.05$). Azithromycin treatment restored values to control levels. Amniotic fluid

PGF_{2a} levels were significantly higher in animals with abnormal umbilical artery pulsatility index (>1.1) than those with normal blood flow ($p<0.05$; Spearman $\rho=0.6$, $p<0.05$). In the IAI group, left ventricular cardiac output was significantly decreased ($p<0.001$) and more animals had abnormal right to left ventricular cardiac output ratios (RCO/LCO defined as >1.6, $p<0.05$). Amniotic fluid IL-6 concentrations were elevated in cases of abnormal RCO/LCO ratio compared to normal cases ($p<0.05$).

Conclusions: Fetal hemodynamic alterations were associated with intra-amniotic *Ureaplasma* infection and ameliorated following maternal antibiotic treatment. Doppler ultrason**o**graphic measurements merit continuing investigation as a diagnostic method to identify fetal cardiovascular and hemodynamic compromise associated with intrauterine infection or inflammation, and in the evaluation of therapeutic interventions or clinical management of preterm labor.

INTRODUCTION

Intrauterine infection is a major cause of early preterm birth, involved in over 70% of births at less than 30 weeks of gestation.[1,2] Intrauterine infection occurs when microbes invade the amniotic cavity, characterized by a robust inflammatory response, chorioamnionitis, fetal inflammatory response syndrome (FIRS) and preterm labor.[1,3,4] FIRS is a risk factor for adverse outcomes in preterm infants and has been linked to hypoxic-ischemic brain damage and neonatal cardio-respiratory failure.[5-7] Increased amniotic fluid (AF) pro-inflammatory mediators double the risk of severe neonatal morbidity, including cortical white matter damage.[8] *Ureaplasma parvum* (*U.parvum*) infection is a prevalent cause of early preterm delivery that causes intrauterine and fetal inflammation, manifested by increased pro-inflammatory mediators in the amniotic fluid, increased fetal cord blood IL-6 and fetal lung injury.[9,10]

Antibiotics and anti-inflammatory agents are therapies for intrauterine infection that can reduce fetal inflammation and delay preterm delivery.[11-13] In the current non-human primate (NHP) model of intrauterine *U. parvum* infection, we have demonstrated that maternal Azithromycin treatment effectively clears intra-amniotic *Ureaplasma* infection in an average of 4 days, inhibits preterm labor, reduces the severity of histological chorioamnionitis and fetal lung injury.[14] Furthermore, recent studies in women have demonstrated that maternal antibiotic treatment can resolve intrauterine infection and delay premature labor without short-term neonatal sequelae.[15,16] However, the risk of long-term adverse consequences continues to be a clinical concern when using antibiotics to treat preterm labor due to the potential for residual inflammation.[17-19] Therefore methods to evaluate fetal wellbeing during

and following treatment could inform the use of antibiotics for intrauterine infection and preterm labor.

Multiple studies have shown that fetal hemodynamic and cardiovascular dysfunction occurs in the setting of intrauterine infection, inflammation and FIRS,[20-25] which could be useful for identifying fetal inflammation by ultrasonography.[26-28] For example, fetal ventricular filling characteristics are considered an indirect measure of cardiac diastolic function, while Tei index takes into account both diastolic and systolic functional properties and is used as an indicator of global cardiac function.[29,30] Abnormalities in fetal hemodynamic indices that indicate blood flow impedance in fetal vessels (e.g. umbilical artery (UA PI) or middle cerebral artery pulsatility index (MCA PI) can be indicative of poor outcomes[31] but are currently not used or well-defined for intra-uterine infection.

Our objective was to utilize Doppler ultrasonography, in a NHP model, to assess the effect of intra-amniotic *Ureaplasma* infection and maternal antibiotic therapy on fetal hemodynamic and cardiac function. We hypothesize that fetal hemodynamic compromise in the setting of intrauterine infection will be improved with antibiotic treatment and that Doppler ultrasound may be a useful aid to the evaluation of therapeutic interventions for preterm labor.

MATERIALS AND METHODS

Ethics Statement

Animal studies were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University West Campus and performed in strict accordance with the Animal Welfare Act and Regulations and the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council. All surgery was performed in keeping with best veterinary practices and all efforts were made to minimize pain and discomfort.

Animal Model

Animals were allocated to the study by assignment from the Oregon National Primate Research Center (ONPRC) breeding colony and divided into control (Control; n=6), intra-amniotic infection with *U. parvum* (IAI; n=15) and intra-amniotic infection plus antibiotic treatment (AZI; n=8) groups and Doppler ultrasound assessments of fetal-placental blood flow and fetal cardiac function were performed.

Using an long established NHP model,[32] time-mated pregnant rhesus monkeys (*Macaca mulatta*) were adapted to a vest and mobile catheter protection system before intra-uterine surgery was performed at 110 ± 8 days gestation to implant catheters into the amniotic cavity and maternal femoral artery and vein.[14,33] A standard post-operative regimen of intravenous antibiotics (Cefazolin sodium) and tocolytic medications (Terbutaline sulfate, Atosiban) were administered.[4,14] Fetuses were delivered by C-section based on signs of imminent preterm labor (uterine activity, cervical dilatation) or gestational age for neonatal survival studies.

Ureaplasma parvum for Intra-amniotic Inoculation

178 At 123 ± 6days gestation (term gestation = 165days), animals in the IAI and AZI groups were
179 inoculated, via intra-amniotic catheters, with a low-passaged clinical isolate of *U. parvum*
180 serovar 1 (1mL of 1.4 x 10^{5-7}CFU/mL in 2SP media supplied by the Mycoplasma Laboratory,
181 University of Alabama at Birmingham, Birmingham, AL, USA). Animals in the control group
182 received sterile media. Following 15 ± 7 days of inoculation animals assigned to the AZI group
183 received maternal Azithromycin treatment (intravenous infusion of 12.5 mg/kg, 12hrly for 10
184 days). For the IAI+AZI group, ultrasound studies were performed in animals following 2-12 days
185 of Azithromycin therapy (average length of treatment when ultrasound was performed was 4
186 days).

187 ***Ultrasound Imaging***

188 All scans were performed at ONPRC by J.P.R. and A.E.F using a standardized protocol and
189 blinded to treatment group. Image-directed pulsed and color Doppler ultrasonography (GE
190 Voluson 730 Expert, Kretztechnik, Zipf, Austria) was utilized as published[34] to obtain feto-
191 placental hemodynamic and cardiac measurements. Pregnant animals were sedated with
192 intramuscular 10mg/kg ketamine and placed in the dorsal recumbency. All animals received the
193 same sedation protocol and vital signs remained stable throughout each procedure.

194 ***Measurements of Fetal Hemodynamics***

195 Blood flow velocity waveforms of the umbilical artery (UA), middle cerebral artery (MCA), the
196 right pulmonary artery (RPA), aortic isthmus, ductus arteriosus, descending aorta, inferior vena
197 cava, the left hepatic vein and ductus venosus were obtained. The pulsatility index [PI =(peak
198 systolic velocity – end diastolic velocity)/time averaged maximum velocity] was determined in
199 each vessel. Cerebro-placental ratio (CPR= MCA PI/UA PI) was also calculated.

UA PI was considered abnormal if it exceeded 1.1, based on data from human studies [35] taking the 90th percentile of the UA PI in the third trimester of human pregnancy as there is no standard available in NHPs. Using this threshold, all control animals were in the normal range.

Measurements of Fetal Cardiac Function

Echocardiography was performed to assess fetal cardiac function.[34] Peak E and A wave velocities were recorded for atrioventricular valves, and the tricuspid and mitral valve E/A ratios calculated. To measure the left ventricular Tei index, mitral and aortic valve blood flow velocity waveforms were simultaneously obtained. The Tei index was calculated as [(isovolumic contraction time + isovolumic relaxation time)/ejection time].[36] To measure ventricular shortening fractions (SF), the end-diastolic dimension (EDD) and end-systolic dimension (ESD) of the left ventricle and right ventricle were measured by M-mode technique.[37] SF of the left ventricle and right ventricle were derived using the following equation: SF = [(EDD-ESD)/EDD] x 100.[37] Cardiac output (CO) was calculated by measuring the diameters (d) of the aortic and pulmonary valves twice on frozen real-time images taken during systole using the leading-edge-to-edge method. Mean values were used in analysis. Aortic and pulmonary valve velocity time integrals (VTI) and heart rates (HR) were calculated. Left and right CO were derived from the equation: CO (ml/min) = VTI (cm) x π x d $(cm)^2$/4 x HR (beats/min).[38] We considered the right to left CO ratio (RCO/LCO) greater than 1.6 as abnormal based on human studies.[39] Fetal biometrics, including biparietal diameter, abdominal circumference, and femur length were also assessed.

Quantification of Amniotic Fluid and Fetal Blood Pro-Inflammatory Cytokines and Prostaglandins

Serial amniotic fluid samples were collected and the concentrations of pro-inflammatory mediators tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and prostaglandins (PGE_2 and $PGF_{2\alpha}$) measured by ELISA following manufacturer's intructions, as previously described.[14,33] For control and IAI groups the peak values were selected from time points between gestational age (GA) 130-150days and, for the AZI group, the peak values from 48hrs after treatment to 2days before delivery were chosen. Fetal cord blood IL-6 concentrations were measured for n=4 per group. ELISA intra- and inter-assay coefficients of variance were <5% and <15%, respectively.

Statistical Analyses

Statistical analyses were performed using SPSS version 19.0 (SPSS Institute, Chicago, IL, USA) and Prism 6 for Mac OSX (GraphPad Software Inc., San Diego, CA, USA). Discrete data was analyzed using Chi-squared or Fisher's exact tests and continuous variables using a One-way ANOVA or Student's t for normally distributed data (as determined by D'Agostino & Pearson omnibus normality test); and Kruskill Wallis or Mann–Whitney U tests, as appropriate for non-parametric data. For analysis of pro-inflammatory mediators measured by ELISA, the lowest detectable value was used for all samples that had values below this level. Post-hoc Bonferroni or Fisher's LSD were performed to identify differences by group in cases where the was $p<0.05$ by one-way ANOVA and Dunn's multiple comparison test was used for non-parametric data. Quartile regression was used to estimate in 25, 50, and 75 quartile of concentrations of fetal and amniotic fluid pro-inflammatory mediators for each group. Comparisons between group at each quartile was based on rank tests. The NORMAL, WILCOXON, and SIGN functions are implemented and suitable for iid error models, and the TAU score function is implemented and

244 appropriate for non-iid error models. Analysis was carried out using SAS 9.4 (SAS Institute Inc.,
245 Cary, NC, USA). Correlations between ultrasound measurements and pro-inflammatory
246 mediator levels were calculated using Spearman's rank correlation. A p-value <0.05 was
247 considered statistically significant.

RESULTS

There were no differences in gestational age, fetal sex, biparietal diameter, abdominal circumference, femur length or fetal heart rate among three groups (Table 1).

Increased Umbilical and Pulmonary Artery Impedance in Animals Exposed to Infection

The umbilical artery (UA) pulsatility index (PI) was significantly elevated in the IAI group when compared to control ($p<0.01$, Table 2). Significantly more IAI animals also demonstrated umbilical artery impedance in the abnormal range (UA PI>1.1) than control animals. Pulmonary artery impedance (RPA PI) was significantly elevated in the IAI group compared to both control and IAI+AZI animals ($p=0.034$, Table 2). Descending aorta PI was significantly elevated in the AZI animals compared to control ($p<0.05$). There were no significant differences in the MCA, the ductus arteriosus and aortic isthmus PI values or in the cerebro-placental ratio among the three groups (Table 2).

Decreased Left Ventricular Output in Fetuses Exposed to Infection

Cardiac output in the left ventricle (LCO) was significantly decreased in IAI group compared with control and AZI groups ($p<0.001$, Table 3). When the ratio of bilateral cardiac output (RCO/LCO) was calculated, there were significantly more animals with abnormal left to right cardiac output ratio (RCO/LCO>1.6) in the IAI verse control and AZI groups ($p<0.05$). The mitral valve E/A ratio was decreased in the IAI group ($p<0.05$, Table 3). There were no significant differences in other measurements of cardiac function among three groups.

Amniotic Fluid and Fetal Blood Pro-Inflammatory Mediators are elevated in Animals with Placental Hemodynamic and Fetal Cardiac Changes

The peak concentrations in amniotic fluid of all pro-inflammatory mediators (Table 4) were significantly increased with infection ($p<0.05$) at the 25, 50 and 75% quartiles. Concentrations of TNFα, IL-1β, IL-6, PGE_2 and $PGF_{2\alpha}$ were also significantly elevated compared to control in the IAI+AZI group at the 50 and 75% quartiles, however, these values were not significantly increased at the 25^{th} percentile.

We also examined intra-amniotic inflammation in reference to umbilical artery impedance (UA PI) values and cardiac output. In those animals with abnormal umbilical artery impedance (defined as UA PI >1.1, Fig. 1A), $PGF_{2\alpha}$ concentrations were significantly higher than in animals with normal UA PI values ($p<0.01$, Fig. 1B). The correlation coefficient (spearman, rho) between UA PI and $PGF_{2\alpha}$ was 0.7 ($p<0.05$). Amniotic fluid PGE_2 was also significantly higher with abnormal UA PI values ($p<0.05$). In animals with abnormal RCO/LCO ratios (shown in Fig. 2A), amniotic fluid IL-6 concentrations were significantly higher than in those with normal output ratios ($p<0.05$, Fig. 2B).

DISCUSSION

Principal Findings

Our Doppler ultrasound study in a non-human primate model of intra-amniotic *U. parvum* infection and preterm labor identified fetal and placental hemodynamic alterations that were associated with intrauterine infection and amniotic fluid pro-inflammatory mediators. Principal findings of the study are that intra-amniotic *Ureaplasma* infection was associated with (1) increased vascular impedance in the umbilical and fetal pulmonary arteries (2) a decrease in fetal left ventricular cardiac output and (3) an association between intrauterine inflammation (amniotic fluid pro-inflammatory mediators) and these fetal

hemodynamic changes. Finally, we demonstrated that maternal Azithromycin treatment partially corrects fetal cardiac output and vascular impedance in the placental and pulmonary circulations. Taken together, these data demonstrate the efficacy of maternal antibiotic treatment to mitigate the fetal hemodynamic consequences of intrauterine infection, as well as the potential clinical utility of Doppler ultrasonography in detecting fetal hemodynamic compromise in the setting of intrauterine infection.

Results in the Context of What is Known

Fetal hemodynamic function has not previously been assessed with reference to treatment of intrauterine infection in animal models or in women. However, clinical and animal studies have previously linked inflammation and chorioamnionitis with fetal hemodynamic alterations and cardiac dysfunction. Furthermore, reports of measurement of fetal hemodynamic outcomes in clinical studies have mostly been limited to pregnancies associated with pPROM [40,41] rather than confirmed cases of intrauterine infection. The main findings in the current study were hemodynamic alterations associated with intrauterine infection (i.e. increased umbilical artery impedance). Cardiac output was altered, although it is suggested that this is due to reductions in venous return in the fetus since functional indices of cardiac function, such as Tei index, were not altered (see Fig. 3).

Fetal Hemodynamic Responses to Intra-Uterine Infection

Given the observations in the current study, we hypothesize that uterine and placental inflammation, which occur in the presence of intrauterine *Ureaplasma* infection, results in perturbation of placental circulation, as evidenced by increased umbilical artery vascular impedance, which we propose leads to abnormal gas exchange and subsequent fetal

hemodynamic responses and reduced cardiac output (conceptual model shown in Fig. 3). Previous studies have linked increased umbilical vascular impedance with increased circulating maternal and fetal cytokines, placental vascular congestion and inflammation in the endothelium of the fetal-placental microcirculation in cases of placental insufficiency.[42-45] Decreased placental blood flow has also been reported in fetal sheep exposed to LPS as an inflammatory stimuli.[46,47] Clinically, increased umbilical artery resistance is a well-established indicator of placental compromise and of poor perinatal outcome, which is routinely monitored in high-risk pregnancies by Doppler ultrasound. Therefore the increase in umbilical artery impedance identified in this study suggests impacts on placental function in the setting of intrauterine *Ureaplasma* infection with potential consequences on fetal wellbeing.

Fetal hypoxemia, is a potential consequence of placental compromise, and an important mediator of fetal hemodynamic responses. We suggest that placental compromise leading to fetal hypoxemia results in the increase in pulmonary artery impedance observed with infection (i.e. elevated RPA PI), attributed to processes of hypoxic pulmonary vasoconstriction.[34] Fetal lung injury caused by *Ureaplasma* infection may also exacerbate these effects.[48,49] Pulmonary vasoconstriction, reduces the available blood volume for venous return to the left atrium and thus decreasing preload of the left ventricle. A reduction in left ventricular preload could then result in the reduced left ventricular cardiac output observed in this study with intra-amniotic *Ureaplasma* infection. The foramen ovale, which shunts oxygenated blood from the right atrium to the left atrium, bypassing the fetal pulmonary circulation, functions close to its maximum capacity[50,51] so cannot increase left atrial flow in order to compensate for this drop in pulmonary venous return. The finding of decreased mitral valve E/A ratio in the infected group

335 (i.e. abnormal left ventricular filling) further supports the concept of reduced left ventricular
336 preload contributing to decreased left cardiac output in this model. Other potential indicators
337 of fetal hypoxemia, such as reduced fetal cerebral blood flow impedance (MCA PI) or reduction
338 in cerebro-placental ratio[52,53] were not observed in this study. However, it has previously been
339 shown in human preterm infants that neither chorioamnionitis nor elevated circulating
340 cytokines are associated with changes in middle cerebral artery Doppler blood flow velocities.[21]
341 Alterations in fetal cardiac function were not identified in animals exposed to intra-
342 amniotic infection, despite previous studies in women and animal models demonstrating that
343 cardiac performance could be impaired with intrauterine infection and inflammation, including
344 signs of fetal congestive heart failure.[20,54,55] In this study, left ventricular Tei index, which detects
345 changes in myocardial performance (e.g. cardiac contractility and relaxation parameters) was
346 similar between the groups suggesting that the reduction in left ventricular cardiac output was
347 not due to direct cardiac dysfunction. The absence of histological evidence of myocardial injury
348 supports this hypothesis. Additionally, measures of right ventricular cardiac function remained
349 normal. Therefore, we propose that the decreases in mitral valve E/A ratio and left ventricular
350 cardiac output observed in this study with infection are due to fetal hemodynamic changes
351 rather than abnormal myocardial performance. Altered fetal blood flow distribution and
352 reduced cardiac output may have significant consequences on fetal wellbeing and by reducing
353 blood flow to peripheral organs may contribute to adverse postnatal outcomes and
354 underdevelopment of organ systems that are particularly vulnerable to injury associated with
355 *Ureaplasma* infection, intrauterine inflammation and prematurity (e.g. necrotizing enterocolitis,
356 bronchopulmonary dysplasia).[56,57]

Proinflammatory mediators associated with fetal hemodynamic responses

In this study, we have demonstrated that abnormal distribution of cardiac output and increased umbilical artery vascular impedance were correlated with increased amniotic fluid levels of IL-6 and $PGF_{2\alpha}$, respectively. Azithromycin treatment of intra-amniotic infection, reduces amniotic fluid concentrations of pro-inflammatory cytokines and fetal cord blood IL-6, which is associated with a delay in preterm labor and diminished signs of chorioamnionitis[14] and improvement in fetal hemodynamic function observed in this study. Intrauterine, placental and fetal inflammation are associated with fetal hemodynamic function in this model.

Antibiotic treatment of Infection-Associated Preterm Labor

Our results extend previous studies of fetal cardiovascular function in cases of intrauterine infection and inflammation to address the question of whether antimicrobial treatment of intrauterine infection – to delay preterm labor – can also improve the negative consequences of fetal inflammation. Randomized controlled trials of antibiotic treatment for preterm labor in women have produced mixed results and identified potential adverse outcomes for the fetus.[19,58,59] More success has been reported in prolonging gestation without negative consequences on the fetus in cases of preterm premature rupture of membranes (pPROM).[60] Infection is a significant cause of preterm labor, present in up to half of preterm births earlier than 28 weeks of gestation. However, trials and epidemiological studies are not always able to select patients with confirmed intrauterine infection, which potentially limits the ability of large clinical studies to identify effective treatments. Azithromycin has demonstrated anti-inflammatory actions,[61] has been shown to cross the placenta and accumulates in amniotic fluid following maternal intravenous administration,[62] which may directly treat inflammation in

379 the placenta and fetal lung and therefore improve the negative fetal hemodynamic
380 consequences of infection. Thus, the observation in this study that maternal Azithromycin
381 treatment mitigates changes in fetal hemodynamics in a translational model of known infection
382 may have important clinical relevance to the use of antibiotic treatment for preterm labor.

383 ***Clinical Implications***

384 The findings of the current study supports the use of maternal antibiotic therapy for
385 intrauterine infection to improve fetal outcomes and suggests that utilization of Doppler
386 ultrasound to monitor fetal wellbeing, as occurs commonly for pregnancies at high-risk for fetal
387 growth restriction, may aid the clinical management of women in preterm labor. However,
388 Doppler ultrasound parameters are not been well defined for the diagnosis of intrauterine
389 infection and/or inflammation. A recent study of ultrasound assessment for the management
390 pPROM found no differences in the MCA PI and CPR and only a slight increase in UA PI values in
391 women with ruptured membranes with suspected chorioamnionitis.[40] Whilst we also found no
392 changes in MCA or CPR indices in the current study, with a known infection in a clinically-
393 relevant NHP model, we did identify significantly increased umbilical artery impedance, with a
394 majority of animals the infection group in a defined abnormal range for this index. Recent
395 clinical studies examining the use of antibiotics for the treatment of preterm labor, again
396 highlighted the importance of direct identification of intrauterine infection by amniocentesis
397 for the evaluation of the efficacy antibiotic treatment to target infection-associated preterm
398 labor.[15,16,63] The choice of antimicrobial agent is also an important consideration, due to
399 microbial sensitivity and development of potential resistance as well as studies that have

suggested that newer generation macrolide antibiotics, such as clarithromycin, may have more efficacy with better neonatal outcomes in studies in women.[64]

Research Implications

Cardiovascular decline in the fetus after intrauterine infection may contribute to injury in multiple organ systems by exacerbating the effects of inflammation on these tissues. Further study of fetal brain, lung, gut and other organs, along with placental vascular changes may provide information about the sensitivity of the fetus to alterations in blood flow during infection and following antibiotic treatment. *In vivo* imaging of placental blood oxygen concentrations – techniques used in a NHP model of Zika virus infection –have potential for evaluating functional placental gas exchange in cases of intrauterine *Ureaplasma* infection.[65,66] *In vivo* measurement of fetal oxygenation during infection and postnatal pulmonary function may also provide evidence of the mechanisms involved in the fetal hemodynamic responses to infection that we identified in this study. Furthermore, Mitchell *et al*[67] recently reported elevated levels of IL-6 and IL-8 in the myocardium and changes in the expression of cardiac genes in fetal monkeys exposed to intrauterine infection despite a lack of histopathological inflammation, suggesting that fetal cardiac inflammation may be present without overt evidence of injury. Evaluation of postnatal cardiovascular function will be important in determining whether programming of postnatal cardiovascular disease occurs in this model, despite absence of histological cardiac injury, and whether the hemodynamic improvements observed in the fetal period with antibiotic treatment persist following birth.

Strengths and Limitations

The strength of this study lies in our utilization of clinically relevant Doppler techniques in a translational animal model to examine direct fetal consequences of antibiotic treatment for intrauterine infection. NHPs share many similarities with human pregnancy (placentation, endocrinology, labor mechanisms). These advantages, our ability to perform serial amniotic fluid sampling, the direct application of Doppler measurements used in human pregnancies and the known timing of intrauterine infection overcome some of the limitations of epidemiological studies (e.g. amniocentesis to confirm infection, retrospective data analysis). Furthermore, our NHP model recapitulates clinical features of infection-associated preterm labor with a robust amniotic and fetal inflammatory response, increased uterine contractility, cervical effacement, chorioamnionitis and fetal lung injury.[4,14] However, due to the clinical nature of this model, a limitation we faced was in coordinating the timing of treatment (based on clinical signs of preterm labor) with ultrasound timepoints. For this reason, sample size was not uniform between the infection and treatment groups. Additionally, the mode of infection utilized in this study of direct amniotic fluid inoculation potentially bypasses the early stages of ascending reproductive tract infection. This is a limitation of this experimental model compared to natural infection in women, however, this is balanced by the known timing of infection which is difficult to ascertain in observational clinical studies of women with intrauterine infection. Ongoing studies by the authors also include choriodecidual *Ureaplasma* inoculation in order to replicate an earlier stage of ascending intrauterine infection in this NHP model. We also acknowledge that the direct mechanisms involved in inducing fetal hemodynamic compromise have not been addressed in this study and in particular the examination of placental vascular alterations – a

potential mediator of the fetal hemodynamic responses to infection we observed – may be important to our understanding and the implementation of therapeutic strategies.

Conclusions

Our study advances the current understanding of mechanisms of FIRS as it evaluates changes in fetal hemodynamic and cardiac function in NHPs exposed to intra-uterine *Ureaplasma* infection, and with subsequent antibiotic therapy. This study supports the use of maternal antibiotic therapy for treatment of intrauterine infection and suggests that Doppler ultrasound may be a useful tool for identifying inflammation-mediated fetal cardiovascular dysfunction. Studies in women with known infection confirmed by amniocentesis have shown varied efficacy of treatment with broad-spectrum antibiotics,[15,16] indicating that the development of prognostic techniques to identify whether individual cases are responding to therapies could be beneficial when defining clinical management plans for intrauterine infection and in improving neonatal and long-term outcomes associated with preterm birth and pPROM.

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632 **Table 1. Fetal biometry, heart rate and sex distribution.**

	Control (N=7)	IAI (N=15)	IAI+AZI (N=8)	p-value
GA(days)	133 ± 3	134 ± 4	136 ± 5	0.433
BPD (mm)	46.0 ± 1.4	44.2 ± 2.1	44.3 ± 1.9	0.156
AC (mm)	134.9 ± 7.1	129.6 ± 11.6	133.2 ± 21.0	0.752
FL (mm)	36.2 ± 1.8	35.5 ± 2.5	37.2 ± 2.1	0.332
Fetal Heart Rate (BPM)	181 ± 29	181 ± 13	179 ± 7	0.960
Fetal Sex (male)	42.9%	53.3%	37.5%	0.749

633 IAI, intra-amniotic infection; IAI+AZI, Azithromycin treatment group. GA, gestational age; BPD,
634 biparietal diameter; AC, abdominal circumference; FL, femur length; BPM, beats per minute.
635 P-value <0.05 was considered significant. Data given as mean ± SD or %.

636 **Table 2. Uteroplacental and fetal hemodynamic parameters**

	Control (N=7)	IAI (N=15)	IAI+AZI (N=8)	p-value
UA PI	0.95 ± 0.09	1.26 ± 1.84*	1.10 ± 0.15	0.008
Abnormal UA PI (>1.1)	0.0%	78.6%*	50.0%	0.017
MCA PI	1.48 ± 0.32	1.45 ± 0.14	1.47 ± 0.12	0.970
CPR	1.30 ± 0.19	1.18 ± 0.19	1.24 ± 0.19	0.471
RPA PI	6.28 ± 3.07	10.05 ±3.52*#	6.71 ± 2.39	0.034
Ductus arteriosus PI	2.30 ± 0.33	2.40 ± 0.39	2.55 ± 0.39	0.551
Aortic isthmus PI	1.96 ± 0.33	3.06 ± 1.32	3.55 ± 1.53	0.110
Descending aorta PI	1.43 ± 0.18	1.89 ± 0.37	2.08 ± 0.52^	0.017

637 IAI, intra-amniotic infection; IAI+AZI, Azithromycin treatment group; UtA, uterine artery; PI,
638 pulsatility index; UA, umbilical artery; MCA, middle cerebral artery; CPR, cerebro-placental
639 ratio; RPA, right pulmonary artery. P-value <0.05 was considered significant by one-way
640 ANOVA. Symbols indicate significant differences between groups by post-hoc analysis: *Control
641 vs IAI; ^ Control vs IAI+AZI; #IAI vs IAI+AZI. Data expressed as mean ± SD or %.

642 **Table 3. Fetal cardiac functional parameters and cardiac outputs**

	Control (N=7)	IAI (N=15)	IAI+AZI (N=8)	p-value
Tricuspid E/A ratio	0.74 ± 0.09	0.80 ± 0.11	0.74 ± 0.16	0.577
Mitral valve E/A ratio	0.73 ± 0.08	0.68 ± 0.09**#**	0.81 ± 0.10	0.045
Left ventricular TEI	0.47 ± 0.04	0.45 ± 0.08	0.40 ± 0.07	0.235
Right SF (%)	29.22 ± 4.22	38.05 ± 10.51	33.34 ± 5.76	0.077
Left SF (%)	29.95 ± 3.18	34.98 ± 9.79	32.89 ± 10.67	0.079
Right SF/Left SF	1.77 ± 1.15	1.16 ± 0.44	1.11 ± 0.44	0.987
RCO (ml/min)	92.61 ± 27.60	100.04 ± 15.82	109.86 ± 26.76	0.989
LCO (ml/min)	93.48 ± 18.03	61.93 ± 12.36***#**	72.98 ± 8.29	<0.001
CCO (ml/min)	189.14 ± 62.67	162.52 ± 24.73	169.92 ± 31.89	0.369
RCO/LCO	1.33 ± 0.21	1.60 ± 0.33	1.38 ± 0.21	0.092
Abnormal RCO/LCO (>1.6)	0.0%	53.3%***#**	27.6%	0.022

643 IAI, intra-amniotic infection; AZI, Azithromycin treatment group; E, peak velocity of the early
644 diastolic transmitral flow; A, peak velocity of the late diastolic transmitral flow; E/A ratio, ratio
645 of peak early vs late transmitral flow velocity; TEI, isovolumic contraction time plus isovolumic
646 relaxation time divided by ejection time; SF, shortening fraction; RCO, right cardiac output; LCO,
647 left cardiac output; CCO, combined cardiac output. P-value <0.05 was considered significant by
648 one-way ANOVA. Symbols indicate significant differences between groups by post-hoc
649 analysis:*Control vs IAI; # IAI vs IAI+AZI. Data expressed as mean ± SD or %.

650 **Table 4. Estimation by Quartile Regression of Amniotic Fluid Pro-Inflammatory Mediators following Intra-Amniotic Infection and**
651 **Azithromycin treatment.**

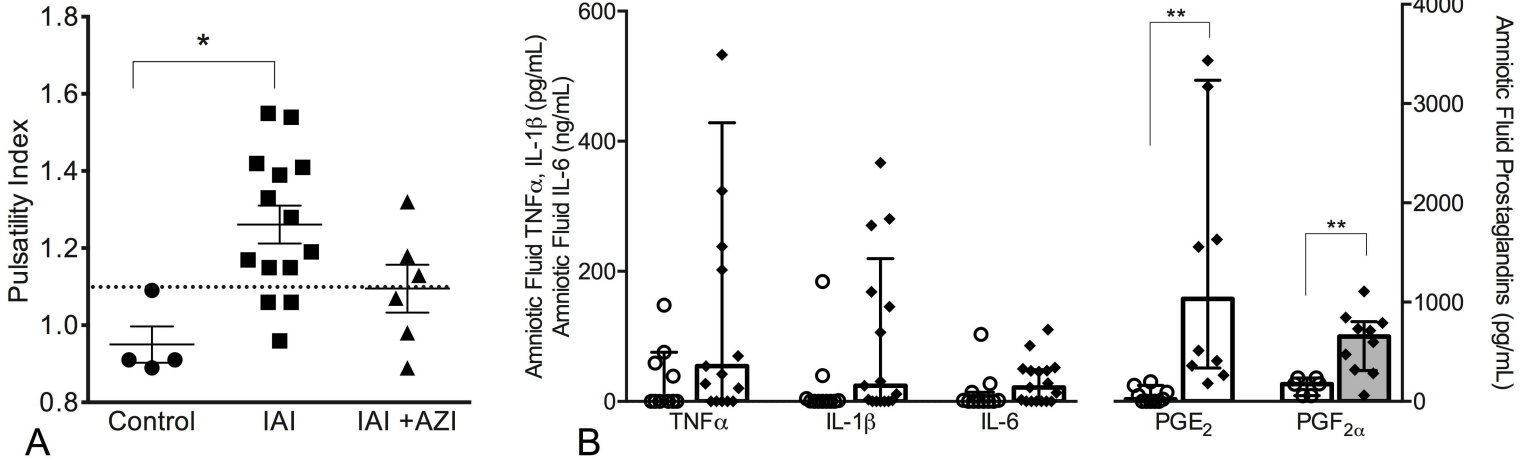
Mediator	Quartile	Control			IAI			IAI+AZI			p-value[1]	
		estimate	lower	upper	estimate	lower	upper	estimate	lower	upper	IAI vs. Con	IAI+AZI vs. Con
TNF-α	25% quartile	0.47	-∞	0.47	0.47	0.47	44.92	20.50	-∞	116.33	0.0155	0.0821
	50% quartile	0.47	0.47	0.47	54.20	18.25	266.45	84.70	24.79	211.68	<0.0001	0.0006
	75% quartile	0.47	0.47	∞	323.60	74.21	1681.11	202.00	116.17	∞	<0.0001	<0.0001
IL-1β	25% quartile	0.17	-∞	0.17	0.17	0.17	6.21	0.40	-∞	20.06	0.0155	0.0821
	50% quartile	0.17	0.17	0.17	11.60	1.26	119.23	17.30	0.51	152.35	<.0001	0.0006
	75% quartile	0.17	0.17	∞	145.60	27.40	468.69	24.00	20.04	∞	<.0001	<.0001
IL-6	25% quartile	1.38	-∞	1.38	1.38	1.38	13638.74	1.38	-∞	1382.55	0.0216	0.0989
	50% quartile	1.38	1.38	1.38	14281.60	57.88	40579.90	1598.20	0.95	76125.50	<.0001	0.0010
	75% quartile	1.38	1.38	∞	47130.60	25674.59	60766.71	45858.60	1483.25	∞	<.0001	<.0001
PGE_2	25% quartile	15.00	-∞	15.00	15.00	15.00	187.41	-0.14	-∞	-0.08	0.0278	0.2736
	50% quartile	15.00	15.00	88.68	196.70	15.91	1006.35	-1.19	-∞	∞	<.0001	0.0363
	75% quartile	15.00	15.00	∞	1134.60	368.62	3258.51	-0.14	-∞	∞	<.0001	0.0001
$PGF_{2\alpha}$	25% quartile	10.00	-∞	10.00	10.00	10.00	90.52	0.10	-∞	216.79	0.0216	0.2636
	50% quartile	10.00	10.00	10.00	176.00	-∞	567.37	0.10	-∞	∞	<.0001	0.0183
	75% quartile	10.00	10.00	∞	596.80	270.47	912.72	173.80	3.36	In	<.0001	<.0001

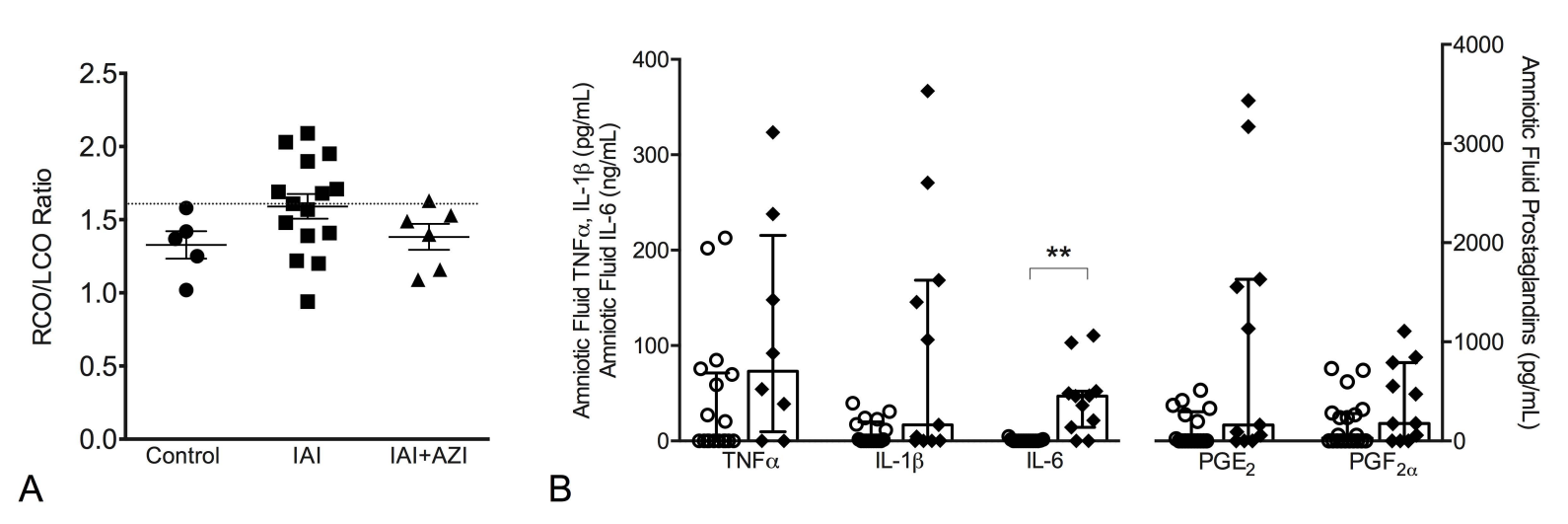
652 [1]Based on rank tests. The NORMAL, WILCOXON, and SIGN functions are implemented and suitable for iid error models, and the TAU
653 score function is implemented and appropriate for non-iid error models.
654 IAI, intra-amniotic infection; AZI, Azithromycin treatment group; ND, not detectable; TNF, tumor necrosis factor; IL, interleukin; PG,
655 prostaglandin; P-value <0.05 was considered significant

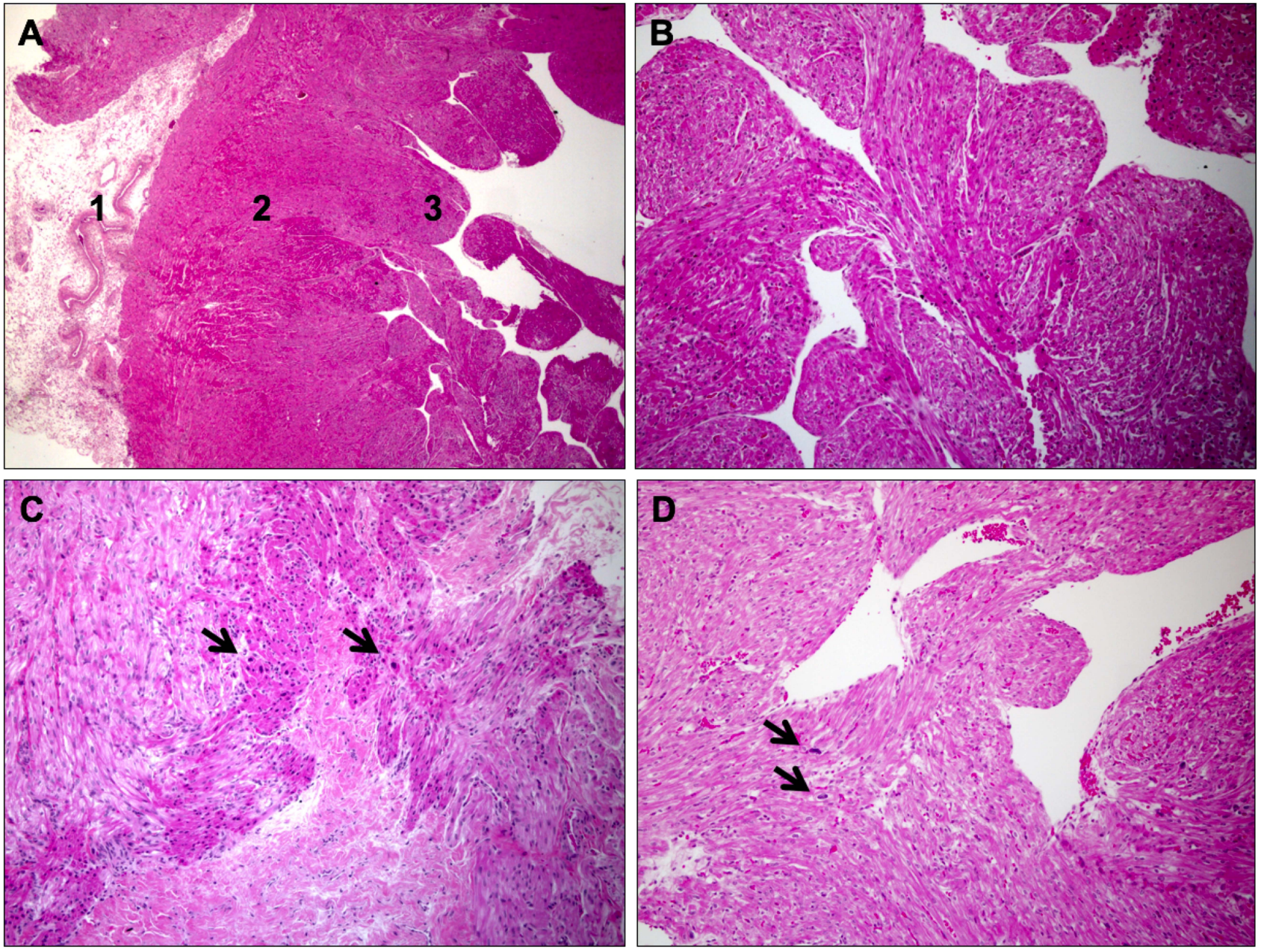
Figure 1 - Amniotic Fluid Pro-Inflammatory Cytokines for animals with Normal and Abnormal Umbilical Artery Pulsatility Index. (A) Umbilical artery pulsatility index is shown for animals in control, intra-amniotic infection (IAI) and in animals with intra-amniotic infection and maternal Azithromycin treatment (IAI + AZI). The threshold for abnormal values was PI >1.1 (indicated by dotted horizontal line). Values are expressed as mean ± SEM; * indicates significant difference between controls and IAI animals ($p<0.05$). **(B)** The concentrations of amniotic fluid pro-inflammatory mediators (TNF, tumor necrosis factor; IL, interleukin; PG, prostaglandin) are shown for animals with normal (open circles) vs abnormal (diamonds) umbilical artery PI values. Values are expressed as median (interquartile range) with statistical difference in mediator concentration indicated by * $p<0.05$ or ** $p<0.01$.

Figure 2 - Concentrations of Amniotic Fluid Pro-Inflammatory Cytokines for animals with Normal and Abnormal Cardiac Output Ratios. (A) The ratio of right to left ventricular cardiac output (RCO:LCO) is shown for animals in control, intra-amniotic infection (IAI) and in animals with intra-amniotic infection and maternal Azithromycin treatment (IAI + AZI). The threshold for abnormal values was PI >1.6 (indicated by dotted horizontal line). Values are expressed as mean ± SEM; **(B)** The concentrations of amniotic fluid pro-inflammatory mediators (TNF, tumor necrosis factor; IL, interleukin; PG, prostaglandin) are shown for animals with normal (open circles) vs abnormal (diamonds) RCO:LCO ratios. Values are expressed as median (interquartile range) with statistical difference in mediator concentration indicated by ** $p<0.01$.

677 **Figure 3**. **Conceptual model of fetal hemodynamic responses to intra-amniotic infection.**
678 Bacteria ascends from the lower reproductive tract and invades the amniotic cavity, resulting in
679 an immune response and production of pro-inflammatory mediators that can be detected in
680 the amniotic fluid. Microbial invasion of the amniotic cavity causes a fetal inflammatory
681 response and fetal hemodynamic changes that likely contributes to an increased risk of poor
682 outcomes for preterm infants exposed to intrauterine infection and inflammation. Maternal
683 antibiotic therapy mitigated the fetal hemodynamic changes observed in our non-human
684 primate model of intra-amniotic *Ureaplasma* infection, suggesting that appropriate
685 antimicrobial treatments may be able to rescue fetal injury in addition to delaying infection-
686 associated preterm labor (*created with Biorender.com*).


Pulsatility Index
1.8
1.6
1.4
1.2
1.0
0.8
*
Control
IAI
IAI +AZI
A
Amniotic Fluid TNFα, IL-1β (pg/mL)
Amniotic Fluid IL-6 (ng/mL)
600
400
200
0
TNFα
IL-1β
IL-6
**
**
PGE2
PGF2α
4000
3000
2000
1000
0
Amniotic Fluid Prostaglandins (pg/mL)
B


RCO/LCO Ratio
2.5
2.0
1.5
1.0
0.5
0.0
Control
IAI
IAI+AZI
A
Amniotic Fluid TNFα, IL-1β (pg/mL)
Amniotic Fluid IL-6 (ng/mL)
400
300
200
100
0
**
TNFα
IL-1β
IL-6
PGE2
PGF2α
4000
3000
2000
1000
0
Amniotic Fluid Prostaglandins (pg/mL)
B


A
1
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3
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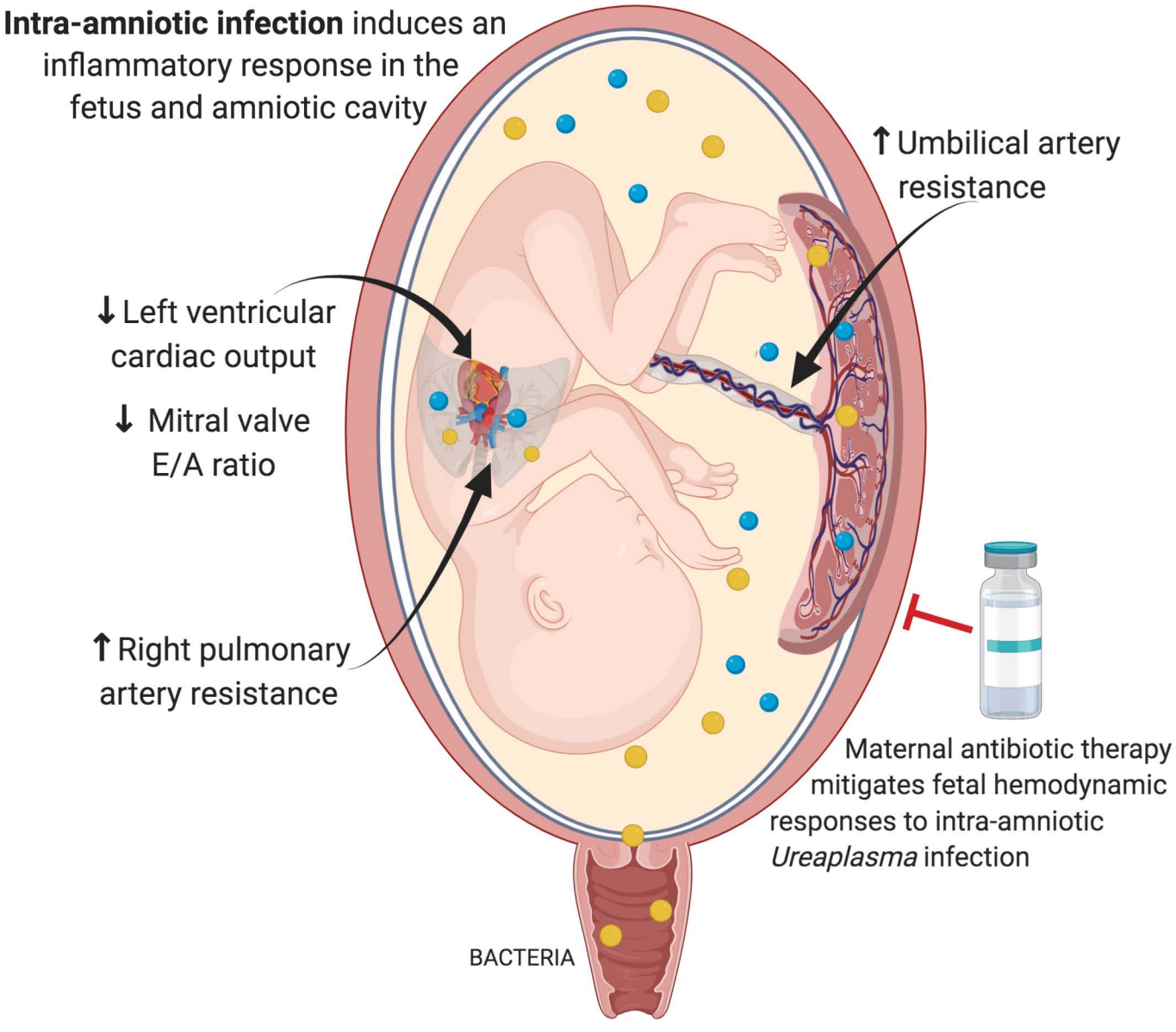

Fetal Hemodynamic Responses to Intra-amniotic *Ureaplasma* Infection

Increased placental vascular impedance

↓

Abnormal placental gas exchange

↓

Fetal hypoxemia

↓

Fetal hypoxic pulmonary vasoconstriction

↓

Reduced pulmonary venous return to the left atrium

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Reduced left ventricular preload and cardiac output