Effect of sublethal prenatal endotoxaemia on murine placental transport systems and lipid homeostasis

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Abstract

Based on accumulating evidence, infection alters the expression of transporters that mediate the placental exchange of xenobiotics, lipids and cytokines. We hypothesized that lipopolysaccharide (LPS) modifies the expression of placental transport systems and lipid homeostasis. LPS (150 µg/kg; i.p.) treatments were administered for 4 h or 24 h, animals were euthanized at gestational days (GD) 15.5 or 18.5, and maternal blood, foetuses and placentae were collected. Increased rates of foetal demise were observed at GD15.5 following LPS treatment, whereas at GD18.5, high rates of early labour occurred and were associated with distinct proinflammatory responses. LPS decreased placental fatty acid binding protein associated to plasma membrane (*Fabppm*) expression at GD15.5 (LPS-4 h), increased *Fat/Cd36* expression at GD18.5 (LPS-4 h) and reduced *Abcb1b*, *Abcc2* and *Abcc5* expression at GD18.5 (LPS-24 h). At the protein level, breast cancer-related protein (BCRP) and Abcg1 levels were decreased in the labyrinth zone (Lz) at both time points, whereas P-glycoprotein (P-gp) levels in the Lz were decreased at GD18.5. Specific maternal plasma and placental changes in triacylglycerol, free fatty acid, cholesterol, cholesterol ester and monoacylglycerol levels were detected in a gestational age-dependent manner. In conclusion, LPS was associated with increased foetal death and early labour by decreasing placental ABC transporter expression and altering maternal plasma and placental lipid homeostasis. These changes likely modify foetal xenobiotic exposure and placental lipid exchange in cases of bacterial infection.

Keywords: LPS; ABC Transporters; P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), cholesterol; ABCG1; *Fabppm; Fat/Cd36*

Introduction

Global estimates indicate that more than 15 million babies are born preterm every year [1]. Preterm birth (PTB) occurs at higher rates in low- and middle-income countries and may range from 5 to 18% of all pregnancies worldwide [2]. Of particular importance, low-income countries have a higher incidence of histological chorioamnionitis (HCA)-related genitourinary (retrograde or ascending bacterial) and malarial (haematogenous) infections. These infections may lead to severe systemic and placental inflammatory response [3–5] and become important triggers of inflammatory PTB pathways [1,6,7]. Other routes/risk factors for infection-associated PTB include maternal periodontal disease, transplacental transfer of pathogens, iatrogenic infection from complicating amniocentesis or chorionic villous sampling [7,8].

The most common microbes observed in HCA are the gram-negative bacteria Ureaplasma urealyticum, Mycoplasma hominis and Escherichia coli [7]. Lipopolysaccharide (LPS), an endotoxin enriched in the cell wall of gram-negative bacteria, binds Toll-like receptor 4 (Tlr-4) and is widely used to model gram-negative bacterial infections [9]. Studies in mice have shown that LPS exposure alters the foetal-placental unit in a gestational age-dependent manner. In early pregnancy, it induces embryonic loss [10] or miscarriage [11], whereas in mid-pregnancy, it causes foetal death, foetal growth restriction [12] and foetal brain injury [13].

Infection and inflammation have the potential to disrupt the syncytiotrophoblast barrier by modulating the expression and function of ATP-binding cassette (ABC) transporters [14–16]. These proteins are active transmembrane efflux transport systems that control the biodistribution of clinically relevant endogenous and exogenous substrates across the maternal-foetal interface. Examples of endogenous substrates include nutrients (cholesterol and other lipids), metabolites (bilirubin- and bile salt-conjugated compounds and oxysterols), steroid hormones (glucocorticoids,

mineralocorticoids, oestrogens, progestogens, and androgens) and immunological factors (cytokines and chemokines). Examples of exogenous substrates include therapeutic drugs (antibiotics, antiretrovirals, synthetic glucocorticoids and NSAIDs) and environmental toxins (organochlorine and organophosphorus pesticides, ivermectin and bisphenol A). As a result, ABC transporters limit the transfer of potentially harmful substrates to the foetus and control transplacental passage of nutrients (mostly lipids) and other maternally derived substances in a gestational age-dependent manner [17]. In addition, the biodistribution of cytokines and chemokines within gestational tissues is modulated by the actions of placental ABC transporters and, as such, may be involved in the pathogenesis of PTB.

Importantly, cultured human primary villous trophoblast cells exposed to LPS and to the viral double-stranded RNA (dsRNA) analogue polyinosinic:polycytidylic acid (polyI:C) exhibit markers of insulin resistance and increased amino acid uptake [18]. Treatment of trophoblast cells with cytokines, such as interleukin (IL)-1β and IL-6, elicited similar responses [19,20] indicating that infection may alter placental nutrient uptake and foetal transfer. However, the effect of infection on placental lipid uptake and foetal transfer is less well understood.

In the present study, we determined whether a bacterial infection at different stages of gestation modulates the levels of selected ABC transporters in the placenta, which in turn might alter foetal exposure to potentially harmful substrates. Furthermore, we investigated the maternal plasma and placental levels of lipid fractions and the mRNA expression of key placental lipid transporters in dams exposed to a sublethal LPS dose to elucidate the possible effects of LPS on altering lipid homeostasis related or unrelated to placental ABC transporter-mediated lipid exchange. We hypothesized that LPS exposure modifies the expression of key placental ABC and lipid transporters, as well as maternal and placental lipid homeostasis in a gestational age-dependent manner.

Methods

Animal experimentation and study design

This study was approved by the Animal Care Committee of the Health Sciences Center, Federal University of Rio de Janeiro (CEUA-190/13) and registered within the Brazilian National Council for Animal Experimentation Control. The study complied with the "Principles of Laboratory Animal Care" generated by the National Society for Medical Research and the U.S. National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

Virgin female and male C57BL/6 mice (8-10 weeks of age) were housed in a temperature-controlled room (23 °C) on a 12/12 h light/dark cycle, with free access to fresh food and water. Female mice in the oestrous phase (identified by vaginal cytology) were time-mated with C57BL/6 males and assigned (gestational day (GD) 0.5) to different groups (Supplementary Figure 1). LPS (150 µg/kg; intraperitoneal injection, i.p., in a single dose) or vehicle (i.p. injection of a single dose) was administered to mice in mid-(GD14.5/15.5) or late-(GD17.5/18.5) pregnancy for 24 or 4 h, respectively. Animals were euthanized at GD15.5 or GD18.5 at the end of the 4 or 24 h treatments with a single dose of LPS/vehicle, and maternal blood, foetuses and placentae were collected. Foetal and placental tissues were weighed, and three placentae per litter were selected for further study. The selection was based on placentae with weights closest to the mean, an approach that we have used previously [5,21–24]. The LPS dose (150 µg/kg) was selected because it has previously been shown to cause an acute maternal inflammatory response with less than 50% foetal death in mid-pregnancy at 4 h after treatment [25].

qPCR

Total placental RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Life Technologies, CA, USA). The total RNA concentration was assessed using a nanophotometer (Implen, Munchen, Germany), and samples with RNA purity (260/280 absorbance) ratios ranging between 1.8 and 2.0 and with proven RNA integrity (confirmed through gel electrophoresis) were included in the study. Total RNA (1 µg) was reverse transcribed into cDNAs using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, São Paulo, Brazil) according to the manufacturer's instructions.

The mRNA levels of selected ABC transporters, lipid metabolism-related genes, proinflammatory cytokines and chemokines (Table 1) were evaluated using qPCR according to the manufacturer's recommendations (EVAGREEN; Solis Byodine, EUA) and using the Master Cycler Realplex system (Eppendorf, Germany) with the following cycling conditions: combined initial denaturation steps at 50°C (2 min) and 95°C (10 min), followed by 40 cycles of denaturation at 95°C (15 s), annealing at 60°C (30 s) and extension at 72°C (45 s). Relative gene expression was quantified using the 2-ΔΔCq method [26]. Assays with 95-105% efficiency were considered acceptable.

Gene expression was normalized to the geometric mean of selected reference genes in each experimental group, which exhibited stable expression levels following LPS challenge (Supplementary Table 1). The geometric mean expression of *B2m* and *β-actin* genes was used to normalize mRNA expression at GD15.5, whereas the geometric mean expression of *Gapdh* and *Ywhaz* reference genes was used to normalize mRNA expression of GD18.5. Intron-spanning primers, reverse transcriptase-negative samples and a melting curve analysis obtained for each qPCR were used to exclude DNA contamination.

Histological and Immunohistochemical analysis

Placental discs were fixed with 4% buffered paraformaldehyde (PFA), dehydrated with increasing concentrations of ethanol, diaphanized in xylene, embedded in paraffin and sectioned (5 µm) using a Rotatory Microtome CUT 5062 (Slee Medical GmbH, Germany) for periodic acid-Schiff (PAS) staining and immunohistochemistry as previously described [5].

Briefly, PAS staining was performed by oxidizing placental sections with 0.5% periodic acid (Sigma-Aldrich, Missouri, USA) for 15 min. Sections were washed with distilled water and incubated (10 min at room temperature) with Schiff's reagent (Merck, Germany), followed by haematoxylin (Proquímios, Rio de Janeiro, Brazil) staining. The junctional zone

(Jz) interface between the maternal and foetal placental cellular components was visually identified, and the area of each region of interest, the Jz and the labyrinth zone (Lz), was measured using ImageJ software (National Institutes of Health, Maryland, USA).

Immunohistochemical staining was performed by incubating sections with Tris-EDTA buffer (pH 9.0) for 15 min, followed by immersion in sodium citrate buffer (pH 6.0) for 8 min (in a microwave). Sections were immersed in a bovine serum albumin (3% - in PBS) solution to block nonspecific antibody binding sites and then incubated with primary antibodies against P-glycoprotein (P-gp-1:500; Santa Cruz Biotechnology, Texas, USA), breast cancer resistance protein (Bcrp-1:100; Merck Millipore, Massachusetts, USA) or Abcg1 (1:200; Abcam Plc, UK) overnight at 4 °C. Normal serum was incubated with negative control sections instead of primary antibodies. Sections were then washed with PBS (3 x 5 min) and incubated with a biotin-conjugated secondary antibody (SPD-060 - Spring Bioscience, California, USA) for 1 h followed by an incubation for 1 h with streptavidin (SPD-060 - Spring Bioscience, California, USA). The reaction was halted with 3,3-diaminobenzidine (DAB) (SPD-060 - Spring Bioscience, California, USA) followed by haematoxylin (Proquímios, Brazil) staining.

Digital images of histological staining were acquired using a high-resolution Olympus DP72 camera (Olympus Corporation, Japan) attached to the Olympus BX53 microscope (Olympus Corporation, Japan). P-gp, Bcrp and Abcg1 staining were quantified using Image-Pro Plus 5.0 software (Media Cybernetics, Maryland, USA), where the percentage of stained tissue area was calculated and negative spaces were excluded. In each experimental group, 30 digital images per placenta (15 digital images for each labyrinthine and spongiotrophoblast area) were evaluated [5].

Lipid analysis

Lipid extraction and analysis were performed using the method described by Bligh and Dyer with some modifications [27]. Maternal plasma (30 µl) and placentae (10 mg) were separately mixed with a solution containing chloroform (1 mL), methanol (2 mL) and water (0.8 mL) with intermittent shaking. After 2 h, the solution was centrifuged (1500 g, 20 min, 4 °C; Sorvall RC-5b; Sorvall Centrifuge, Newtown, CT, USA), the supernatant was collected, and a water-chloroform solution (1:1 v/v) was added. The mixture was shaken and centrifuged (1500 g, 20 min, 4°C), and the organic phase was removed and dried under nitrogen gas. The lipid classes were separated by one-dimensional thin layer chromatography (TLC) for neutral lipids using a solution containing hexane, diethyl ether and acetic acid (60:40:1 v/v). Plates were immersed in a solution composed of 3% C_uSO_4 and 8% H3PO4 (v/v; 10 s) and then heated (110°C, 10 min) [28]. TLC plates were analysed by densitometry using ImageMaster Total Lab 4.1 software (Total Lab Ltd, Newcastle, UK). Standards for each lipid species were used to identify different lipid classes (Sigma-Aldrich, Sao Paulo, Brazil). Their IDs and catalogue numbers are as follows: triacylglycerol, 1,3-dipalmitoyl-2-oleoylglycerol, #D2157; cholesteryl ester, cholesteryl oleate, #C9253; free fatty acids, palmitic acid, #76119; cholesterol, #C8667; and monoacylglycerol, #M2140.

A TLC plate with a standard curve for each of the lipid species was developed to quantify the lipid classes. Densitometric units from the standard curve were compared with densitometric units from the samples (which were already normalized to the unit of tissue or amount of plasma). TLC plates were developed in the mobile phase for 1 h. Supplementary Figures 3 and 4 show the TLC plates with plasma and placenta samples from different groups.

Measurement of plasma cytokine and chemokine levels

Maternal plasma was collected by cardiac puncture, transferred into heparinized tubes on ice, centrifuged (1,077 g, 15 min) and frozen (−80 °C). The levels of interleukin (Il)-1β, Il-6, monocyte chemoattractant protein-1 (Mcp-1/Ccl2) and chemokine (C-X-C motif) ligand 1 (Cxcl1) were assessed using the MILLIPLEX-MAP kit Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, USA) according to the manufacturer's recommendations. Fluorescence intensity was detected using a Luminex 200™ system (Merck Millipore, Massachusetts, USA).

Statistical Analysis

Normality tests were applied followed by Student's t-test or the nonparametric Mann-Whitney test to compare two variables. Pregnancy parameters were evaluated using the mean value of placentae/fetuses in each litter and not individuals [22]. For qPCR, PAS/immunostaining, lipid fraction analysis and cytokine/chemokine measurements, three placentae with the closest weight to the mean placental weight within each litter were selected. Thus, "n" represents the number of litters [5,21–23,25]. Values for all data are presented as the means ± SEM. GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA) was used to conduct statistical analyses, and differences were considered significant when $P<0.05$.

Results

Acute sub-lethal effects of LPS on pregnancy outcome

The sublethal LPS treatment elicited different pregnancy outcomes that varied according to the time of exposure and gestational age. At GD15.5, LPS at 4 h induced the death of 26% of foetuses, whereas at 24 h, an 84% foetal death rate was observed. This high percentage of foetal death prevented us from conducting a further placental analysis in the 24 h group. Conversely, the same LPS dosage induced only 1 and 2% foetal death rates at GD18.5 after 4 h and 24 h of LPS exposure, respectively. However, a sublethal LPS treatment for 24 h in late pregnancy (GD18.5) induced a 64% increase in early labour compared to controls, i.e., fourteen of twenty-two dams exhibited signs of labour within 24 h (table 1) and, therefore, were not included in the study. Importantly, the average gestation length for C57BL/6 mice is 19.25 days (range GD18-22) [5,29], whereas PTB in C57BL/6 mice may occur prior to GD18 [5]. Since we were unable to determine the precise birth time within the 24 h limit of LPS treatment (i.e., GD17.5 or GD18.5) in our cohort, we

Table 1. Pregnancy outcomes following sublethal LPS challenge in mid and late pregnancy.

LPS Injection Day	Groups	Exposure (h)	N (Dams)	Early Labor %	Foetal Death%	Foetal Weight (mg)	Placental Weight (mg)	F:P Ratio
E15.5	Control LPS	4	8 8	0 0	10 (8/81) 36 (19/53)	410±30 400±30	95±2 90±3	4.3±0.35 4.3±0.12
E14.5	Control LPS	24	6 4	0 0	3 (1/30) 87 (20/23)	------ ------	------ ------	------ ------
E18.5	Control LPS	4	9 10	0 0	0 (0/35) 1 (1/68)	1149±50 1145±40	80±0.9 90*±2.0	14.16±0.6 12.80±0.64
E17.5	Control LPS	24	12 22	0 64 (14/22)	2.5 (2/80) 2.0 (1//51)	1130±50 950*±60	82±0.9 92*±3	13.39±0.54 10.72**±0.72

Fetal death %, fetal weight, placental and fetal:placental weight (F:P) ratio values are extracted from pregnancies that did not undergo early labor, or from fetuses that did not show signs of demise. T-student test. *P<0.05; **P<0.01.

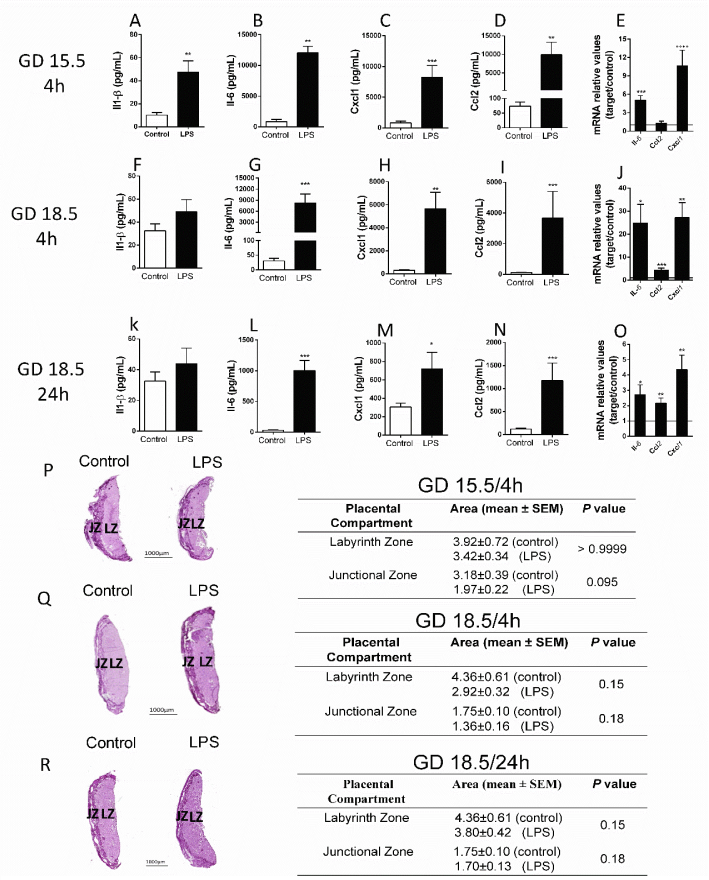

Figure 1. L**PS challenge elicited an acute maternal inflammatory response at GD 15.5 and 18.5**. Plasma levels of (A) Il-1β, (B) Il-6, (C) Cxcl1 and (D) Ccl2 in dams at GD15.5, 4 h after LPS exposure (control group, n=8, LPS group, n=8). (E) Placental levels of the *Il6*, *Ccl2* and *Cxcl1* mRNAs in the same animals (control group, n=8, LPS group, n=8). Plasma levels of (F) Il-1β, (G) Il-6, (H) Cxcl1, and (I) Ccl2 in dams at GD18.5, 4 h after LPS exposure (control group, n=9, LPS group, n=10). (J) Placental levels of the *Il6*, *Ccl2* and *Cxcl1* mRNAs in the same animals (control group, n=9, LPS group, n=10). Plasma levels of (K) Il-1β, (L) Il-6, (M) Cxcl1, and (N) Ccl2 in dams at GD18.5 24 h after LPS exposure (control group, n=8, LPS group, n=10). (O) Placental levels of the *Il6*, *Ccl2* and *Cxcl1* mRNAs in the same animals (control group, n=8, LPS group, n=10). (P) Analysis of the gross placental morphology of dams at GD15.5, 4 h after LPS exposure, (Q) at GD18.5, 4 h after LPS exposure, and (R) at GD18.5, 24 h after LPS exposure. Periodic acid-Schiff staining of control and LPS-treated mice evaluating labyrinth zone (Lz) and junctional Zone (Jz) areas. n=5/group. Gene expression was normalized to the levels of the reference genes *B2m* and β*actin* (E), or *Gapdh* and *Ywhaz* (J and O).

opted to designate this mode of labor as early rather than preterm as a precaution. Importantly, all following placental analyses were performed in tissues from dams that did not undergo early labor or from fetuses that did not show signs of death. Supplementary Figure 1 depicts the actual number of animals included in the subsequent analyses.

Maternal and placental pro-inflammatory response to acute sublethal LPS exposure

Maternal plasma levels of cytokines/chemokines associated with the pathogenesis of PTB, including Il-1β, Il-6, chemokine (C-X-C motif) ligand 1 (Cxcl1) and monocyte chemoattractant protein-1 (Mcp-1/Ccl2) [30,31], were assessed in dams following sublethal LPS exposure at GD15.5 (LPS-4 h) and GD18.5 (LPS-4 and 24 h). At GD15.5 (LPS-4 h), maternal plasma levels of Il-1β, Il-6, Cxcl1 and Ccl2 (P<0.01) were increased compared to the control group (Figure 1A-D). At GD18.5, 4 and 24 h of LPS exposure increased maternal plasma Il-6, Cxcl1 and Ccl2 levels (P<0.05) (Figure 1F-I and 1K-N, respectively), but a significant increase in Il-1β levels was not observed (Figure 1F and 1K). In the placenta, the expression of the *Il-6* and *Cxcl1* mRNAs was significantly increased (P<0.05) following LPS challenge at all time points investigated. In contrast, *Ccl2* mRNA expression was unchanged at GD15.5 (4 h), but increased at GD18.5 (4 h and 24 h; P<0. 05) (Figure 1E, 1J and 1O).

Placental weight was decreased 4 h and 24 h after LPS exposure at GD18.5, while foetal body weight and the foetal:placental (F:P) weight ratio were reduced 24 h after LPS exposure at GD18.5 compared to controls (Table 1). No differences in gross placental morphology, including changes in Lz and Jz areas, were identified in any of the groups investigated (Figure 1P-R).

Gestational age-dependent sub-lethal LPS effects on placental ABC transporters

To investigate how bacterial infection impacts placental efflux transport potential, we investigated the expression of the of *Abca1*, *Abcb1a*, *Abcb1b*, *Abcb4*, *Abcc2*, *Abcc5*, *Abcf2*, *Abcg1 and Abcg2* mRNAs in the mouse placenta at GD15.5 (LPS-4 h) or

GD18.5 (LPS-4 and 24 h) following LPS challenge to determine the effects of bacterial infection on the placental efflux transport potential.

These specific ABC transporter genes were selected based on evidence showing their sensitivity to infection in other models and/or based on their importance to placental and yolk sac barrier function [5,14,15,32,33] . We did not observe significant differences 4 h after LPS treatments at GD15.5 and GD18.5 (Figure 2A-B); however, at GD18.5, the levels of the *Abcb1b (P*<0.05), *Abcc2 (P*<0.01) and *Abcc5 (P*<0.05) mRNAs were reduced (*P*<0.05 and <0.01) 24 h after the LPS insult (Figure 2C).

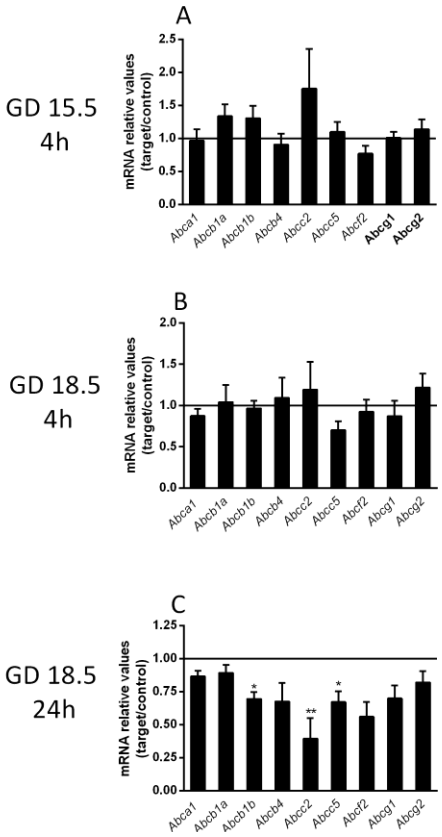

Figure 2. LPS challenge downregulated the expression of key ABC transporter genes at GD 18.5, 24 h after the insult. (A) Placental levels of the *Abca1*, *Abcb1a*, *Abcb1b*, *Abcb4*, *Abcc2*, *Abcc5*, *Abcf2*, and *Abcg2* mRNAs at GD15.5, 4 h after LPS exposure (A) and at GD18.5, 4 h (B) and 24 h (C) after LPS administration. 15.5/4 h: n=8 (control group); n=8 (LPS group). 18.5/4 h: n=9 (control group)/n=10 (LPS group). 18.5/24 h: n=12 (control group)/n=8 (LPS group). Gene expression was normalized to the levels of the reference genes (A) *B2m* and β*actin* or (B and C) *Gapdh* and *Ywhaz*.

P-gp and Bcrp are key multidrug resistance transporters that have been shown to play roles in foetal protection, whereas the lipid transporter Abcg1 is important for foetal nutrient transfer. P-gp immunostaining was detected in the cellular membrane of labyrinthine cells, with variable staining in the cellular membrane and cytoplasm of spongiotrophoblast cells (Figure 3A-F). Bcrp exhibited a similar placental distribution pattern, but with faint and variable nuclear staining in labyrinthine cells. Generally, greater Bcrp cytoplasmic labelling was observed in labyrinthine and spongiotrophoblast cells compared to P-gp-labelled placental cells (Figure 3G-L). The lipid transporter Abcg1 was predominantly localized to cellular membranes in the Lz and Jz, with some variable cytoplasmic staining throughout these layers (Figure 3M-R).

Using a semiquantitative analysis, we identified reduced P-gp staining intensity in the Lz at GD18.5, 4 h and 24 h (*P*<0.05) after LPS administration, compared to controls. No differences were noted after LPS treatment at GD15.5 (Figure 3A-F). The Bcrp staining intensity in the Lz was reduced in all LPS groups investigated (P<0.05). In contrast, Bcrp levels in spongiotrophoblast cells were higher at GD18.5, 4 h and 24 h (P<0.001) after LPS exposure. No differences in the levels of Bcrp in spongiotrophoblast cells were identified at GD15.5 after LPS treatment (Figure 3G-L). A lower Abcg1 staining intensity was observed in labyrinth cells at GD 15.5 (4 h) and 18.5 (24 h) (P<0.05), but the intensity increased in spongiotrophoblast cells at GD 18.5, 4 h and 24 h (P<0.01) after LPS exposure (Figure 3M-R).

LPS impacts maternal and placental lipid homeostasis throughout pregnancy

Since we observed an inhibitory effect of LPS on Abcg1 expression (a lipid transporter) in the Lz, we investigated the impact of sublethal LPS exposure on the maternal plasma and placental levels of various lipid classes (triacylglycerol, free fatty acids, esterified cholesterol, cholesterol, monoacylglycerol, and phospholipids) at GDs 15.5 and 18.5. These analyses were only performed at the 4 h time point at both gestational ages due to the lack of foetal viability after 24 h of LPS treatment at GD15.5.

We observed significant alterations in placental lipid levels following sublethal LPS challenge. Triacylglycerol, free fatty acid, cholesterol ester and free cholesterol levels were decreased at GD15.5 compared to the control groups (*P*<0.01, Table 2), whereas triacylglycerol, free fatty acid, free cholesterol, monoacylglycerol and phospholipid levels were increased at GD18.5 compared to the control groups (P<0.05, Table 3). The levels of the lipid classes monoacylglycerol and phospholipids remained unchanged at GD15.5 (Table 2), whereas cholesterol ester levels did not exhibit alterations at GD18.5 (Table 3).

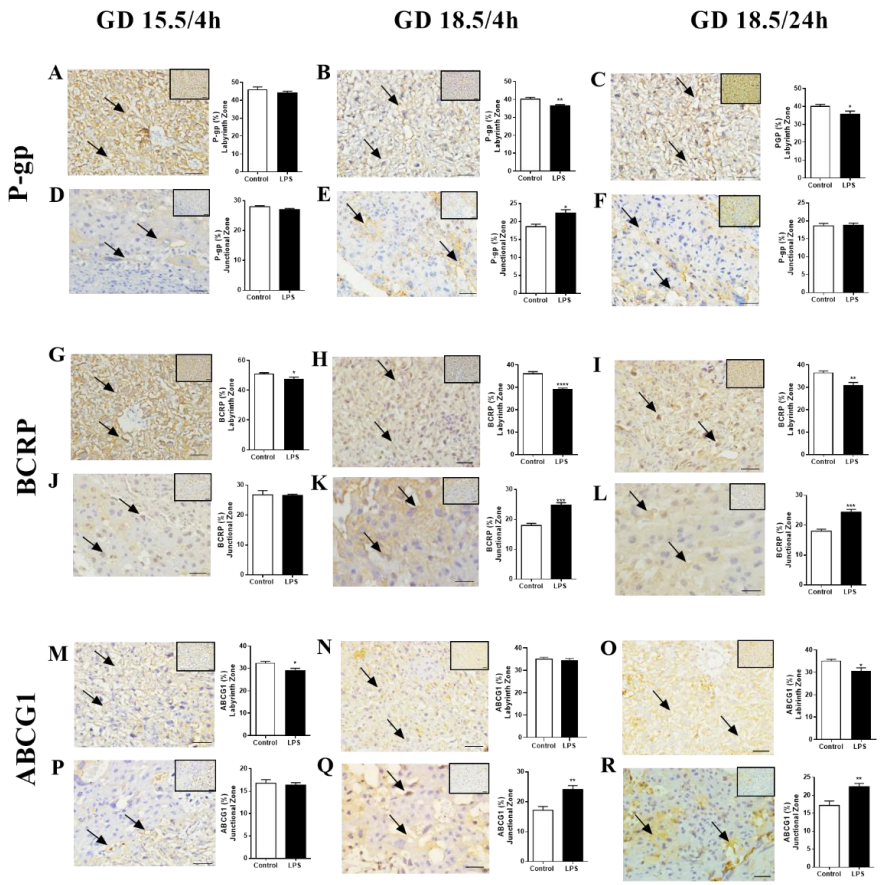

Figure 3. LPS challenge modulates key ABC transporter proteins in the mouse placenta. Representative photomicrographies of immunohistochemistry staining and semiquantitative analysis of P-gp in the labyrinth zone (Lz) (A-C) and junctional zone (Jz) (D-F) at GD 15.5 (4 h) and GD 18.5 (4 h and 24 h) after LPS administration. Bcrp expression in the Lz (G-I) and Jz (J-L), and Abcg1 expression in the Lz (M-O) and Jz (P-R) are shown. The pictures in the inserts represent the control to each immunostaining. The black arrows represent P-gp/Bcrp/Abcg1-stained cells. Graphs represent the % of positively stained cells. n=5/group. Scale bar = 50 μm.

We detected increased triacylglycerol levels and decreased free fatty acid and cholesterol ester levels in the maternal plasma from the LPS-exposed group at GD15.5 compared to the control group (P<0.001, table 4). At GD18.5, the triacylglycerol levels were decreased in the LPS group compared to the control group (*P*<0.05, table 5). No differences were observed in the levels of free cholesterol, monoacylglycerol and phospholipid classes at either gestational age, whereas the free fatty acid and cholesterol ester contents remained unchanged at GD18.5 (Tables 4 and 5).

Placental mRNA expression of key lipid transporters and lipid metabolism-related genes following sublethal LPS treatments was assessed. At GD15.5 (4 h), LPS exposure resulted in lower fatty acid binding protein associated with plasma membrane (*Fabp$_p$m*) mRNA levels (*p*<0.05), whereas at GD18.5 (4 h), increased placental Fat/*Cd36* translocase levels (*p*<0.01) were detected (Figure 4A-B). The mRNA expression of other lipid transporters and metabolism-related genes, such as *Pparg*, fatty acid transporter family protein (*Fatp1*) and lipase lipoprotein (*Lpl)*, remained unchanged throughout pregnancy.

Discussion

We have documented the effects of bacterial infection on placental ABC transporters and lipid homeostasis. LPS caused substantial foetal death at GD15.5 and triggered early labour in late pregnancy (GD18.5) without inducing foetal demise. These effects were likely mediated by distinct maternal and placental cytokine/chemokine responses to LPS throughout pregnancy and were associated with distinct expression profiles of placental efflux and lipid transporters, as well as changes in maternal and placental lipid levels.

Our present findings showing a greater susceptibility to LPS at GD15.5 are consistent with previous studies from our group [25]. However, here, we also show that sublethal LPS challenge in late pregnancy increased early labor, probably by intensifying the maternal and placental output of labor-inducing cytokines and chemokines. Our data are consistent with one study conducted in rats showing that LPS treatment (24 h) at GD18.5

induced early labur in 55% of pregnancies [34], a percentage similar to the value reported in our study (64%). Furthermore, LPS administration in late gestation decreased the foetal weight, stimulated placental growth and reduced the F:P weight ratio. These changes might indicate an impairment of placental nutrient transport efficiency that prevents foetuses from attaining optimal growth potential [30,31,34,35] and predisposing them to poor long-term health outcomes [35].

Table 2: LPS challenge modulates placental lipid fractions at GD15.5 after LPS administration

Placental lipid fractions	Groups	mean ± SEM (µg/mg)	*P* value
Triacylglycerols	Control LPS	0.05±0.002 0.03±0.002	0.0001****
Free Fatty Acids	Control LPS	0.014±0.001 0.008±0.001	0.0043**
Cholesterol Esters	Control LPS	0.18±0.02 0.10±0.01	0.005**
Cholesterol	Control LPS	0.05±0.003 0.03±0.004	0.0043**
Monoacylglycerols	Control LPS	0.02±0.0002 0.02±0.002	0.66
Phospholipids	Control LPS	0.073±0.003 0.077±0.005	0.51

15.5/4h: n=6 (control group); n=5 (LPS group).

Acute sublethal LPS exposure during pregnancy elicited an intense maternal and placental proinflammatory response, which varied across pregnancy. We observed a marked increase in a number of key proinflammatory factors (IL-6 and IL-8 and CCL2) related to PTB. In contrast, plasma Il-1β levels and placental levels of the *Ccl2* mRNA were not consistently affected by LPS, since they did exhibit a gestational age-dependent expression profile. IL-1β, IL-6 and IL-8 are commonly upregulated by infection and induce prostaglandin (PG)E2 and $PGF_{2\alpha}$ production to stimulate myometrial contractility and PTB [31,36,37]. CCL2 is subsequently upregulated in the human myometrium during preterm and term labor and recruits infiltrating leukocytes into the myometrium to amplify local inflammation and trigger the onset of labor [38]. CCL2 also induces the expression of proinflammatory cytokines, prostaglandins and leukotrienes in the myometrium during late pregnancy [39,40]. Importantly, Tlr4 activation induces the myometrial production of Il-1β, Il-6 and Ccl2 via nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) activation [41].

In the present study, LPS stimulated placental *Ccl2* expression in a gestational time-dependent manner, suggesting that this cytokine may participate in the pathogenesis of infective chorioamnionitis and the induction of early labor. Furthermore, our data extracted from pregnancies that did not undergo early labor indicate that a lack of Il-1β upregulation in these pregnancies may have been important in preventing an earlier onset of labor. Future studies comparing the maternal and placental inflammatory responses of pregnancies that did and did not undergo LPS-induced early labor are required to test this hypothesis. We evaluated the mRNA and protein expression/localization of selected ABC transporters involved in the biodisposition of clinically relevant substrates across pregnancy to understand how a sublethal bacterial infection alters placental efflux transport potential.

Table 3: LPS challenge modulates placental lipid fractions at GD18.5 after LPS administration

Placental lipid fractions	Groups	mean ± SEM (µg/mg)	*P* value
Triacylglycerols	Control LPS	0.04±0.009 0.08±0.008	0.03*
Free Fatty Acids	Control LPS	0.004±0.0005 0.03± 0.001	<0.0001****
Cholesterol Esters	Control LPS	0.23±0.02 0.19±0.01	0.09
Cholesterol	Control LPS	0.026±0.001 0.04±0.0008	<0.0001****
Monoacylglycerols	Control LPS	0.03±0.002 0.04±0.003	0.0002***
Phospholipids	Control LPS	0.065±0.002 0.071±0.002	0.04*

18.5/4h: n=9 (control group); n=7 (LPS group).

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As shown in our previous study, a sublethal LPS challenge (150 µg/kg) impairs placental P-gp activity at GD15.5 (4 h) in C57BL/6 mice, with no changes in placental *Abcb1a* and *Abcb1b* mRNA levels [25]. In the present study, no changes in P-gp-encoding genes were observed at GD15.5 (4 h) after LPS exposure. Rather, we found lower *Abcb1b* expression at GD18.5 (LPS-24 h) and a lower labyrinthine P-gp staining intensity at GD18.5 (LPS-4 and 24 h). Placental levels of P-gp and its

18.5/4h: n=9 (control group); n=7 (LPS group).

encoding genes are developmentally regulated in both rodents and humans, i.e., expression decreases towards term [42,43], resulting in reduced foetal protection against P-gp substrates in late pregnancy. Based on our results, a late-term bacterial infection may further decrease this already limited P-gp-mediated barrier function. These changes will likely increase the levels of cytokines and chemokines within gestational tissues, causing foetal demise and morbidity and/or inducing preterm/early labor pathways.

The results from the present and previous studies suggest that bacterial infection has the potential to increase foetal accumulation of P-gp substrates (cytokines, chemokines, endogenous and synthetic glucocorticoids, antibiotics, antiretrovirals, etc.) [14] both in earlier stages of pregnancy due to impaired placental P-gp activity [25] and in later stages of pregnancy by decreasing labyrinthine-P-gp expression. These changes will likely increase the levels of cytokines and chemokines within gestational tissues, causing foetal demise and morbidity and/or inducing preterm/early labour pathways.

Consistent with our data, P-gp is downregulated in a) human first trimester placental explants exposed to LPS [16], b) human PTB placentae with chorioamnionitis [15] and c) placentae from pregnant malaria-infected C57BL/6 mice exhibiting high rates of PTB [5]. Furthermore, P-gp activity is impaired in the developing foetal blood brain barrier of pregnant C57BL/6 mouse exposed to PolyI:C, a TLR3 viral mimic [44]. Together, these studies suggest that lower placental *Abcb1b* and P-gp levels are associated with infection-driven PTB [5,15] and/or earlier labor and delivery. The effects of lower placental P-gp levels on foetal health and postnatal development require further investigation.

Table 4: LPS challenge modulates plasma lipid fractions at GD15.5 after LPS administration

Plasma lipid fractions	Groups	mean ± SEM (µg/µl)	*P* value
Triacylglycerols	Control LPS	0.31±0.019 0.56± 0.02	<0.0001****
Free Fatty Acids	Control LPS	0.14±0.01 0.06±0.003	<0.0001****
Cholesterol Esters	Control LPS	0.31±0.02 0.19±0.01	0.0003***
Cholesterol	Control LPS	0.11±0.02 0.13±0.02	0.59
Monoacylglycerols	Control LPS	0.044±0.003 0.058±0.006	0.095
Phospholipids	Control LPS	0.30±0.07 0.38±0.03	0.26

15.5/4h: n=7 (control group); n=8 (LPS group).

Table 5: LPS challenge modulates plasma lipid fractions at GD15.5 after LPS administration

Plasma lipid fractions	Groups	mean ± SEM (µg/µl)	*P* value
Triacylglycerols	Control LPS	0.66±0.07 0.62±0.03	0.04*
Free Fatty Acids	Control LPS	0.06±0.01 0.08±0.01	0.44
Cholesterol Esters	Control LPS	0.16±0.03 0.22±0.07	0.4
Cholesterol	Control LPS	0.12±0.02 0.15±0.02	0.23
Monoacylglycerols	Control LPS	0.07±0.009 0.06± 0.007	0.26
Phospholipids	Control LPS	0.19±0.01 0.20±0.008	0.19

18.5/4h: n=9 (control group); n=7 (LPS group).

A lower level of Bcrp immunostaining was observed in the Lz at all time points investigated following LPS exposure. Placental Bcrp levels have been shown to be downregulated in human first trimester placental explants treated with LPS [16], in malaria-infected murine placentae [5] and in HTR8/SVneo (human extravillous trophoblast (EVT)-like) cells exposed to either LPS or to the TLR8 viral mimic single stranded RNA (ssRNA) [45]. Thus, different infective agents/challenges during pregnancy, including bacterial infection, have the potential to increase foetal accumulation of Bcrp substrates during pregnancy. However, BCRP was upregulated in human PTB placentae with chorioamnionitis [15] which is commonly induced by polymicrobial infection [7], and after treatment of trophoblastic cells with PGE2 [46], indicating that the nature of infective/inflammatory stimuli determines the trophoblastic-BCRP modulatory response.

Interestingly, the Bcrp and Abcg1 staining intensity in the spongiotrophoblast was higher at GD18.5 following LPS administration but not different at GD15.5. The role of ABC transporters in spongiotrophoblast cells is far less understood. Bcrp immunolocalization has been previously reported in spongiotrophoblasts, which remained unaltered in pregnancies in which the mother underwent nutritional manipulations [21]. Spongiotrophoblasts comprise the mouse placental Jz and provide structural support for the growth of the labyrinthine villi and limit foetal endothelium overgrowth [47]. However, very little is known about the possible functions of ABC transporters in the mouse Jz and how infection and inflammation impact Jz function.

Labyrinthine Abcg1 expression was also downregulated by LPS at GD15.5 and GD18.5. Abcg1 is a cholesterol and phospholipid efflux transporter predominantly localized to the basolateral membrane of the syncytiotrophoblast and in the endothelium of the foetal capillaries of the human placenta, suggesting that it mediates lipid efflux from the maternal compartment to the foetal compartment [14].

However, the directionality of Abcg1-mediated lipid exchange in the mouse placenta has yet to be described, preventing us from postulating on the effects of sublethal LPS exposure on placental Abcg1-mediated biodisposition of cholesterol and other lipids.

Maternal plasma and placental lipid levels were subsequently investigated to better understand how a bacterial infection alters lipid homeostasis at the maternal-foetal interface. Due to the high level of foetal demise in the GD 15.5 dams at the 24 h time point, we conducted lipid analysis at the 4 h time point at both gestational ages. Maternal levels of triacylglycerols and free fatty acids were altered after LPS exposure at GD15.5. Similar results have been reported in hepatic tissue following LPS exposure [48], which occurs in a gestational time-dependent manner. In fact, relevant alterations in plasma lipoproteins occur during injury or infection [49] or in patients with sepsis who occasionally present with hypertriglyceridemia [49]. The higher plasma triacylglycerol levels may be directly related to the inflammatory status in pregnant mice and may be associated with the high rates of foetal death at GD15.5. Nevertheless, higher placental triacylglycerol and free fatty acid levels were detected at GD18.5. This later effect may be an attempt to circumvent the lower foetal weight and one possible mechanism responsible for the higher placental weight observed in this group.

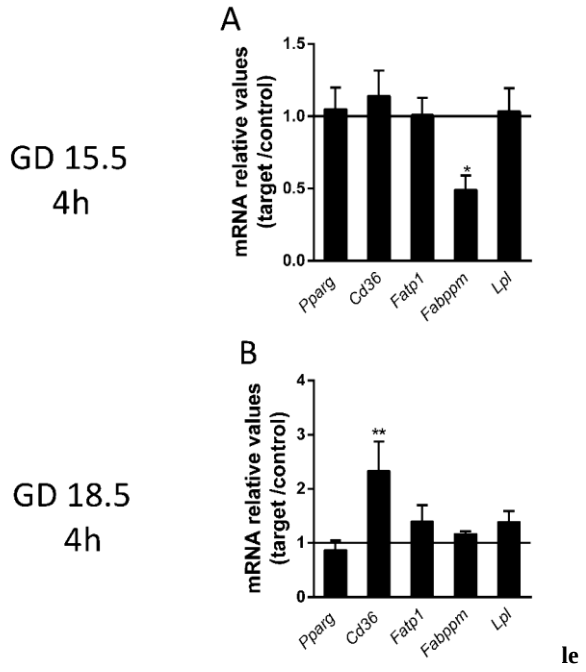

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Figure 4. LPS challenge modulates the mRNA expression of lipid transporters and associated proteins at GDs 15.5 and 18.5, 4 h after the insult. (A-B) Quantification of the relative mRNA expression of selected lipid transporters (*Pparg* and *Cd36*) and associated proteins (*Fatp1*, *Fabppm* and *Lpl*) at GD 15.5 and 18.5 4 h after LPS challenge. 15.5/4 h n=8 (control group); n=8 (LPS group). 18.5/4 h: n=9 (control group)/n=10 (LPS group). Gene expression was normalized to the levels of the reference genes (A) *B2m* and β*actin*, or (B) *Gapdh* and *Ywhaz*.

Placental fatty acid transport is modulated by different transport systems, including fatty acid transporter family proteins (FATPs), fatty acid binding proteins (FABPs), fatty acid binding protein associated with plasma membrane (FABPpm), lipase lipoproteins (LPL) and Fat/cd36 translocase located on both apical and basolateral membranes of the trophoblast [50,51] We observed decreased placental $Fabp_{pm}$ at GD15.5 and increased *Fat/cd36* mRNA expression at GD18.5 after LPS exposure. This finding may at least partially explain the different patterns of free fatty acid accumulation we observed in the present study. Foetuses (and intrauterine tissues) in pregnancies complicated by maternal bacterial infection may be exposed to higher levels of cytokines/chemokines, drugs and environmental toxins present in the maternal circulation. These changes may also be associated with suboptimal placental lipid storage. Combined, these changes may contribute to the inflammatory PTB pathways and lead to the onset of PTB or early labor.

In conclusion, this sublethal LPS model of bacterial infection during pregnancy may induce foetal death or early labor, depending on the gestational age. These outcomes are associated with specific maternal and placental inflammatory responses, altered expression of ABC and lipid transporters and altered maternal and placental lipid homeostasis

Acknowledgments:

This study was supported by the Bill & Melinda Gates Foundation (MCTI/CNPq/MS/SCTIE/Decit/Bill and Melinda Gates 05/2013; OPP1107597), the Canadian Institutes for Health Research (SGM: Foundation-148368), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 304667/2016-1, 422441/2016-3, 303734/2012-4, 422410/2016-0); Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES, finance Code 001); Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, CNE 2015/E26/203.190/2015); and PRPq-Universidade Federal de Minas Gerais (PRPq-UFMG, 26048).

Competing Interests

The authors declare no competing interests.

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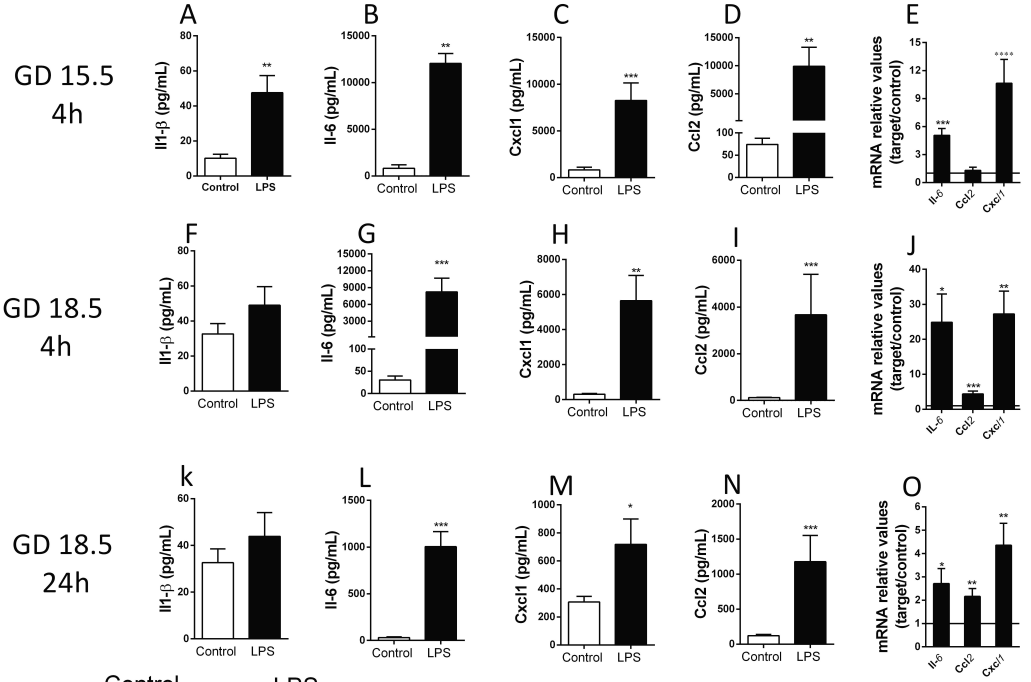
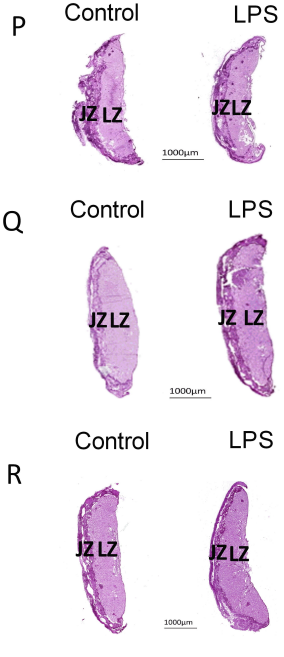




GD 15.5/4h

Placental Compartment	Area (mean ± SEM)	*P* value
Labyrinth Zone	3.92±0.72 (control) 3.42±0.34 (LPS)	> 0.9999
Junctional Zone	3.18±0.39 (control) 1.97±0.22 (LPS)	0.095

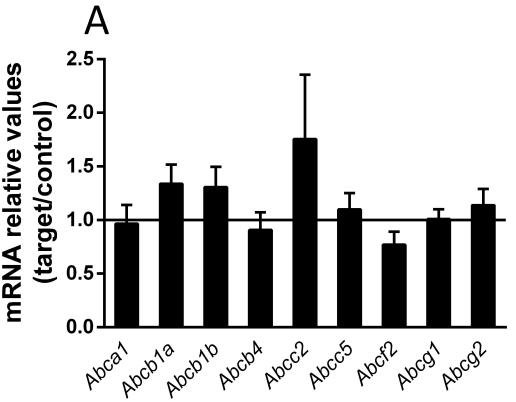
GD 18.5/4h

Placental Compartment	Area (mean ± SEM)	*P* value
Labyrinth Zone	4.36±0.61 (control) 2.92±0.32 (LPS)	0.15
Junctional Zone	1.75±0.10 (control) 1.36±0.16 (LPS)	0.18

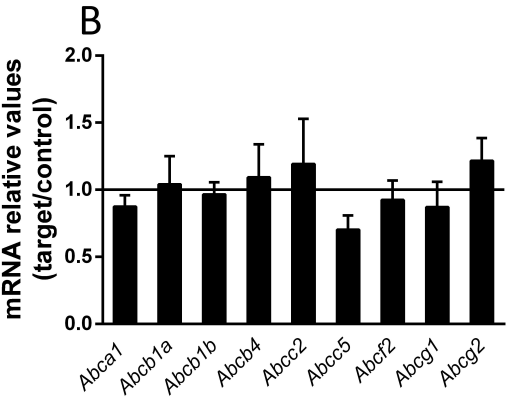
GD 18.5/24h

Placental Compartment	Area (mean ± SEM)	*P* value
Labyrinth Zone	4.36±0.61 (control) 3.80±0.42 (LPS)	0.15
Junctional Zone	1.75±0.10 (control) 1.70±0.13 (LPS)	0.18

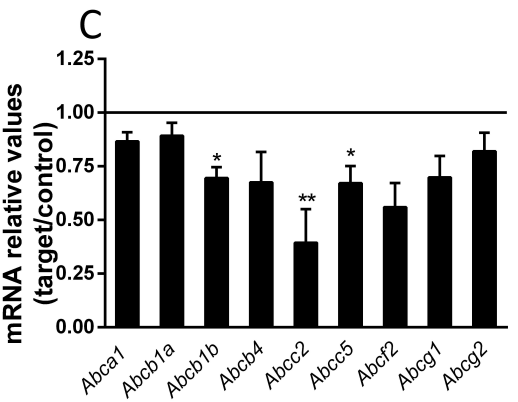
GD 15.5
4h

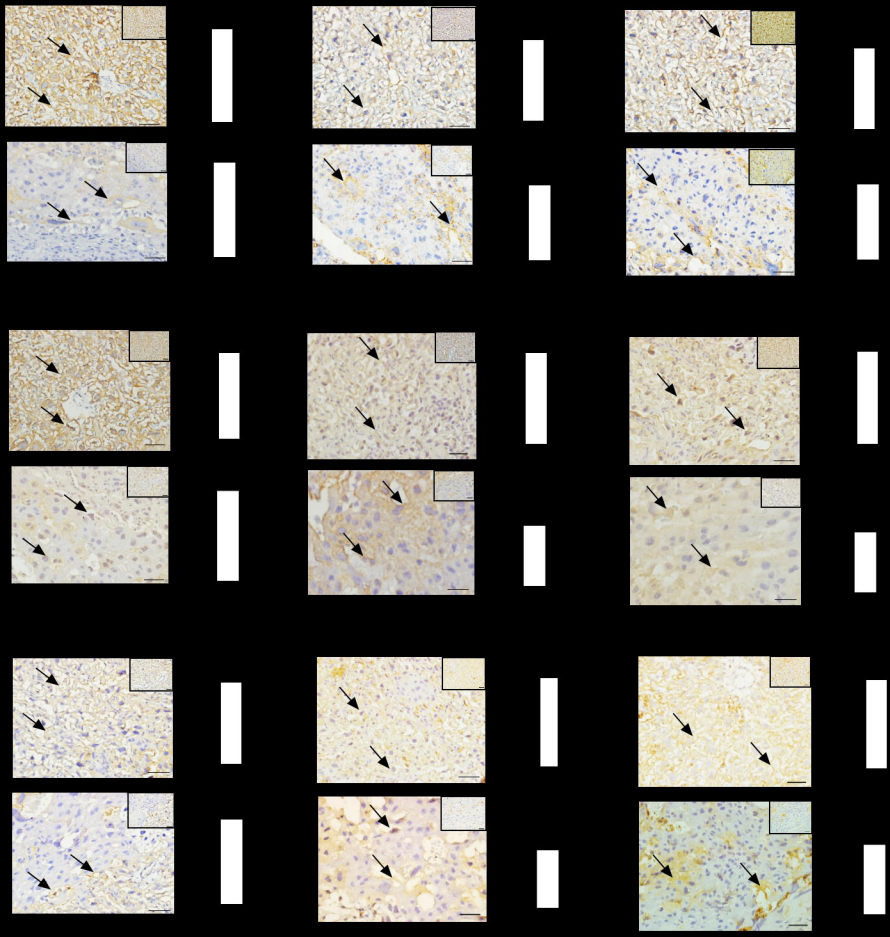


GD 18.5
4h

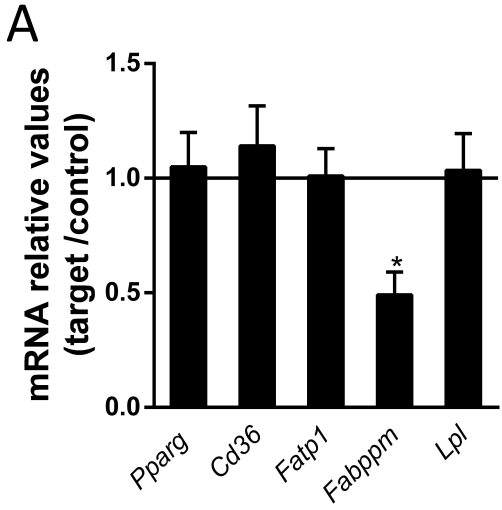
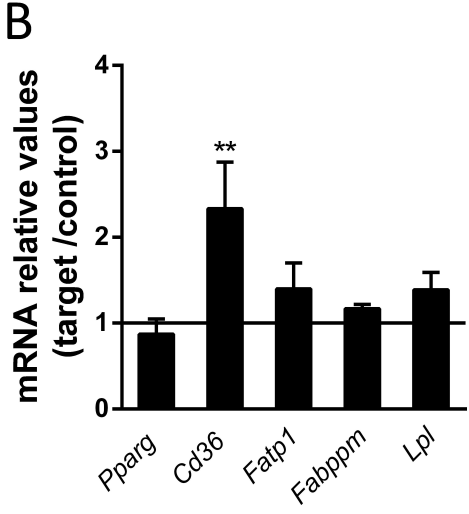


GD 18.5
24h





GD 15.5
4h



GD 18.5
4h