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Obeticholic acid improves fetal bile acid profile in a mouse model of gestational hypercholanemia

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Running title: Obeticholic acid improves fetal hypercholanemia

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Abstract

Intrahepatic cholestasis of pregnancy (ICP) is characterized by elevated maternal circulating bile acid levels and associated dyslipidemia. ICP leads to accumulation of bile acids in the fetal compartment and the elevated bile acid concentrations are associated with an increased risk of adverse fetal outcomes. The farnesoid X receptor agonist, obeticholic acid (OCA) is efficient in the treatment of cholestatic conditions such as primary biliary cholangitis. We hypothesized that OCA administration during hypercholanemic pregnancy will improve maternal and fetal bile acid and lipid profiles. Female C57BL/6J mice were fed either: a normal chow diet, a 0.5% cholic acid (CA)-supplemented diet, a 0.03% OCA-supplemented diet, or a 0.5% CA + 0.03% OCA-supplemented diet for 1 week prior to mating and throughout pregnancy until euthanization on day 18. The effects of CA and OCA feeding on maternal and fetal morphometry, bile acid and lipid levels, and cecal microbiota were investigated. OCA administration during gestation did not alter the maternal or fetal body weight or organ morphometry. OCA treatment during hypercholanemic pregnancy reduced bile acid levels in the fetal compartment. However, fetal dyslipidemia was not reversed, and OCA did not impact maternal bile acid levels or dyslipidemia. In conclusion, OCA administration during gestation had no apparent detrimental impact on maternal or fetal morphometry and improved fetal hypercholanemia. As high serum bile acid concentrations in ICP are associated with increased rates of adverse fetal outcomes, further investigations into the potential use of OCA during cholestatic gestation are warranted.

46

47 **New and noteworthy**

48 We used a mouse model of gestational hypercholanemia to investigate the use of obeticholic acid
49 (OCA), a potent FXR agonist, as a treatment for the hypercholanemia of intrahepatic cholestasis of
50 pregnancy (ICP). The results demonstrate that OCA can improve the fetal bile acid profile. This is
51 relevant not only to women with ICP, but also for women who become pregnant while receiving
52 OCA treatment for other conditions such as primary biliary cholangitis and non-alcoholic
53 steatohepatitis.

54

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a cholestatic condition that affects 0.4-2.2% of pregnancies in North America and Western Europe, but is more common in Chile and Bolivia where it can affect 1.5-4% of pregnancies (11, 13, 44). ICP typically presents from 30 weeks of gestation and the main symptom is persistent generalized itch. Diagnosis is made in women with an elevation of serum bile acids. ICP is associated with maternal dyslipidemia (12, 27) and increased risk of gestational diabetes mellitus (26, 27, 49). The most common treatment for ICP is ursodeoxycholic acid (UDCA) administration, but not all patients respond (8, 9, 18) and a recent trial revealed no benefit for adverse perinatal outcomes (8).

The adverse fetal outcomes that occur in ICP include preterm birth, fetal hypoxia, meconium-stained amniotic fluid, stillbirth and prolonged admission to the neonatal unit (19). Maternal bile acid levels have been reported to be positively correlated to fetal bile acid levels, and incremental rises in maternal serum bile acids above 40 μmol/l are associated with higher risk of adverse fetal outcomes (7, 19, 21). The fetal lipid profile has also been shown to be affected by maternal cholestasis, with increased cholesterol accumulation in the fetal liver and placenta in a mouse model of gestational cholestasis and in the umbilical cord of neonates exposed to maternal ICP (41).

It has previously been described that during normal pregnancy, the activity of farnesoid X receptor (FXR), the master nuclear receptor regulating bile acid homeostasis, is decreased allowing for a maternal pro-cholestatic profile even during normal gestation (31, 33, 39). However, it is thought that in ICP, the combination of genetic susceptibility, elevated reproductive hormones and environmental factors may lead to an exacerbation of the pro-cholestatic profile found in pregnancy and result in a pathological rise of bile acid levels (17).

In recent years, synthetic FXR agonists have been developed. In particular, the semi-synthetic bile acid, obeticholic acid (OCA) has over 100x higher affinity for FXR than its most potent natural ligand, chenodeoxycholic acid (CDCA), and has been shown to promote bile acid efflux and reduce bile acid

synthesis (51). Clinical trials of OCA have shown promising results for the treatment of primary biliary cholangitis (PBC) and non-alcoholic steatohepatitis (NASH) (2).

In this study, we used a previously established model of 0.5% cholic acid (CA) feeding in pregnancy to mimic the hypercholanemia of ICP (32, 41). Due to the key role of FXR in bile acid synthesis, transport and excretion, as well as regulation of lipid metabolism, we hypothesized that activation of FXR by OCA could improve maternal and fetal hypercholanemia and dyslipidemia.

Materials and methods

Animal experiments

Six to eight-week-old C57BL/6J mice were purchased from Envigo, UK and allowed to acclimatize for one week before any experimental procedures were carried out. All mice were kept on a 12h/12h light/dark cycle with access to food and water *ad libitum*. All procedures were approved by the Animal Welfare and Ethical Review Body at King's College London and carried out according to the UK Animals (Scientific Procedures) Act 1986. All diets were supplied by Special Diet Services, UK.

We have previously shown that cholic acid (CA) feeding can induce maternal hypercholanemia in mice (32, 41). Female mice were assigned to either standard maintenance and breeding diet (CRM), referred to as normal chow diet (NC), a 0.5% CA-supplemented CRM diet, a 0.03% obeticholic acid (Intercept Pharmaceuticals, USA) (OCA)-supplemented CRM diet, or a 0.5% CA + 0.03% OCA (CA+OCA)-supplemented CRM diet one week prior to mating, and maintained on their assigned diet for the duration of the experimental procedures. The dose of OCA was selected based on previously published literature (5), and was equivalent to approximately 42 mg/kg/day. Females were mated to control males and checked daily for the presence of a copulatory plug. The day of identification of the copulatory plug was considered day 1 of pregnancy (D1). Body weight of pregnant females was measured on days 7, 14 and 18 of pregnancy (D7, D14, D18). On D18, females were fasted for 4 hours and euthanized by CO_2 inhalation. Maternal and fetal sera were collected and pup number per litter was assessed. Maternal liver, subcutaneous white adipose tissue (sWAT), gonadal white adipose tissue (gWAT), brown adipose tissue (BAT), fetal and placental weight were measured. Maternal liver, terminal ileum, fetal liver and placenta were collected and snap-frozen. Non-pregnant control female mice were maintained on the same diets as pregnant females for an equivalent length of time and were assessed for the same parameters.

Gene expression studies

Total RNA was extracted from frozen tissue samples using the RNeasy Mini kit (Qiagen, UK) according to the manufacturer's guidance. Following RNA extraction, 1 µg of total RNA was reversed transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen, UK). RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, UK) was used as an RNase inhibition step. Assessment of the expression of target genes of interest was assessed using quantitative RT-PCR with a ViiA™ 7 Real Time PCR System (Thermo Fisher Scientific, UK) by adding cDNA in duplicate to a 384-well plate followed by a reaction mix of 1X SYBR Green Jumpstart Readymix (Sigma-Aldrich, UK) and 1 µM of forward/reverse primers. The housekeeping gene *cyclophilin b* was used as an internal reference for quantification of relative gene expression. Primer sequences of genes of interest are provided in Supplementary Table S1 (Private sharing link for Figshare data https://figshare.com/s/d95fdf67ee4829c114df).

Serum and tissue lipid quantification

Serum and tissue lipid content were extracted and measured as previously described (38). In brief, frozen tissues of interest were homogenized in Hank's Balanced Salt Solution using a TissueLyser II (Qiagen, UK) system. Samples were then centrifuged at 12000 rpm for 15 minutes at 4^{o}C (Rotina 420R Benchtop Centrifuge, Hettich, Germany). The supernatant was discarded. The pellet was re-suspended in 500 µL of lysis buffer containing 0.125 M potassium phosphate, 1 mM EDTA and 0.1% Triton-X 100 at pH 7.4. Samples were sonicated at 4^{o}C for 8 minutes in a Bioruptor Plus (4 cycles of sonication for 30 seconds followed by 4 cycles of resting for 30 seconds). Samples were subsequently centrifuged at 10000 rpm for 15 minutes at 4^{o}C. Total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides (TGs), free fatty acids (FFAs) and total protein were measured in plasma and tissue extracts with an Unicel DxC 800 autoanalyzer (Beckman-Coulter, the Netherlands) using dedicated kits, with the exception of FFAs which were measured using a kit from Wako

Diagnostics (Germany). The measurements in the tissue extracts were normalized with the protein content of each individual tissue sample.

Serum and cecal bile acid quantification

Measurements of serum and cecal bile acids were performed on an ultra-performance liquid chromatography Alliance 2695 system coupled to a Xevo TQ mass spectrometer using a SunFire C18 column as previously described (1, 45). Analytes were detected using selected ion monitoring and quantified against deuterium-labelled internal standards. Quantification was achieved by comparison of peak height of molecular anions or negative daughter to the peak height of the deuterated internal standards.

16S rRNA gene sequencing analysis

Cecal samples were homogenized and DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, UK), according to the manufacturers' protocol. Sample libraries were prepared as previously described (28) using the V1-V2 primers (35). An Illumina MiSeq platform was used to perform the sequencing with the MiSeq Reagent Kit v3 and paired-end 300 bp chemistry (Illumina Inc, USA). Mothur software (v1.35.1; www.mothur.org) was used for data analysis, following the MiSeq SOP Pipeline (47). The Silva bacterial database (www.arb-sliva.de) were used for sequence alignments and sequences were classified according to the RDP database reference sequence files using the Wang method (16). The UniFrac weighted distance matrix created by Mothur was used to produce non-metric multidimensional scaling (NMDS) plots and PERMANOVA (permutational multivariate analysis of variance) p-values and analysis carried out using the Vegan library (6) within the R statistical software (www.r-project.org). Bacterial relative abundance was expressed as extended error bar plots using the Statistical Analysis of Metagenomic Profiles software package and

analyzed by White's non-parametric t-test with Benjamini-Hochberg False Discovery Rate (FDR). The alpha diversity (Shannon diversity index, H') was calculated using Mothur and Tukey's Honest Significant Difference test was performed using IBM SPSS Statistics Software version 23. P- and q-values of 0.05 were considered to be significant.

Statistical analysis

All values are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 7 software. One-way ANOVA followed by a Newman-Keuls post-hoc test was used, with a significance cut-off of $P \leq 0.05$. Statistical analysis of 16S rRNA gene sequencing data is detailed in the relevant section above.

Results

OCA administration during pregnancy does not negatively impact maternal or fetal morphometry

We first aimed to establish the effect of hypercholanemia and OCA supplementation during pregnancy on body weight and organ morphometry. During pregnancy, no body weight differences were seen between groups, except on D7 when CA and CA+OCA-fed females were significantly lighter than OCA-fed females (Figure 1A). Although no body weight differences were registered on D18 gestation, pregnant females fed a CA diet had increased liver weight and decreased gWAT weight, regardless of OCA co-feeding (Figure 1B). A trend for decreased sWAT weight was also seen in pregnant CA and CA+OCA groups (Figure 1B). OCA supplementation alone did not affect body weight or organ morphometry (Figure 1B).

Despite the changes in maternal liver and gWAT morphometry in the CA and CA+OCA-fed groups, no changes in pup number, pup weight or placental weight were registered (Figure 1C).

Outside of pregnancy, both CA and CA+OCA non-pregnant females were lighter than NC- and OCA-supplemented females on D18 (Supplementary Fig. S1A, Private sharing link for Figshare data https://figshare.com/s/d95fdf67ee4829c114df). This weight difference likely reflected a decrease in gWAT, sWAT and BAT depot weight, despite an increase in liver weight (Supplementary Fig. S1B).

These results demonstrate that OCA administration either alone or to hypercholanemic pregnant females did not negatively impact maternal or fetal body or organ morphometry.

OCA administration during hypercholanemic pregnancy reduces fetal hypercholanemia

We next investigated whether OCA administration ameliorated the maternal and fetal bile acid profiles during hypercholanemic gestation. In pregnant females, CA feeding led to a significant increase in total serum bile acid levels, CA, deoxycholic acid (DCA), taurocholic acid (TCA) and taurodeoxycholic acid (TDCA) compared to NC controls, confirming that CA-feeding induces

maternal hypercholanemia, as has previously been described (32, 41). CA+OCA co-supplementation did not ameliorate total serum bile acid levels, although total unconjugated bile acids were significantly reduced compared to CA alone, due to changes in CA ($P > 0.05$) and DCA ($P \leq 0.05$) (Figure 2A).

In non-pregnant females, total bile acids, DCA, TCA and TDCA levels were significantly elevated by CA feeding and were not reduced by CA+OCA co-feeding (Supplementary Fig. S2).

In the fetal compartment, maternal hypercholanemia led to a significant rise in fetal serum total bile acids (Figure 2B). However, total serum bile acid levels were 29.9% lower in fetuses from mothers fed a CA+OCA diet compared to CA alone, although still higher than NC controls (Figure 2B). This was due to decreased concentrations of DCA, TCA, TDCA and in particular, CA (Figure 2B). Maternal OCA feeding alone did not change fetal bile acid concentrations although the presence of OCA and T-OCA in the fetal circulation suggests that OCA is able to cross the placenta (Figure 2B).

Overall, OCA administration to hypercholanemic females did not significantly ameliorate maternal hypercholanemia, but improved the fetal bile acid profile.

OCA administration alone reduces cecal bile acid levels

Cecal bile acid concentrations were also measured. As expected in the cecum, bile acids were largely unconjugated (Figure 3). Total cecal bile acid levels were significantly increased in mice fed CA+OCA compared to CA alone, however this was largely due to enrichment with OCA, and also with DCA that also increased in the CA-fed group (Figure 3A,B). Muricholic acids levels were markedly reduced in both CA and CA+OCA groups (Figure 3B,C). OCA administration alone significantly reduced total cecal bile acid levels compared to all other groups, which was due to an overall reduction in bile acids (Figure 3A). Interestingly, as seen in the serum, T-OCA levels were significantly

lower in CA+OCA co-fed mice compared to females supplemented with OCA only, while OCA levels were increased (Figure 3B,C).

Bile acid supplementation impacts the cecal microbiome's microbiota composition

Conversion of primary to secondary bile acids, as well as bile acid deconjugation, are performed by intestinal bacteria. Since changes in bile metabolizing bacteria will affect the host bile acid pool, the cecal bacterial community was investigated by 16S rRNA gene sequencing. Non-metric multidimensional scaling (NMDS) analysis of weights UniFrac distances, which shows how the microbial communities vary between the groups, demonstrates significant differences between all the dietary groups in pregnant mice (Figure 4A, Supplementary Table S2). OCA supplementation alone was the least different to NC, with CA and then CA+OCA being more dissimilar. Differences in the relative proportion of phyla were observed between pregnant groups (Figure 4B); specifically, both CA feeding and CA+OCA co-feeding significantly increased the relative abundance of *Proteobacteria* in the cecum of pregnant mice, compared to NC groups (Figure 4C). OCA feeding alone did not significantly impact *Proteobacteria*, but the relative abundance of *Bacteroidetes* was significantly decreased in pregnant females (Figure 4C). Significant changes were also observed at genus level, with an increase in the relative proportion of *Bilophila* and *Bacteroides* in CA+OCA-fed mice compared to all other groups (Figure 4D). This was reinforced by correlation analysis between microbiota and bile acid concentrations in the cecum, which showed that Proteobacteria and Bacteroidetes positively correlated with OCA, and negatively with T-OCA, concentrations (Supplementary Fig. S3A). Alpha diversity (Shannon diversity index) plots showed that CA supplementation alone or co-fed with OCA resulted in decreased bacterial diversity (Supplementary Fig. S3B). Pregnancy caused a significant increase in an unclassified class of *Bacteroidetes* in NC controls (Supplementary Fig. S3C). In non-pregnant mice, NMDS analysis and alpha diversity plots were similar to pregnant mice (Supplementary Fig. S4A,B). However, changes between the dietary

groups differed at phylum level; in particular, significant differences were observed in *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Supplementary Fig. S4C).

OCA administration represses maternal hepatic Cyp7a1 expression via intestinal FXR

To further assess the effects of hypercholanemia and OCA administration on bile acid homeostasis during pregnancy, the expression of key genes for bile acid homeostasis in the liver and terminal ileum was investigated.

The hepatic FXR target *Shp* was significantly upregulated in pregnant females fed a CA or a CA+OCA diet and this change was concomitant with the repression of hepatic *Cyp7a1* (Figure 5A). Both CA and CA+OCA diet increased the hepatic expression of the bile acid transporters *Bsep, Mrp3* and *Mrp4* in pregnant females (Figure 5A). Whilst OCA supplementation alone did not induce significant hepatic *Shp* upregulation, *Cyp7a1* expression was significantly decreased in D18 pregnant females (Figure 5A). In parallel, intestinal *Shp* expression was upregulated in OCA-fed females and intestinal *Fgf15* expression was significantly increased by maternal CA, OCA and CA+OCA supplementation (Figure 5B).

In non-pregnant females, relative mRNA expression followed a very similar pattern to pregnant mice (Supplementary Fig. S5A,B). Of note, lower hepatic gene expression of several FXR targets was observed in pregnant mice compared to non-pregnant, regardless of diet (Table 1). Expression of FXR targets in the terminal ileum was similarly affected by pregnancy. In pregnant CA-fed females, *Shp* and *Fgf15* expression was lower than outside pregnancy (Table 2). *Shp* expression levels were also lower in CA+OCA-fed pregnant females compared to non-pregnant (Table 2).

Overall, we conclude that despite decreased expression of FXR target genes during pregnancy, activation of intestinal rather than hepatic FXR can mediate OCA-induced suppression of hepatic *Cyp7a1* expression.

Maternal OCA administration represses fetal hepatic Cyp7a1 expression

Given the decrease in fetal serum bile acid concentrations in maternal CA+OCA feeding groups, the expression of key bile acid homeostasis genes in the fetal liver and placenta were assessed. Maternal CA feeding alone or co-supplemented with OCA induced an upregulation of *Shp* expression, and a concomitant reduction in *Cyp7a1* and *Ntcp,* in the fetal liver (Figure 6A). Of note, while maternal OCA diet alone did not have an impact on fetal hepatic *Shp* expression, a significant downregulation of hepatic *Cyp7a1* expression was observed, although to a lesser extent than in groups with maternal CA supplementation (Figure 6A). Maternal bile acid feeding did not have an impact on hepatic fetal *Mrp3*, *Mrp4* or *Oatp1b2* expression (Figure 6A).

As the placenta plays a crucial role in bile acid transport between maternal and fetal circulations, we further sought to determine whether maternal OCA administration had an impact on placental bile acid transporter gene expression. Interestingly, all maternal bile acid feeding groups showed a significant upregulation of *Abcg2* expression in the placenta (Figure 6B). Moreover, maternal CA+OCA feeding increased placental *Mrp2* expression when compared against all other feeding groups, and *Oatp1b2* expression was increased compared to NC and CA groups (Figure 6B). Overall, we conclude that OCA modulates the expression of *Cyp7a1* in the fetal liver and bile acid transporters in the placenta.

OCA administration during hypercholanemic pregnancy does not reverse maternal dyslipidemia

Cholestasis is commonly accompanied by dyslipidemia. Hence, we next studied the effect of OCA administration during hypercholanemic pregnancy on maternal and fetal serum and hepatic lipid levels. No changes in total serum cholesterol levels were seen in pregnant CA and CA+OCA-supplemented groups (Figure 7A). However, females exposed to a CA or CA+OCA diet had raised

serum LDL-cholesterol and decreased HDL-cholesterol levels compared to NC females (Figure 7A), also outside of pregnancy (Supplementary Fig. S6A). Conversely, OCA feeding resulted in decreased total serum cholesterol levels compared to NC controls which was associated with a reduction in serum HDL-cholesterol concentrations (Figure 7A). Serum HDL-cholesterol was also reduced in non-pregnant OCA-fed mice (Supplementary Fig. S6A). CA feeding did not alter serum triglyceride levels in pregnant females, but OCA diet reduced serum triglyceride levels and a further decrease was observed in CA+OCA fed females (Figure 7A). In contrast, no significant changes were observed in serum triglyceride levels in non-pregnant females (Supplementary Figure S6A).

In the liver, CA, OCA and CA+OCA supplementation of pregnant females led to hepatic cholesterol accumulation compared to NC control group (Figure 7B). In non-pregnant females, hepatic cholesterol levels were significantly lower with OCA supplementation alone compared to CA and CA+OCA-fed mice (Supplementary Fig. S6B).

Taken together, these data lead us to conclude that OCA administration does not ameliorate maternal dyslipidemia during hypercholanemic gestation.

OCA administration during hypercholanemic pregnancy does not reverse fetal dyslipidemia

As maternal dyslipidemia is commonly associated with fetal dyslipidemia, we next investigated the fetal lipid profile. Maternal CA feeding significantly increased fetal serum cholesterol levels, including LDL-cholesterol, and this was not altered by maternal CA+OCA supplementation (Figure 8A). In parallel, fetal serum HDL-cholesterol concentrations were reduced in maternal CA and CA+OCA supplementation groups. Fetal circulating triglycerides were increased in fetuses from CA-fed mothers and were not improved by maternal CA+OCA feeding (Figure 8A). Of note, maternal OCA-feeding alone had no effect on fetal total and LDL- or HDL-cholesterol levels or triglyceride and FFA concentrations (Figure 8A).

Fetal hepatic cholesterol and FFA content were increased in fetuses from CA+OCA-fed mothers compared to NC mothers (Figure 8B). However, maternal OCA diet alone did not affect fetal cholesterol and FFA accumulation in the liver (Figure 8B). A trend for increased hepatic cholesterol and FFAs was also observed in fetuses from CA-fed mothers compared to NC controls, albeit not reaching statistical significance (Figure 8B).

To assess a potential relationship between fetal and placental lipid levels, the placental lipid content on D18 of gestation was also evaluated. However, no significant changes in placental cholesterol, triglycerides or FFAs content were registered between different groups (Figure 8C).

We subsequently aimed to establish whether the changes in the fetal lipid profile on D18 of gestation were due to shifts in lipid *de novo* biosynthesis and transport in the fetal liver or placenta. Maternal bile acid feeding did not impact fetal hepatic *Hmgcr, Fas* or *Fatp4* expression (Figure 9A). However, maternal CA+OCA feeding led to a significant increase in placental expression of the cholesterol transporter *Abca1* compared to NC placentas (Figure 9B). Interestingly, maternal CA and CA+OCA supplementation, but not maternal OCA alone, resulted in a significant increase in *Fatp4* placental expression compared to NC controls (Figure 9B). Taken together, these data lead us to conclude that OCA administration does not ameliorate fetal dyslipidemia during hypercholanemic gestation.

Discussion

ICP is the commonest gestational liver disease and can lead to adverse fetal outcomes (19, 21, 40). Increased rates of stillbirth, spontaneous preterm birth, and meconium-stained amniotic fluid have been reported in pregnancies with high maternal serum concentrations of bile acids (19, 21, 40), likely related to fetal exposure to high bile acid concentrations (7). While UDCA treatment of ICP has been shown to reduce maternal bile acid levels in some studies (23), it is not effective in all patients (8), and it does not return fetal bile acid levels to normal concentrations (20). The present study shows that OCA administration in a mouse model of hypercholanemia, as seen in ICP, is not detrimental to the mother or fetus and improves fetal hypercholanemia.

In our model, CA-feeding led to significantly raised total bile acids in fetal serum. This was largely due to an increase in taurine-conjugated CA and DCA. While the fetus synthesizes bile acids from early pregnancy onwards, maternal bile acids can also cross the placenta and contribute to the fetal bile acid pool (29). Unconjugated and, at much lower levels, taurine-conjugated CA and DCA were also raised in the serum of CA-fed mothers. In the fetal compartment, DCA must be maternally derived since the fetus cannot synthesize secondary bile acids due to the absence of gut flora, and it is possible that CA is also being transferred from the mother. However, it is not known whether there is preferential transport of more hydrophilic taurine conjugates across the placenta, or increased taurine conjugation occurring in the fetal liver. We have previously observed in humans that the ratio of conjugated to unconjugated bile acids is higher in umbilical cord blood than in maternal serum (20).

OCA treatment during hypercholanemic gestation significantly reduced fetal total serum bile acid levels, due to a reduction in DCA, TDCA and TCA, compared to fetuses of untreated hypercholanemic mothers. Furthermore, analysis of fetal serum showed that OCA crosses the placenta and is present in the fetal compartment, predominantly as T-OCA. In line with this, hepatic *Cyp7a1* expression was reduced in fetuses from OCA-fed mice, and further reduced in both CA and CA+OCA-fed groups.

Interestingly, OCA treatment of hypercholanemic mothers was associated with an upregulation of placental transporters *Mrp2* (at the maternal-facing apical membrane) and *Oatp1b2* (basolateral membrane), which suggests enhanced elimination of fetal bile acids via the placenta. Increased placental expression of MRP2 has previously been associated with reduced bile acids in the fetal compartment in ICP pregnancies following UDCA treatment (3). Protein expression and bile acid transport studies would be required to confirm whether enhanced placental bile acid detoxification is responsible for this reduction in serum bile acids. The impact of OCA on fetal bile acid levels is of clinical interest due to the recent approval of OCA as a treatment for patients with PBC, as women with PBC may already be receiving OCA treatment when they become pregnant. In our study, we did not observe any detrimental effect of OCA on the fetus, in agreement with a previous study that found no impact on resorptions, number of fetuses, or fetal growth (10). However, detailed pathological investigations are required to assess the safety of fetal exposure to OCA.

In contrast to the fetus, maternal total serum bile acid levels were not reduced by OCA treatment. Furthermore, OCA treatment did not induce significant shifts in hepatic mRNA expression of bile acid homeostasis genes. These findings differ from a previous study of an estrogen-induced cholestasis rodent model reporting that OCA treatment induced bile flow and hepatocyte expression of *Shp*, *Bsep* and *Mrp-2*, while repressing *Ntcp* and *Cyp7a1* expression (15). A more recent study of estrogen-induced cholestasis in mice showed that OCA treatment did not upregulate mRNA expression of FXR targets in the liver or placenta but did increase hepatic FXR protein levels. Total serum bile acid levels were reduced in mothers, however serum bile acids were only mildly elevated in this model (10). In contrast, a study investigating the effect of OCA administration to *Mdr2*$^{-/-}$ mice found that dietary 0.03% OCA supplementation failed to exert any effect on bile flow and composition. This study further reported that both OCA and INT-767, a dual FXR and TGR5 agonist, were effective in reducing *Cyp7a1* and *Cyp8b1* gene expression, but only INT-767 administration resulted in increased hepatic *Shp* gene expression and BSEP protein expression (4). A possible explanation is that despite a far higher affinity of FXR for OCA, due to the activation of FXR by CA-

feeding, this limited the impact of OCA in our study. This is perhaps surprising given that CA is a weak agonist of FXR (EC_{50} = 586 µM (25)) in comparison to OCA (EC_{50} = 99nM (42)). In line, CA has previously been shown to only partially induce BSEP *in vitro*, in comparison to the natural FXR ligand, CDCA (25). A possible explanation is the 10-times higher abundance of CA as compared to OCA, at least as measured in serum, which limited the impact of OCA. Regardless, OCA administration alone did not cause the expected robust upregulation of hepatic FXR targets. Of note, OCA alone downregulated hepatic *Cyp7a1* expression and this change was associated with an upregulation of *Shp* and *Fgf15* in the terminal ileum rather than hepatic *Shp* induction. Indeed, previous studies have demonstrated that OCA administration in rats leads to upregulation of *Shp* in the terminal ileum (46) and that in mice lacking intestinal *Fxr*, OCA supplementation does not result in repression of hepatic *Cyp7a1* expression (50). Taken together with these studies, our findings suggest OCA acts primarily through ileal FXR to stimulate FGF15 secretion into the portal circulation and repress hepatic *Cyp7a1* expression in the maternal liver, rather than via hepatic FXR to modulate the expression of other hepatic genes involved in bile acid homeostasis. Our study did not assess the effect of OCA on markers of liver damage. However, we are aware that CA feeding in twice the dose in male Swiss Albino mice has previously been shown to increase serum AST, ALT and AP levels, as well as hepatocyte size, mitosis and necrosis (14).

Of note, the expression of FXR target genes was decreased overall by pregnancy, both in the liver and terminal ileum, which likely reflects the previously documented decreased gestational FXR activity (31, 33, 39). Nonetheless, in the liver of pregnant NC-fed females, OCA administration did not appear to efficiently overcome the reduction of FXR activity, and gene expression levels of FXR targets were similar. Conversely, in the maternal terminal ileum, the upregulation of *Shp* and *Fgf15* expression suggests an increase in FXR activity induced by OCA administration to NC-fed mice, but levels remained below those observed outside of pregnancy and so similarly indicate that OCA is unable to fully activate FXR in the terminal ileum. In support of this data, we also observed in a mouse model of gestational diabetes mellitus a diminished effect of OCA in pregnant mice compared

to non-pregnant controls (30). This highlights the issue that limited efficacy of FXR agonists should be taken into account in treatment of pregnant women.

OCA was predominately unconjugated in the serum and the cecum, in contrast to mice fed OCA alone where T-OCA predominated. This indicated a different pattern or activity of bile acid deconjugating microbiota. Indeed, 16S rRNA gene sequencing showed that there was an increase in relative abundance of *Bacteroidetes* and *Proteobacteria* (and also *Bacteroides* and *Bilophila*, when analysed at genus level) in the cecum of CA+OCA-fed females. We recently reported in pregnant mice that bile salt hydrolase, involved in deconjugation of bile acids, was exclusively detected in *Bacteroidetes*, with *Proteobacteria* also enriched in pregnancy, likely secondary to increased taurine made available after bile acid deconjugation (39). *Bilophila Wadsworthia* is known to be taurine-metabolizing (24). These findings suggest that the predominance of unconjugated OCA in the serum of CA+OCA-fed mice could be due to an increase of *Bacteroidetes* and *Proteobacteria* in the gut.

OCA administration during hypercholanemic gestation did not reverse maternal dyslipidemia. Of note, maternal OCA supplementation alone resulted in a decrease in serum total cholesterol, due to a reduction in HDL-cholesterol. A similar decrease in serum HDL-cholesterol was seen in non-pregnant females. This decrease is not unexpected as OCA has previously been shown to reduce HDL-cholesterol in healthy humans, PBC and NASH patients (22, 37, 43), and we recently reported that OCA reduced serum cholesterol in a mouse model of gestational diabetes mellitus (30). Furthermore, hepatic cholesterol content was raised in all bile acid-supplemented mice, although to a lesser extent in non-pregnant females fed an OCA diet. Dyslipidemia with hepatic cholesterol accumulation has previously been suggested to be associated with *Cyp7a1* repression found in cholestasis, as downregulation of bile acid synthesis from cholesterol leads to cholesterol accumulation in the liver (36, 48), suggesting that cholesterol accumulation in the liver may be proportional to hepatic *Cyp7a1* repression in our model.

Notably, serum triglycerides were reduced in pregnant mice that received OCA. This change is in line with previous studies showing that FXR activation reduces circulating triglycerides in *db/db* mice (52). Additionally, in patients with non-alcoholic fatty liver disease and type 2 diabetes, administration of 50 mg OCA daily for 6 weeks resulted in decreased serum triglyceride concentrations (34). However, OCA administration did not improve fetal dyslipidemia. In fact, maternal CA+OCA co-administration resulted in accumulation of cholesterol and FFAs in the fetal liver compared to fetuses of control mothers. Further investigations are needed to establish whether the upregulation of expression of placental lipid transporters *Abca1* and *Fatp4* may play a role.

In conclusion, OCA administration during hypercholanemic pregnancy, mimicking the raised serum bile acids observed in ICP, ameliorated fetal hypercholanemia although maternal bile acid levels were not significantly decreased, and maternal and fetal dyslipidemia was not resolved. Significantly, no negative effects of maternal OCA treatment on maternal and fetal morphology, and most importantly, fetal survival, were observed. As OCA may be used to treat women of reproductive age with PBC and NASH, further investigations into the safety of maternal and fetal exposure to OCA during pregnancy are warranted.

Author contributions

VP, GP and CW were responsible for study conception and design. VP, JAKM, AW, EJ and HUM generated experimental data. VP, SM and JAKM performed data analysis. CW supervised the research and acquired funding. VP, SM and CW drafted the article. GP, CO, JAKM, AW, EJ, LA, DS, JRM and HUM provided critical revision of the article.

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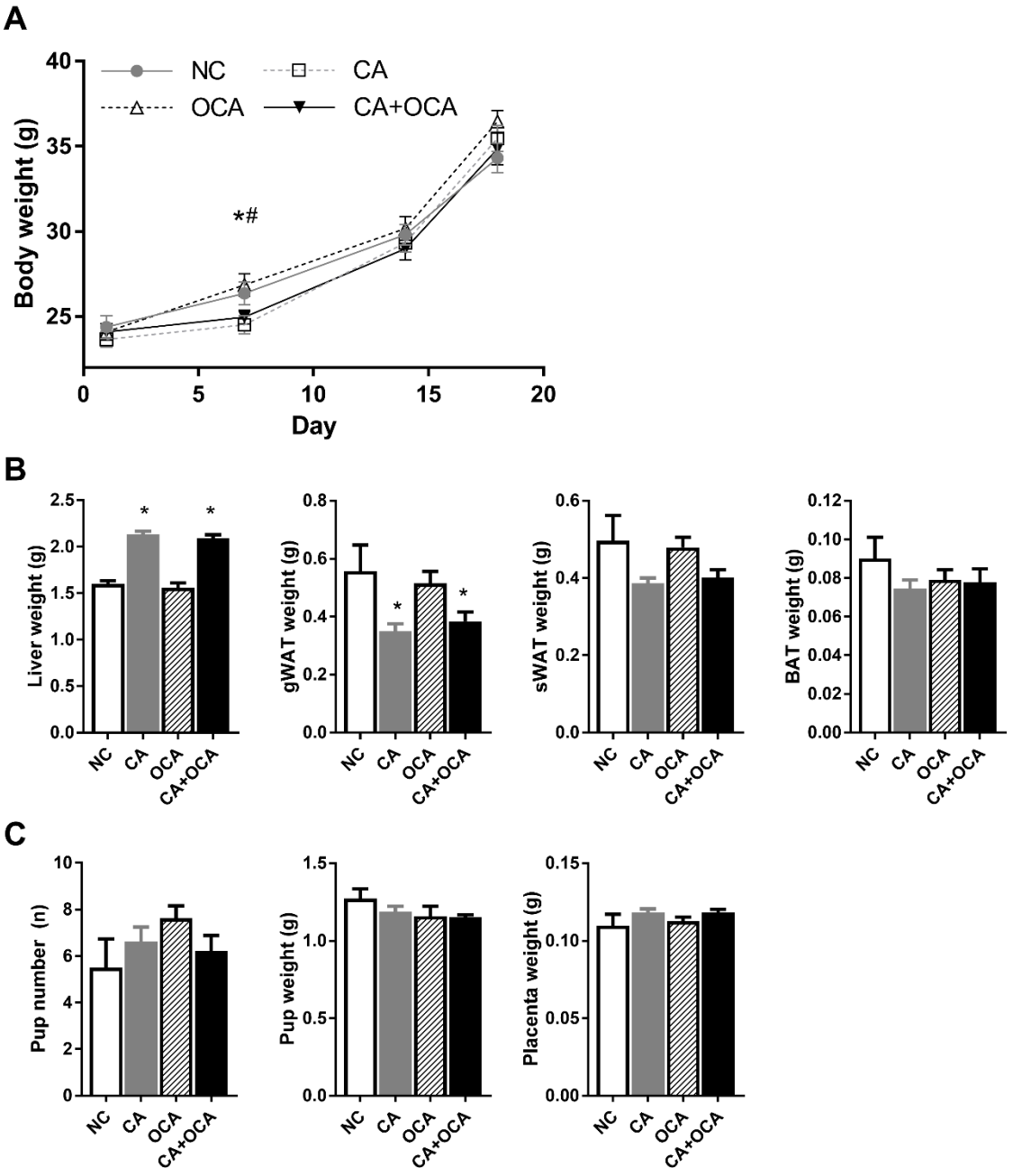
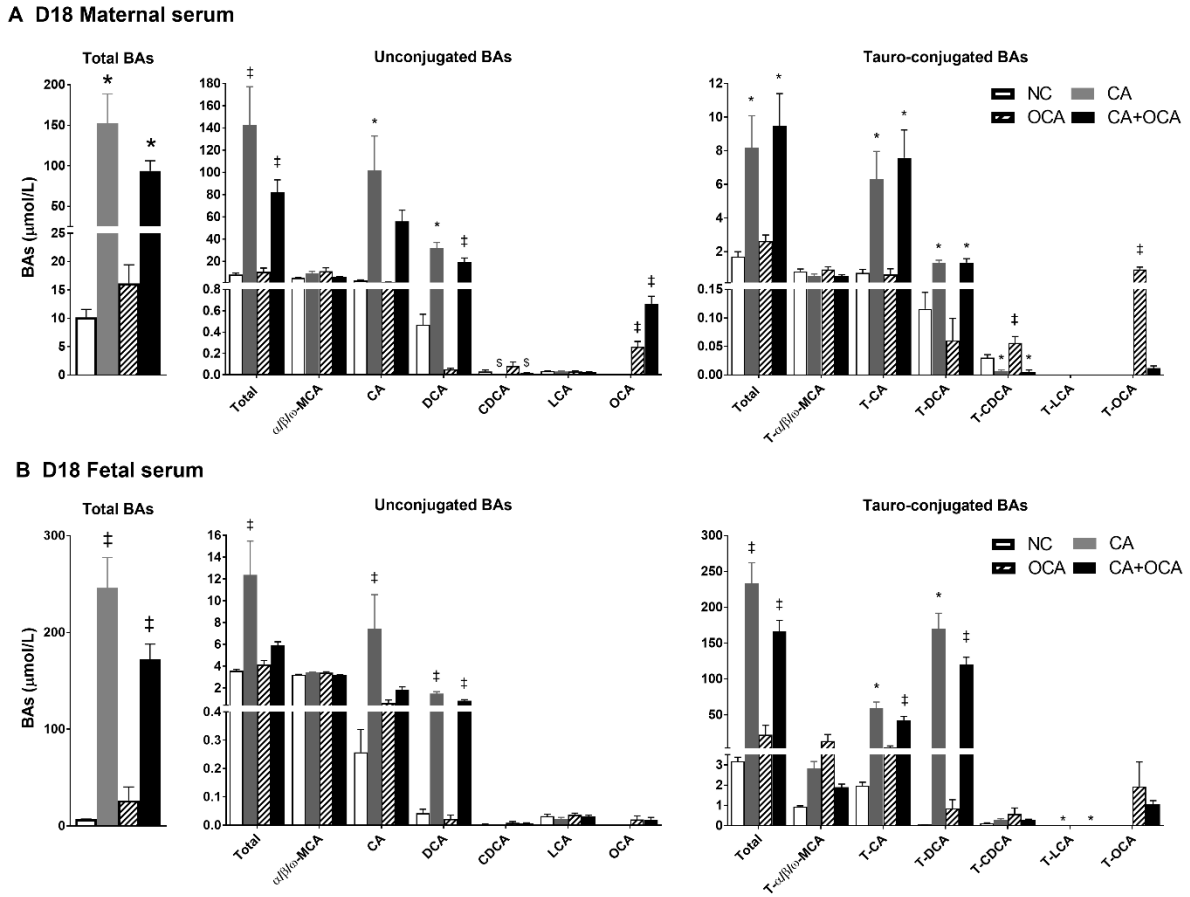
643

644 **Figure Legends**



645
646 **Figure 1 – Effects of hypercholanemia and OCA treatment during pregnancy on body and organ**
647 **morphometry.** (A) Body weight of pregnant females on D1, D7, D14 and D18. # P ≤ 0.05 for CA vs
648 OCA, * P ≤ 0.05 for CA+OCA vs OCA groups. (B) Weight of liver, gonadal white adipose tissue
649 (gWAT), subcutaneous white adipose tissue (sWAT) and brown adipose tissue (BAT) of pregnant
650 females at D18. (C) Pup number, pup weight and placenta weight of D18 fetuses. * P ≤ 0.05 in
651 comparisons vs NC and OCA groups. Data are presented as mean ± SEM. n = 6-9



652

653 **Figure 2 – Effects of hypercholanemia and OCA treatment during pregnancy on serum bile acid**
654 **profile.** (A) Serum total bile acid (BAs), unconjugated bile acid, and taurine-conjugated bile acid
655 levels in D18 pregnant females. n = 6 per group. (B) Serum total bile acid, unconjugated bile acid,
656 and taurine-conjugated bile acid levels in D18 fetuses. n = 4-6 per group. * P ≤ 0.05 in comparisons
657 vs NC and OCA groups. ‡ P ≤ 0.05 in comparisons vs all groups. $ P ≤ 0.05 in comparisons vs OCA. † P
658 ≤ 0.05 in comparisons vs NC and CA groups. Data are presented as mean ± SEM.

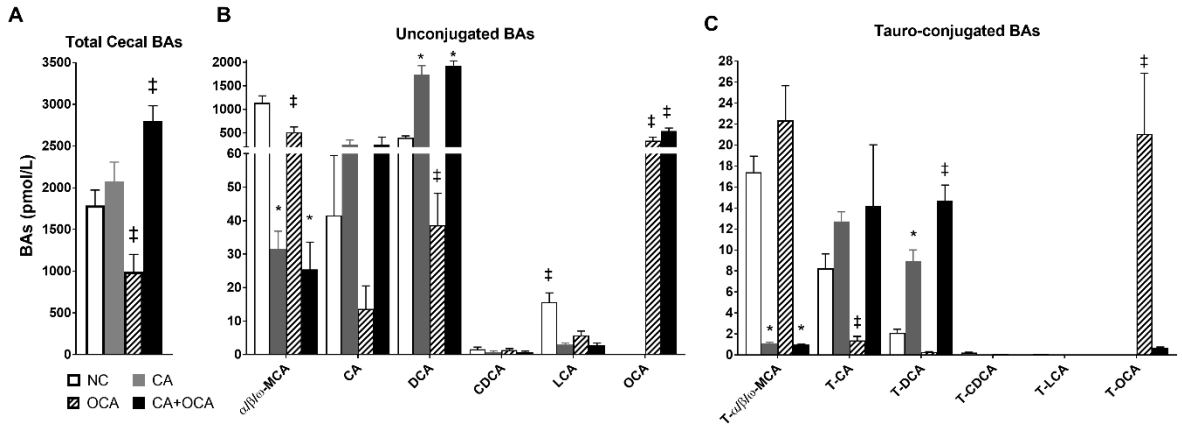


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Figure 3 - Effects of hypercholanemia and OCA treatment during pregnancy on cecal bile acid profile. Bile acid levels in cecum of D18 pregnant females. (A) Total cecal bile acids (BAs). (B) Unconjugated bile acids. (C) Taurine-conjugated bile acids. Data are presented as mean ± SEM. n = 6-9. ‡ P ≤ 0.05 in comparisons vs all groups. * P ≤ 0.05 in comparisons vs NC and OCA groups.

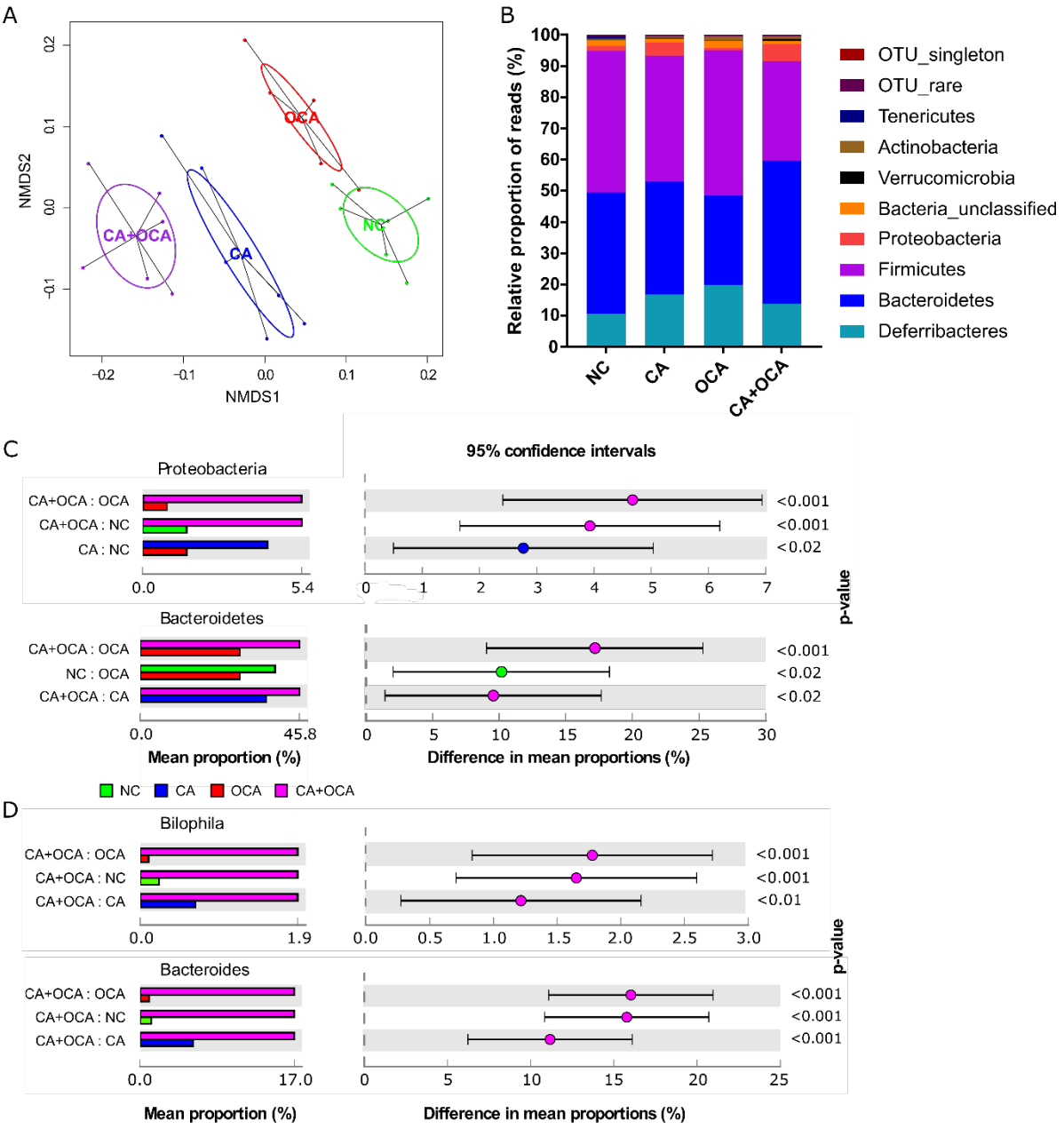

Figure 4 – Changes in cecal microbiota in pregnant mice measured by 16S rRNA gene sequencing. (A) Nonmetric multidimensional scaling (NMDS) plot showing differences in bacterial community structure based on the weighted UniFrac distance metric. For p-values see Supplementary Table S2. (B) Changes in relative proportion of reads at phylum level. (C and D) Significant changes in the average relative proportion of sequences assigned to each taxa for each dietary group, at phylum (C) and genus level (D). Data presented as extended error bar plots showing p-value, effect size and

confidence interval for each taxa. Analyzed by Kruskal-Wallis H-test with Benjamini-Hochberg False Discovery Rate. n = 6 mice per group.

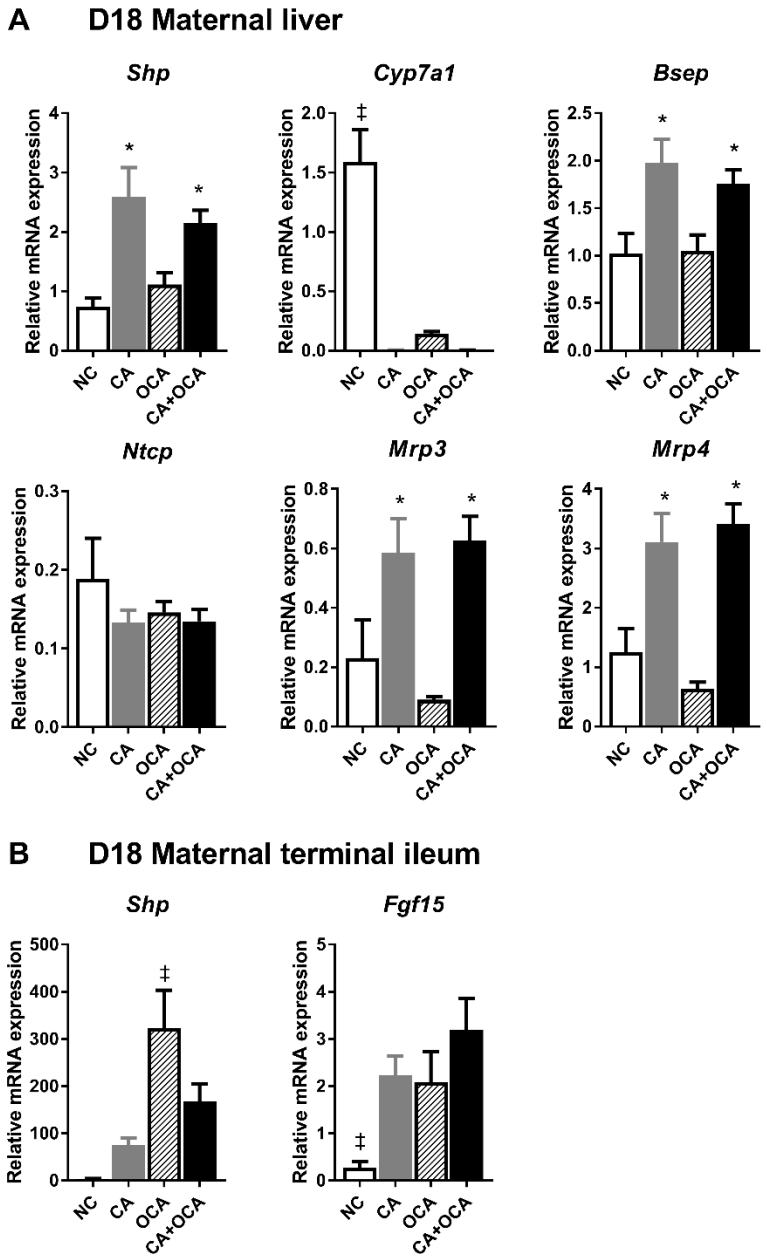

Figure 5 – Expression of key bile acid homeostasis genes in pregnant females. (A) mRNA expression of genes regulating bile acid synthesis and transport in the liver. (B) mRNA expression of genes regulating bile acid synthesis and transport in the terminal ileum. Data are presented as mean ± SEM. n = 4-6. * P ≤ 0.05 in comparisons vs NC and OCA groups. ‡ P ≤ 0.05 in comparisons vs all groups.

680

Table 1 - Effect of pregnancy on hepatic mRNA expression of key bile acid homeostasis genes.

	NC		CA		OCA		CA+OCA	
	NP	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**
Shp	2.06 ± 0.35	0.74 ± 0.14*****	3.36 ± 0.63	2.59 ± 0.49	1.78 ± 0.39	1.12 ± 0.19	3.46 ± 0.49	2.15 ± 0.21*****
Cyp7a1	1.37 ± 0.37	1.59 ± 0.27	0.004 ± 0.001	0.004 ± 0.002	0.66 ± 0.21	0.14 ± 0.02*****	0.006 ± 0.0023	0.005 ± 0.001
Ntcp	0.77 ± 0.21	0.19 ± 0.05*****	0.29 ± 0.06*	0.13 ± 0.02*****	0.36 ± 0.03	0.15 ± 0.01*****	0.28 ± 0.05	0.14 ± 0.01*****
Bsep	1.74 ± 0.38	1.02 ± 0.21	2.78 ± 0.36	1.98 ± 0.25	2.05 ± 0.37	1.05 ± 0.17*****	2.62 ± 0.34	1.76 ± 0.15*****
Mrp3	1.31 ± 0.12	0.23 ± 0.13*****	2.68 ± 0.41	0.59 ± 0.11*****	1.07 ± 0.17	0.09 ± 0.01*****	2.28 ± 0.29	0.63 ± 0.08*****
Mrp4	1.17 ± 0.12	1.25 ± 0.40	4.87 ± 0.21	3.11 ± 0.48*****	1.56 ± 0.28	0.63 ± 0.11*****	4.58 ± 0.61	3.412 ± 0.34

Relative mRNA expression of target genes in non-pregnant (NP) and pregnant (P) females fed the same diet. Data are presented as mean ± SEM. n = 3-6 * P ≤ 0.05 in comparisons vs non-pregnant females fed the same diet.

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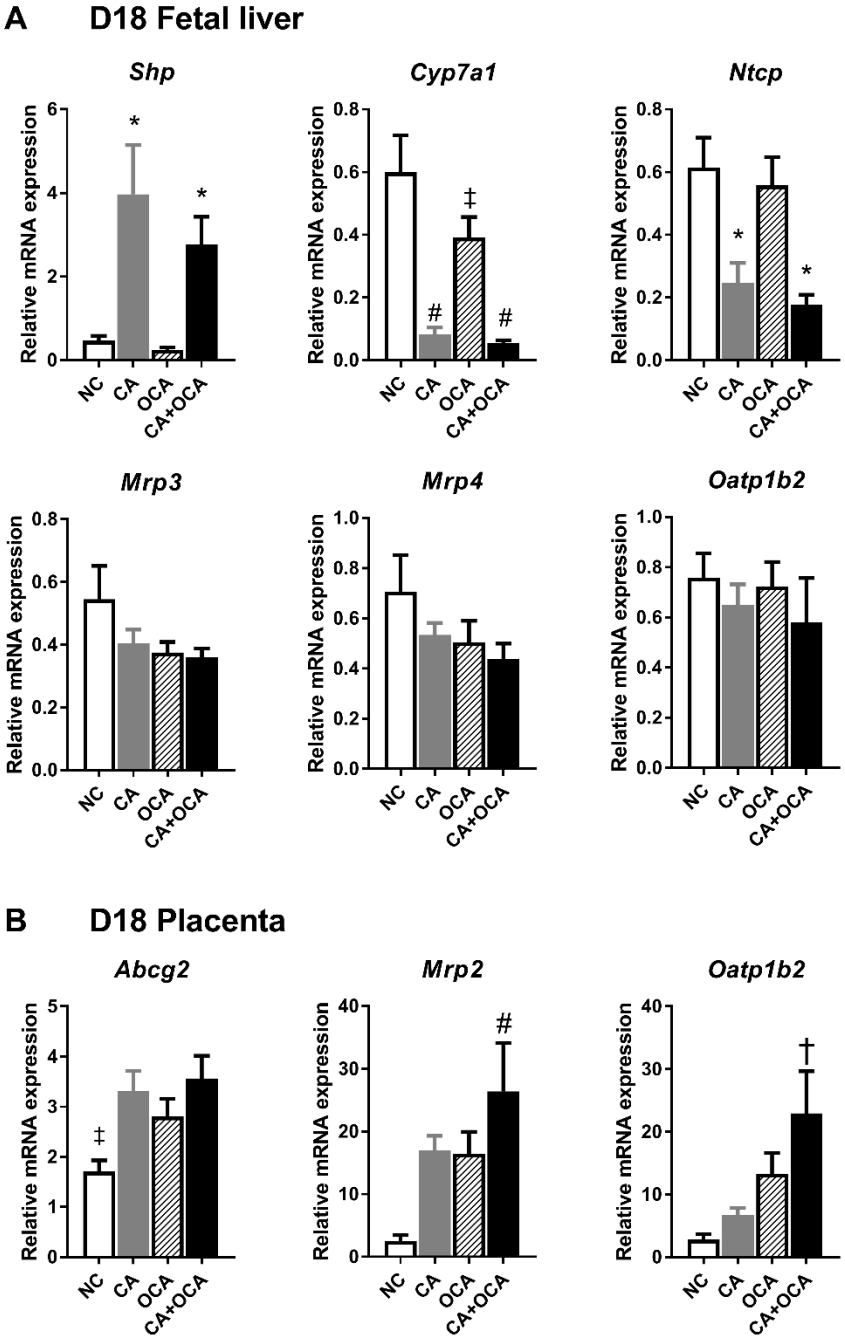
Table 2 - Effect of pregnancy on mRNA expression of key bile acid homeostasis genes in the terminal ileum.

	NC		CA		OCA		CA+OCA	
	NP	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**
Shp	3.17 ± 1.96	3.93 ± 0.36	229.70 ± 57.30	76.18 ± 14.53*****	670.4 ± 211.6	322.50 ± 80.76	398.90 ± 73.87	167.70 ± 36.85*****
Fgfr15	1.47 ± 0.57	0.27 ± 0.14	4.29 ± 0.27	2.23 ± 0.40*****	2.88 ± 0.55	2.08 ± 0.64	4.10 ± 0.62	3.20 ± 0.66

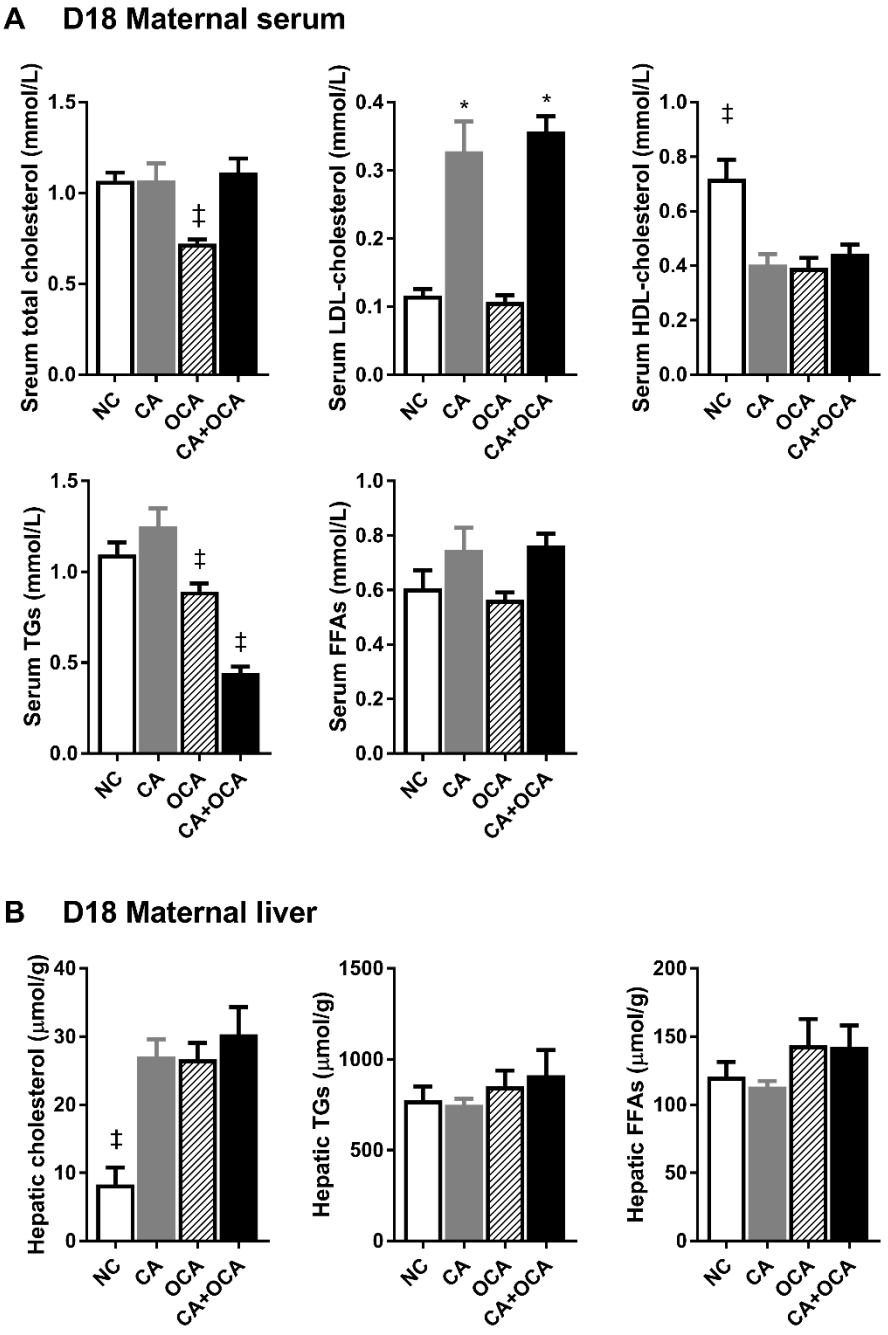
Relative mRNA expression of target genes in non-pregnant (NP) and pregnant (P) females fed the same diet. Data are presented as mean ± SEM. n = 3-6 * P ≤ 0.05 in comparisons vs non-pregnant females fed the same diet.



683

684 **Figure 6 - Expression of key bile acid homeostasis genes in the fetoplacental unit.** (A) mRNA
685 expression of genes regulating bile acid synthesis and transport in the fetal liver. (B) mRNA
686 expression of genes regulating bile acid transport in the placenta. Data are presented as mean ±
687 SEM. n = 5-6. * $P \leq 0.05$ in comparisons vs NC and OCA groups. # $P \leq 0.05$ in comparisons vs NC. ‡ $P \leq$
688 0.05 in comparisons vs all groups.† $P \leq 0.05$ in comparisons vs NC and CA groups.

689



690

691 **Figure 7 – Effects of hypercholanemia and OCA treatment during pregnancy on serum and hepatic**
692 **lipid levels.** (A) Serum lipid levels. (B) Hepatic lipid levels. Data are presented as mean ± SEM. n = 4-
693 6. ‡ P ≤ 0.05 in comparisons vs all groups. # P ≤ 0.05 in comparisons vs NC. * P ≤ 0.05 in comparisons
694 vs NC and OCA groups. TGs, triglycerides; FFAs, free fatty acids.

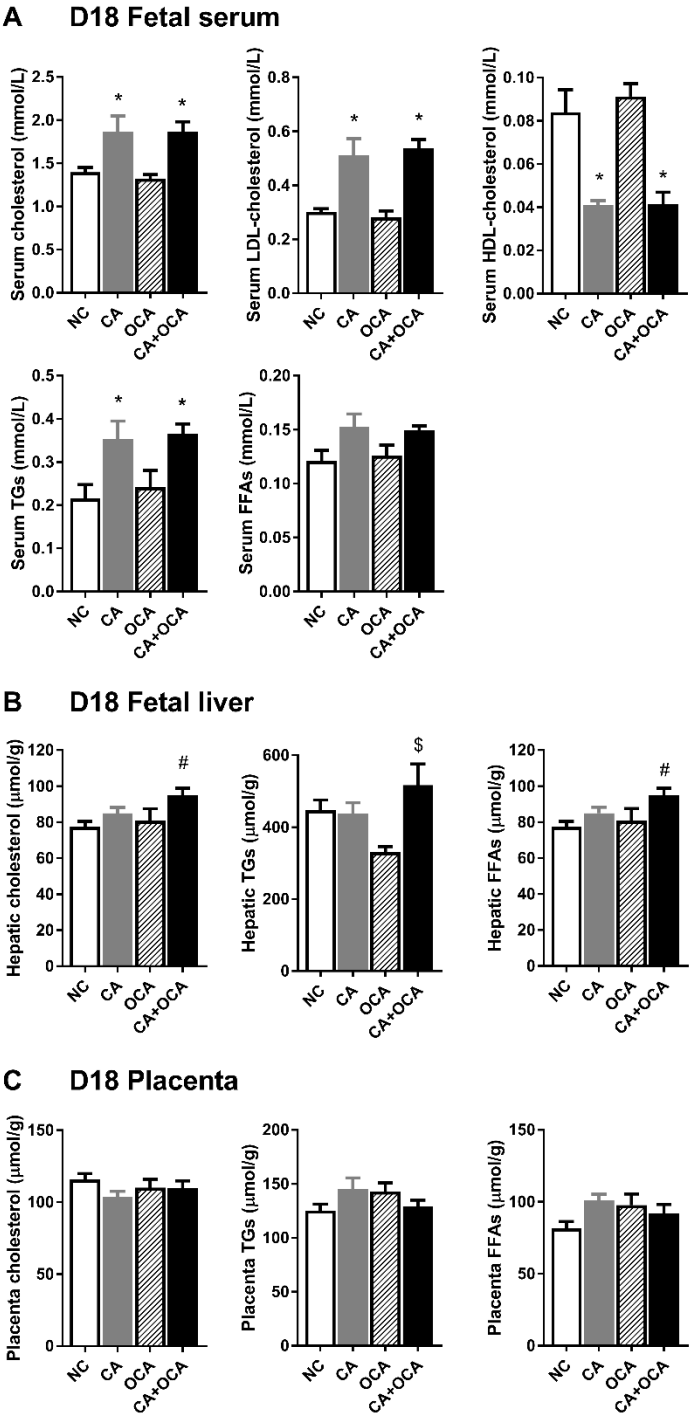


Figure 8 – Effects of hypercholanemia and OCA treatment on lipid levels in the fetoplacental unit. (A) Fetal serum lipid levels. (B) Fetal hepatic lipid levels. (C) Placental lipid levels. Data are presented as mean ± SEM. n = 4-6. * $P \leq 0.05$ in comparisons vs NC and OCA groups. # $P \leq 0.05$ in comparisons vs NC. $ $P \leq 0.05$ in comparisons vs OCA group. TGs, triglycerides; FFAs, free fatty acids.

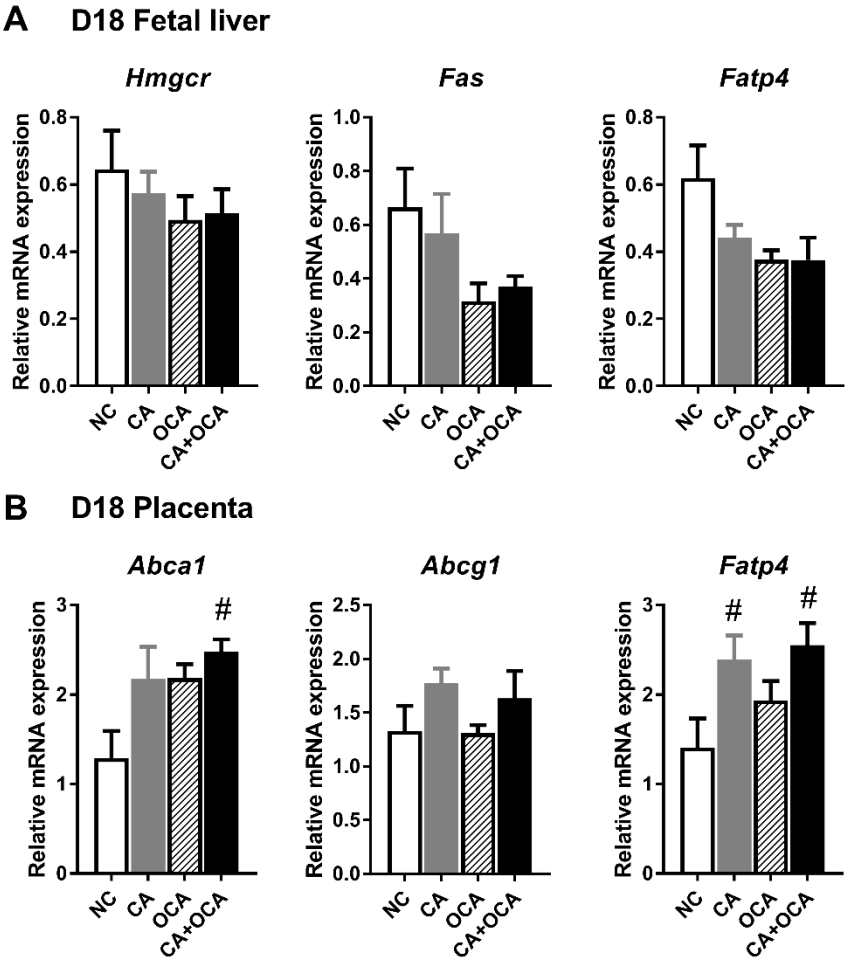


Figure 9 - Effects of hypercholanemia and OCA treatment on lipid homeostasis genes in the fetoplacental unit. (A) Expression of key hepatic lipid biosynthesis and transport genes in the fetal liver**.** (B) Placental expression of lipid transport genes. Data are presented as mean ± SEM. n = 4-6. # P ≤ 0.05 in comparisons vs NC.