Effect of sub-lethal prenatal endotoxemia on murine placental transport systems and lipid homeostasis

Reginatto MW[1], Fontes KN[1], Monteiro VRS[1], Silva NL[1], Andrade CBV[1], Gomes HR[1], Imperio GE[1,3,6], Bloise FF[1], Kluck G[2], Atella GC[2], Matthews SG[3,4,5,6], Bloise E[3,6,7], Ortiga-Carvalho TM*[1].

[1]Laboratory of Translational Endocrinology; Institute of Biophysics Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.
[2]Laboratory of Lipids and Lipoproteins Biochemistry; Institute of Medical Biochemistry, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.
[3]Department of Physiology; Faculty of Medicine, University of Toronto, Toronto, Canada.
[4]Department of Obstetrics & Gynaecology; Faculty of Medicine, University of Toronto, Toronto, Canada.
[5]Department of Medicine, Faculty of Medicine, University of Toronto, Toronto, Canada.
[6]Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, Canada.
[7] Laboratory of Molecular Pathology, Department of Morphology, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.
*Correspondent author: taniaort@biof.ufrj.br

Abstract

There is growing evidence that infection impacts the expression of transporters that mediate placental exchange of xenobiotics, lipids and cytokines. We hypothesized that lipopolysaccharide (LPS), modifies the expression of placental transport systems and lipid homeostasis. LPS (150µg/Kg;ip.) treatments were conducted for 4h or 24h and animals were euthanized at gestational-days (GD) 15.5 or 18.5 and maternal blood, fetuses and placentae collected. Increased rates of fetal demise were observed at GD15.5 following LPS, whereas at GD18.5, high rates of early labor occurred; associated with distinct pro-inflammatory responses. LPS decreased placental *Fabppm* at GD15.5 (LPS-4h); increased *Fat/Cd36* at GD18.5 (LPS-4h) and reduced *Abcb1b, Abcc2* and *Abcc5* at GD18.5 (LPS-24h). At the level of protein, breast cancer-related protein (BCRP) and Abcg1 were decreased in the labyrinth (L) at both time-points, whereas P-glycoprotein (P-gp) in the L was decreased at GD18.5. Specific maternal plasma and placental changes in triacylglycerol, free fatty acids, cholesterol, cholesterol ester and monoacylglycerol were detected in a gestational-age dependent manner. In conclusion, LPS associated with increased fetal death and early labor decreased placental ABC transporter expression and altered maternal plasma and placental lipid homeostasis. These changes likely modify fetal 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

Globally, it is estimated that over 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 higher incidence of histological chorioamnionitis (HCA)-related to genitourinary (retrograde or ascending bacterial) and malarial (hematogenous) infections. These can lead to severe systemic and placental inflammatory responses[3–5] and become important triggers of inflammatory PTB pathways[1,6,7]. Other routes/risk factors of 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 found 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 infection[9]. Studies in mice have shown that LPS exposure impacts the fetal-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 fetal death, fetal growth restriction[12] and fetal brain injury[13].

Infection and inflammation have the potential to disrupt the syncytiotrophoblast barrier, via modulation of the expression and function of ATP-binding cassete (ABC) transporters[14–16]. These are active transmembrane efflux transport systems that control the biodistribution of clinically relevant endogenous and exogenous substrates, across the maternal-fetal interface. Examples of endogenous substrates include nutrients (cholesterol and other lipids), metabolites (bilirubin- and bile salts-conjugated compounds and oxysterols), steroid hormones (glucocorticoids, mineralocorticoids, estrogens, 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 fetus 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 can be modulated through the actions of placental ABC transporters and, as such, 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), exhibited markers of insulin resistance and increased amino acid uptake[18]. Treatment of trophoblast cells with cytokines, interleukin (IL)-1β and IL-6, elicited similar responses[19,20]; demonstrating that infection may alter placental nutrient uptake and fetal transfer. However, the effect of infection on placental lipid uptake and fetal transfer is less well understood.

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, which is in compliance 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) under a 12/12 h light/dark cycle, with free access to fresh food and water. Female mice in the estrous 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). Treatment with LPS (150µg/Kg; i.p.) or vehicle was undertaken in mid- (GD14.5/15.5) or in late- (GD17.5/18.5) pregnant mice for 24 or 4h respectively. Animals were euthanized at GD15.5 or GD18.5, 4 or 24h following LPS treatments and maternal blood, fetuses and placentae collected. Fetal and placental tissues were weighed and three placentae per litter were selected for further study. Selection was based on placentae with weights closest to the mean; an approach that we have used previously. [5,38,45–47]. The LPS dose (150µg/Kg) was selected as it has previously been shown to cause an acute maternal inflammatory response with less than 50% fetal death in mid-pregnancy, 4h after treatment [22].

qPCR

Total placental RNA was extracted using TRIzol reagent, according to the manufacturer's instructions (Life Technologies, CA, USA). Total RNA concentration was assessed using nanophotometer (Implen, Munchen, Germany) and samples with RNA purity (260/280 absorbance) ratio 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 converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, São Paulo, Brazil) according to the manufacturer's instructions.

Levels of mRNA for selected ABC transporters, lipid metabolism related genes, pro-inflammatory cytokines and chemokines (Table 1) were evaluated by qPCR following manufacturer's recommendations (EVAGREEN; Solis Byodine, EUA) and using the Master Cycler Realplex system (Eppendorf, Germany), with the following cycling conditions: combined initial denaturation at 50ºC (2 min) and 95ºC (10 min), followed by 40 cycles of denaturation at 95ºC (15s), annealing at 60ºC (30s) and extension at 72ºC (45s). Quantification of relative gene expression was calculated using 2-ΔΔCq method [48]. The assay with 95-105% efficiency was considered acceptable.

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

Histological and Immunohistochemical analysis

Placental discs were fixed in 4% buffered paraformaldehyde (PFA), dehydrated with increasing concentrations of ethanol, diaphanized in xylene, paraffin-embedded and sectioned (5µm), 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 undertaken by oxidizing placental sections with 0.5% periodic acid (Sigma-Aldrich, Missouri, USA) for 15 minutes. Sections were washed in distilled water and incubated (10 mins at room temperature) with Schiff's reagent (Merck, Germany), followed by hematoxylin (Proquímios, Rio de Janeiro, Brazil) staining. The junctional zone (Jz) interface between the maternal and fetal placental cellular components was visually identified and the area of each region of interest, the junctional zone (Lz) and labyrinth (L) was measured using Image J software (National Institutes of Health, Maryland, USA).

Immunohistochemical analyses were performed by incubating sections with Tris-EDTA buffer (pH 9.0) for 15 mins, followed by an immersion in sodium citrate buffer (pH 6.0) for 8 mins (in microwave). In order to block non-specific antibody binding sites, slides were immersed in bovine serum albumin (3% - in PBS) solution, prior to primary antibody incubation with 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. For negative control sections, normal serum was incubated instead of primary antibodies. Sections were then washed in PBS (3 x 5 minutes) and incubated with biotin-conjugated secondary antibody (SPD-060 - Spring Bioscience, California, USA) for 1 hour, followed by incubation for 1 hour with streptavidin (SPD-060 - Spring Bioscience, California, USA). The reaction was halted with 3,3-diamino-benzidine (DAB) (SPD-060 - Spring Bioscience, California, USA) followed by hematoxylin (Proquímios, Brazil) staining.

Histological digital images 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 quantification was undertaken using Image Pro Plus 5.0 software (Media Cybernetics, Maryland, USA), where 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 areas) were evaluated[5].

Lipid analysis

Lipid extraction and analysis was performed using the method of Bligh and Dyer modified[49]. Maternal plasma (30 µl) and placentae (10 mg) were separately mixed to a solution containing chloroform–methanol–water (2:1:0.8 v/v) with intermittent shaking. After two hours, the solution was centrifuged (1500g, 20 mins, 4 °C; Sorvall RC-5b; Sorvall Centrifuge, Newtown, CT, USA), and the supernatant collected, and water-chloroform solution (1:1 v/v) added. The mixture was shaken and centrifuged (1500 g, 20 mins, 4°C), and the organic phase removed and dried using gas nitrogen. 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% CuSO4 and 8% H3PO4 (v/v; 10s) and then heated (110°C, 10 mins)[50]. TLC plates were analyzed by densitometry using the ImageMaster Total Lab 4.1 software (Total Lab Ltd, Newcastle, UK).

Measurement of plasma cytokine and chemokine levels

Maternal plasma was collected by cardiac puncture, transferred into heparinized tubes on ice, centrifuged (1,077g, 15 min) and frozen (−80 °C). Determination of interleukin (Il)-1β, Il-6, monocyte chemoattractant protein-1 (Mcp-1/Ccl2) and the 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 Luminex 200 ™ system (Merck Millipore, Massachusetts, USA).

Statistical Analysis

Normality tests were applied followed by Student's T-test or non-parametric Mann-Whitney test, to compare two variables. Pregnancy parameters were evaluated using the mean value of placentae/fetuses in each litter and not individuals[45]. 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[5,38,45–47]. Values for all data are expressed as mean ± SEM. The GraphPad prism6 software (GraphPad Software, Inc., San Diego, CA, USA) was used to conduct statistical analysis and differences were considered significant when $P<0.05$.

Results

Acute sub-lethal effects of LPS on pregnancy outcome

Sub-lethal LPS treatment elicited different pregnancy outcomes that varied according to time of exposure and gestational age. At GD15.5, LPS-4h induced 26% fetal death, whereas at 24h there was 84% fetal demise. This high percentage of fetal death prevented us from undertaking further placental analysis in the 24h group. Conversely, the same LPS dosage induced only 1 and 2% of fetal death at GD18.5, 4h and 24h after LPS exposure, respectively. However, sub-lethal LPS treatment for 24h in late pregnancy (GD18.5), induced a 64% increase of early labor compared to controls, i.e. 14 out of 22 dams exhibited signs of labor within 24 h (table 1) and, therefore, were not included in the study. It is important to note that average gestation length for C57BL/6 is 19.25 days (range GD18-22)[5,21], whereas PTB in C57BL/6 mice may occur prior to GD18[5]. Since we could not determine, in our cohort, the precise birth time within the 24h limit of LPS treatment (i.e. 17.5 or 18.5), as a precaution, we opted to designate this mode of labor as early, rather than preterm.

Importantly, all placental analyses were performed in tissue from dams that did not undergo early labor, or from fetuses that did not show signs of demise. Supplementary figure 1 depicts the actual number of animals included in the following analysis.

Pro-inflammatory maternal and placental response to acute sub-lethal LPS exposure

Maternal plasma levels of cytokines/chemokines associated with the pathogenesis of PTB, Il-1β, Il-6, chemokine (C-X-C motif) ligand 1 (Cxcl1) and monocyte chemoattractant protein-1 (Mcp-1/Ccl2) [25,26], were assessed in dams following sub-lethal LPS exposure at GD15.5 (LPS-4h) and at GD18.5 (LPS-4 and 24h). At GD15.5 (LPS-4h), maternal plasma levels of Il-1β, Il-6, Cxcl1 and Ccl2 ($P<0.01$) were increased when compared to the control group (Figure 1A-D). At GD18.5, 4 and 24h LPS exposure, increased maternal plasma Il-6, Cxcl1 and Ccl2 ($P<0.05$) (Figure 1F-I and 1K-N, respectively), but there was no significant increase in Il-1β (Figure 1F and 1K). At the level of the placenta, *Il-6* and *Cxcl1* mRNA was significantly

increased (P<0.05) following LPS challenge at all time points investigated. In contrast, *Ccl2* mRNA expression was unchanged at GD15.5 (4h) but increased at GD18.5 (4h and 24h; P<0. 05) (Figure 1E, 1J and 1O).

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

Table 1: Pregnancy outcome following sublethal LPS challenge in mid- and late- pregnancies.

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

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

In order to investigate how bacterial infection impact placental efflux transport potential, we investigated levels of *Abca1*, *Abcb1a*, *Abcb1b*, *Abcb4*, *Abcc2*, *Abcc5*, *Abcf2*, *Abcg1 and Abcg2* mRNA in the mouse placenta at GD15.5 (LPS-4h), or at GD18.5 (LPS-4 and 24h), following LPS challenge. These specific ABC transporters genes were selected based on evidence showing their sensitivity to infection in other models and or based on their importance to placental barrier function[5,14,15]. We did not observe significant differences 4h following LPS treatments at GD15.5 and GD18.5 (Figure 2A-B), however, at GD18.5 levels of *Abcb1b (P*<0.05) , *Abcc2 (P*<0.01) and *Abcc5 (P*<0.05) mRNA were reduced (P<0.05, <0.01) 24h after LPS insult (Figure 2C).

P-gp and Bcrp are key multidrug resistance transporters that have been shown to play roles in fetal protection, whereas the lipid transporter Abcg1 is important for fetal 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 with the addition of faint and variable nuclear staining in labyrinthine cells. Generally, there was greater Bcrp cytoplasmic labelling in labyrinthine and spongiotrophoblast cells compared to P-gp (Figure 3G-L). The lipid transporter Abcg1, was predominantly localized to cellular membranes of the labyrinth (L) and junctional zone (JZ), with some variable cytoplasmic staining throughout these layers (Figure 3M-R).

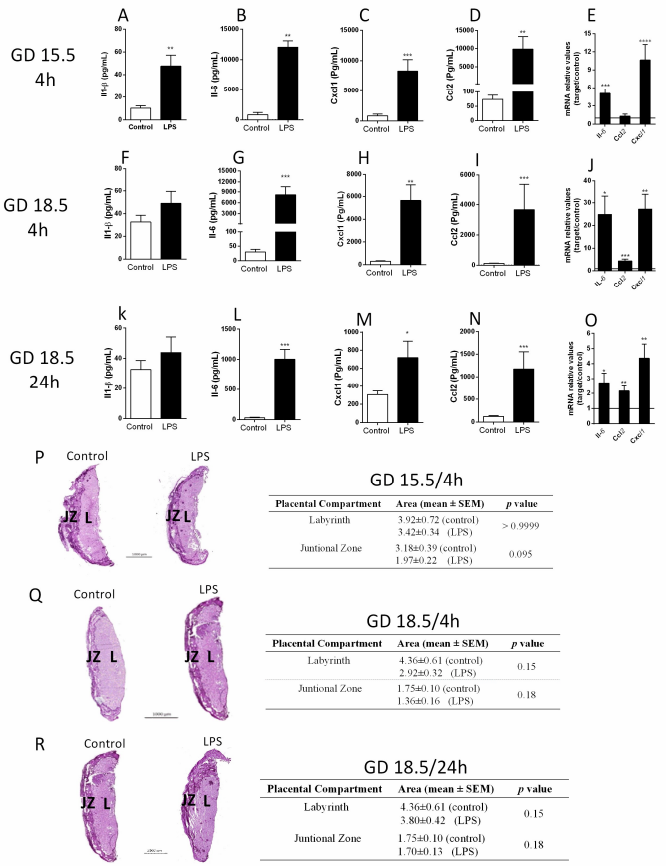

Fig. 1. LPS challenge elicited an acute maternal inflammatory response at GD 15.5 and 18.5. Plasma levels of (A) Il-1β, (B) Il-6, (C) Cxcl1 and (D) Ccl2 in dams at GD15.5, 4h after LPS exposure (control group, n=8, LPS group, n=8). (E) Placental mRNA levels of *Il6*, *Ccl2* and *Cxcl1* mRNA in the same animals (control group, n=11, LPS group, n=8). Plasma levels of (F) Il-1β, (G) Il-6, (H) Cxcl1, (I) Ccl2 in dams at GD18.5, 4h after LPS exposure (control group, n=9, LPS group, n=10). (J) Placental mRNA levels of *Il6*, *Ccl2* and *Cxcl1* mRNA in the same animals (control group, n=9, LPS group, n=10). Plasma levels of (K) Il-1β, (L) Il-6, (M) Cxcl1, (N) Ccl2 in dams at GD18.5, 24h after LPS exposure (control group, n=8, LPS group, n=10). (O) Placental mRNA levels of *Il6*, *Ccl2* and *Cxcl1* mRNA in the same animals (control group, n=8, LPS group, n=10). (P) Placental gross morphological analysis dams at GD15.5, 4h after LPS exposure, (Q) at GD18.5, 4h after LPS exposure, (R) at GD18.5, 24h after LPS exposure. Periodic acid-Schiff staining of control and LPS-treated mice, evaluating Labyrinth (L) and Junctional Zone (JZ) areas. n=5/group. Gene expression related to media of housekeeping genes (E) B2m and Bactin, (J, O) *Gapdh* and *Ywhaz*.

Using semi-quantitative analysis, we identified reduced P-gp staining intensity in the L at GD18.5, 4h and 24h (P<0.05) after LPS administration compared to controls. No differences were noted after LPS at GD15.5 (Figure 3A-F). Bcrp staining intensity in the L was reduced in all LPS groups investigated (P<0.05). In contrast, Bcrp levels in the spongiotrophoblast cells were higher at GD18.5, 4h and 24h (P<0.001) after LPS. No differences in the levels of Bcrp in the spongiotrophoblast cells were identified at GD15.5 after LPS treatment (Figure 3G-L).

Abcg1 staining intensity was lower in labyrinth cells at GD 15.5 (4h) and 18.5 (24h) (P<0.05) but increased in spongiotrophoblast cells at GD18.5 4h and 24h (P<0.01) after LPS exposure (Figure 3M-R).

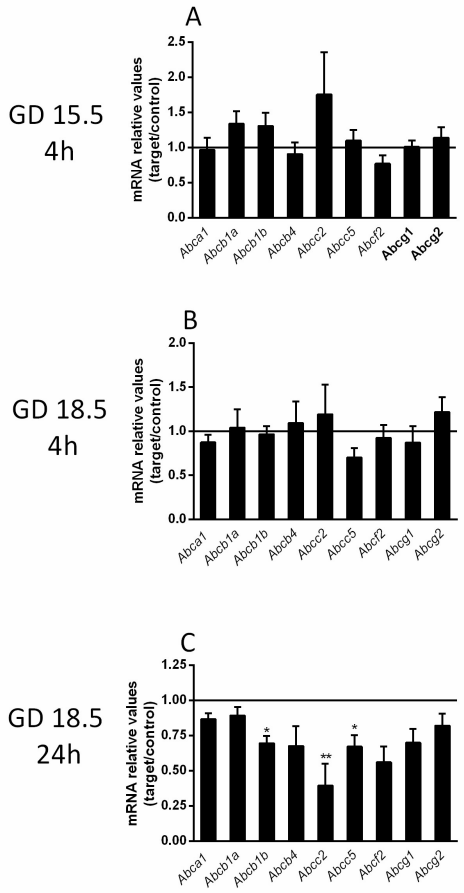

Fig. 2. LPS challenge downregulates the expression of key ABC transporter genes at GD 18.5, 24h after LPS administration. (A) Placental levels of *Abca1*, *Abcb1a*, *Abcb1b, Abcb4*, *Abcc2*, *Abcc5*, *Abcf2*, *Abcg2* mRNA at GD15.5, 4 hours after LPS exposure (A) and at GD18.5, 4h (B) and 24h (C) after LPS administration. 15.5/4h: n=12 (control group); n=8 (LPS group). 18.5/4h: n=9 (control group)/ n=10 (LPS group). 18.5/24h: n=12 (control group)/n=8 (LPS group). Gene expression related to media of housekeeping genes (A) B2m and Bactin, (B,C) *Gapdh* and *Ywhaz*.

LPS impacts maternal and placental lipid homeostasis throughout pregnancy

Since we observed an inhibitory effect of LPS on Abcg1 (a lipid transporter) in the L, we investigated the impact of sub-lethal LPS exposure on levels of maternal plasma and placental lipid classes (triacylglycerol, free fatty acids, esterified cholesterol, cholesterol, monoacylglycerol, and phospholipids) at GD 15.5 and 18.5. These analyses were undertaken only in the 4h time point, in both gestational ages, due to the lack of fetal viability in the 24h LPS treatment at GD15.5.

We observed significant alterations in placental lipid levels following sub-lethal LPS challenge. Triacylglycerol, free fatty acids, cholesterol ester and free cholesterol, were decreased at GD15.5, comparing to control groups (*P*<0.01, Table 2), whereas triacylglycerol, free fatty acids, free cholesterol, monoacylglycerol and phospholipids were increased at GD18.5, comparing to control groups (P<0.05; Table 3). The lipid classes monoacylglycerol and phospholipids remained unchanged at GD15.5 (Table 2) whereas cholesterol ester didn't exhibit alterations at GD18.5 (Table 3).

In the maternal plasma, we detected increased plasma levels of triacylglycerol and decreased free fatty acids and cholesterol ester at GD15.5 in the LPS exposed group compared to control group (P<0.001, table 4). At GD18.5, the triacylglycerol levels were decreased in the LPS group compared to control (P<0.05, table 5). No differences were found for free cholesterol, monoacylglycerol and phospholipids classes in both gestational ages whereas, free fatty acids, cholesterol ester, cholesterol, monoacylglycerol and phospholipids contents remained unchanged at GD18.5 (Tables 4 and 5).

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

Discussion

We have demonstrated the effects of bacterial infection on placental ABC transporters and lipid homeostasis. LPS caused substantial fetal death at GD15.5 and triggered early labor in late-pregnancy (GD18.5), without inducing fetal demise. These effects were likely mediated by distinct maternal and placental cytokine/chemokine responses to LPS throughout pregnancy and were associated with distinct expression 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[22]. However, here we also show that sub-lethal 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 (24h) at GD18.5, induced early labor in 55% of pregnancies [23], a percentage similar to that found in our study (64%).

Furthermore, LPS in late gestation decreased fetal weight, stimulated placental growth and reduced the F:P weight ratio. This might indicate an impairment of placental nutrient transport efficiency preventing fetuses from attaining optimal growth potential [24,25] and predisposing them to long term poor health outcomes[24].

CCL2 also induces the expression of proinflammatory cytokines, prostaglandins and leukotrienes in the myometrium in late pregnancy[30,31]. Importantly, Tlr4 activation induces myometrial production of Il-1β, Il-6 and Ccl2 via nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) activation [32].

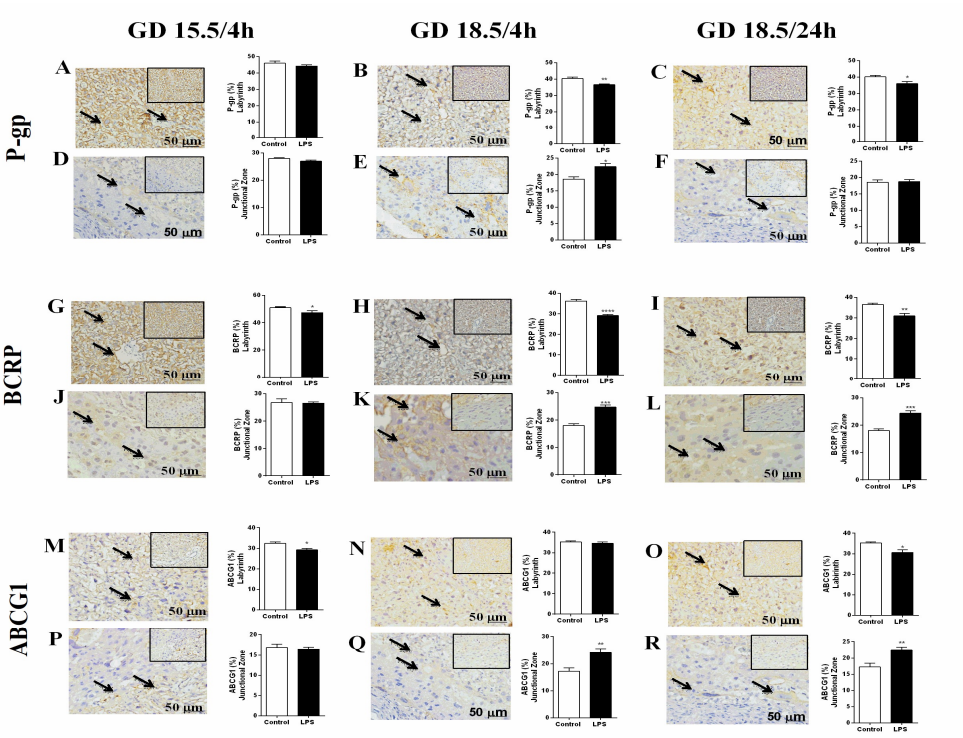

Fig. 3. LPS challenge modulates key ABC transporters proteins in the placenta. Representative immunohistochemistry images and semi-quantitative analysis of P-gp in the labyrinth (L) (A, B, C) and junctional zone (JZ) (D, E, F) at GD 15.5 (4h) and GD 18.5 (4h and 24h) after LPS administration. Bcrp in the L (G, H, I) and JZ (J, K, L), and Abcg1 in the L (M, N, O) and JZ (P, Q, R). The black arrows represent P-gp/Bcrp/Abcg1 stained cells. Graphs represents the % of positively stained cells. n=5/group. scale bar = 50 µm.

Acute sub-lethal LPS exposure during pregnancy, elicited an intense maternal and placental pro-inflammatory response, which varied across pregnancy. We observed a marked increase in a number of key pro-inflammatory factors related to PTB. In contrast, plasma Il-1β and placental levels of *Ccl2* mRNA were no affected by LPS, though they did exhibit a gestational-age dependent expression profile. IL-1β, IL-6 and IL-8 are commonly up-regulated by infection and induce prostaglandin (PG)E2 and $PGF_{2\alpha}$ production to stimulate myometrial contractility and PTB[26–28]. CCL2 in turn, is up-regulated in human myometrium during pre-term and term labor and recruit's leukocyte influx into the myometrium to amplify local inflammation and trigger the onset of labor[29].

In the present study, LPS stimulated placental *Ccl2* in a gestational-time dependent manner suggesting that this cytokine may participate in the pathogenesis of infective chorioamnionitis and the induction of early labor. Further, our data extracted from pregnancies that did not undergo early labor, indicate that 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.

In order to understand how sub-lethal bacterial infection impacts placental efflux transport, we evaluated the mRNA and protein expression/localization of selected ABC transporters involved in the biodisposition of clinically relevant substrates

across pregnancy. Previously, we have shown that sub-lethal LPS challenge (150 µg/kg) impairs placental P-gp activity at GD15.5 (4h) in C57BL/6 mice, with no changes in placental *Abcb1a* and *Abcb1b* mRNA levels[22]. In the present study, there were no changes in P-gp encoding genes at GD15.5 (4h) after LPS. Rather, we found lower *Abcb1b* at GD18.5 (LPS-24h) with less labyrinthine P-gp staining intensity at GD18.5 (LPS-4 and 24h). Placental P-gp and its encoding genes are developmentally regulated in both rodents and humans, i.e. expression decreases towards term[33,34] resulting in reduced fetal protection against P-gp substrates in late pregnancy. Our results indicate that late term bacterial infection may further decrease this already limited P-gp-mediated barrier function.

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

Placental Lipid fractions	Groups	mean ± SEM (µg/mg)	*p* value
Triacylglycerol	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 Ester	Control LPS	0.18±0.02 0.10±0.01	0.005**
Cholesterol		0.05±0.003 0.03±0.004	0.0043**
Monoacylglycerol	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).

Bcrp immunostaining was lower in the L at all time-points investigated following LPS exposure. Placental Bcrp has been shown to be down-regulated in human first trimester placental explants treated with LPS[16], in malarial-infected murine placentae[5] and in HTR8/SVneo (human extra villous trophoblast (EVT)-like) cells exposed to either LPS or to the TLR8-viral mimic, single stranded RNA (ssRNA)[36]. This indicates that different infective agents/challenges during pregnancy, including bacterial infection, have the potential to increase fetal accumulation of Bcrp substrates during pregnancy.

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

Placental Lipid fractions	Groups	mean ± SEM (µg/mg)	*p* value
Triacylglycerol	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 Ester	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****
Monoacylglycerol	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).

Results from this and previous studies would suggest that bacterial infection has the potential to increase fetal accumulation of P-gp substrates (cytokines, chemokines, endogenous and synthetic glucocorticoids, antibiotics, antiretrovirals, etc.)[14] both in earlier, due to impaired P-gp activity[22] and in later stages of pregnancy, via decreased L-P-gp. This will likely increase the levels of cytokines and chemokines within gestational tissues, causing fetal demise, morbidity, and / or inducing preterm/early labor pathways.

In line with our data, previous reports have shown P-gp to be downregulated in: a) human first trimester placental explants exposed to LPS[16], b) human PTB placentae with chorioamnionitis[15] and c) placentae from malarial infected C57BL/6-pregnancies exhibiting high rates of PTB [5]. Further, P-gp activity was impaired in the developing fetal blood brain barrier of C57BL/6-pregnancies exposed to PolyI:C, a TLR3-viral mimic[35]. Together, these studies suggest that lower placental *Abcb1b* and P-gp, are associated with the infection-driven PTB[5,15] and or an earlier labor delivery. Determination of the impact of lower placental P-gp on fetal health and postnatal development requires further investigation.

However, BCRP was upregulated in human PTB placentae with chorioamnionitis[15], which is commonly induced by polymicrobial infection[7] and following treatment of trophoblastic cells with PGE2[37], which demonstrates that the nature of infective/inflammatory stimuli determines the trophoblastic-BCRP modulatory response.

Interestingly, 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 are far less understood. Spongiotrophoblasts make up the mouse placental Jz and provide structural support for the growth of the labyrinthine villi and to limit fetal endothelium overgrowth[39]. However, very little is known about possible functions of ABC transporters in the mouse Jz, and how infection and inflammation impact Jz function.

Labyrinthine Abcg1 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 fetal capillaries of the human placenta; suggesting that it can efflux lipids from the maternal to the fetal compartment[14]

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

Plasma Lipid fractions	Groups	mean ± SEM (µg/µl)	*p* value
Triacylglycerol	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 Ester	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
Monoacylglycerol	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 GD18.5 after LPS administration

Plasma Lipid fractions	Groups	mean ± SEM (µg/µl)	*p* value
Triacylglycerol	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 Ester	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
Monoacylglycerol	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).

However, directionality of Abcg1-mediated lipid exchange in the mouse placenta is yet to be described, preventing us from postulating how placental Abcg1-mediated biodisposition of cholesterol and other lipids would be impacted by sublethal LPS exposure.

Maternal plasma and placental lipid levels were, then investigated in order to better understand how bacterial infection could alter lipid homeostasis at the maternal fetal interface. Due to the high level of fetal demise in the GD 15.5 dams at the 24h time-point, we to conducted the lipid analysis at the 4h time-point in both gestational ages. Maternal levels of triacylglycerol and free fatty acids were altered after LPS at GD15.5. Similar results have been reported in hepatic tissue following LPS exposure[40], which occurs in a gestational time-dependent manner. In fact, relevant alterations in plasma lipoproteins occur during injury or infection[41] or in septic patients who can sometimes present with hypertriglyceridemia[41]. The higher plasma triacylglycerol levels can be directly related to inflammatory status in pregnant mice and may be connected to the high rates of fetal death at GD15.5. Notwithstanding, placental triacylglycerol and free fatty acid levels were higher at GD18.5. This later effect may be an attempt to circumvent lower fetal weight and one possible mechanism responsible for the higher placental weight observed at in this group.

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 to plasma membrane (FABPpm), lipase lipoproteins (LPL) and Fat/cd36 translocase located on both apical and basolateral membrane of the trophoblast [42,43].

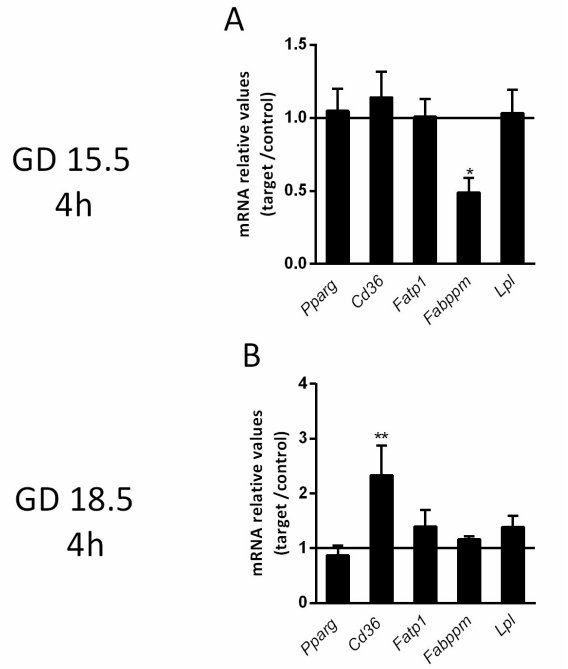

Fig. 4. LPS challenge modulates mRNA expression of lipid transporters and associated proteins at GD 15.5 and 18.5, 4h after LPS administration. (A, B) Relative mRNA quantification of selected lipid transporters (*Pparg* and *Cd36*) and associated proteins (*Fatp1*, *Fabppm* and *Lpl*) of GD 15.5 and 18.5, 4h after LPS challenge, respectively. 15.5/4h n=12 (control group); n=8 (LPS group). 18.5/4h: n=9 (control group)/ n=10 (LPS group). Gene expression related to media of housekeeping genes (A) B2m and Bactin, (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 to plasma membrane (FABPpm), lipase lipoproteins (LPL) and Fat/cd36 translocase located on both apical and basolateral membrane of the trophoblast [42,43]. We observed decreased placental *Fabp$_{pm}$* at GD15.5 and increased *Fat/cd36* gene expression at GD18.5 after LPS exposure. This may explain, at least in part, the different patterns of free fatty acids accumulation that we observed in the present study.

The effect of LPS on the placental accumulation of free cholesterol and cholesterol ester was also dependent on gestational age. Free cholesterol uptake at the apical membrane, cytoplasmic transport and basolateral syncytiotrophoblast extrusion into the fetal compartment are regulated by a complex interplay of receptors, lysosome/endosome pathways, sterol carrier proteins and efflux transporters[44]. It is possible that components of this machinery are likely regulated by infection, an effect that is gestational-age dependent.

In conclusion, this sub-lethal LPS model of bacterial infection during pregnancy may induce fetal death or early labor depending on 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. Fetuses (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. This may also be associated with sub-optimal placental lipid storage. Combined, these changes may contribute to the inflammatory PTB pathways and lead to the onset of PTB or early labor.

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Competing Interests

The authors declare no competing interests.

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