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

2 Vanessa Pataia<sup>1</sup>, Saraïd McIlvride<sup>1</sup>, Georgia Papacleovoulou<sup>1</sup>, Caroline Ovadia<sup>1</sup>, Julie A. K. McDonald<sup>2</sup>,  
3 Annika Wahlström<sup>3</sup>, Eugène Jansen<sup>4</sup>, Luciano Adorini<sup>5</sup>, David Shapiro<sup>5</sup>, Julian R. Marchesi<sup>6,7</sup>, Hanns-  
4 Ulrich Marschall<sup>3</sup> and Catherine Williamson<sup>1\*</sup>

5 <sup>1</sup>Department of Women and Children's Health, King's College London, United Kingdom; <sup>2</sup>MRC Centre  
6 for Molecular Bacteriology and Infection, Imperial College London, London, UK; <sup>3</sup>Department of  
7 Molecular and Clinical Medicine/Wallenberg Laboratory, Sahlgrenska Academy, University of  
8 Gothenburg, Sweden; <sup>4</sup>Centre for Health Protection, National Institute for Public Health and the  
9 Environment, the Netherlands; <sup>5</sup>Intercept Pharmaceuticals, San Diego, CA, USA; <sup>6</sup>Department of  
10 Metabolism, Digestion and Reproduction, Imperial College London, UK; <sup>7</sup>School of Biosciences,  
11 Cardiff University, Cardiff, UK.

12 Running title: Obeticholic acid improves fetal hypercholanemia

13 \*Corresponding author:

14 Professor Catherine Williamson

15 2.30W Hodgkin Building,

16 King's College London,

17 Guy's Campus,

18 London SE1 1UL,

19 United Kingdom.

20 Email address: [catherine.williamson@kcl.ac.uk](mailto:catherine.williamson@kcl.ac.uk)

21 Telephone: +44 20 7848 6350

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26

## 27 **Abstract**

28 Intrahepatic cholestasis of pregnancy (ICP) is characterized by elevated maternal circulating bile acid  
29 levels and associated dyslipidemia. ICP leads to accumulation of bile acids in the fetal compartment  
30 and the elevated bile acid concentrations are associated with an increased risk of adverse fetal  
31 outcomes. The farnesoid X receptor agonist, obeticholic acid (OCA) is efficient in the treatment of  
32 cholestatic conditions such as primary biliary cholangitis. We hypothesized that OCA administration  
33 during hypercholanemic pregnancy will improve maternal and fetal bile acid and lipid profiles.  
34 Female C57BL/6J mice were fed either: a normal chow diet, a 0.5% cholic acid (CA)-supplemented  
35 diet, a 0.03% OCA-supplemented diet, or a 0.5% CA + 0.03% OCA-supplemented diet for 1 week prior  
36 to mating and throughout pregnancy until euthanization on day 18. The effects of CA and OCA  
37 feeding on maternal and fetal morphometry, bile acid and lipid levels, and cecal microbiota were  
38 investigated. OCA administration during gestation did not alter the maternal or fetal body weight or  
39 organ morphometry. OCA treatment during hypercholanemic pregnancy reduced bile acid levels in  
40 the fetal compartment. However, fetal dyslipidemia was not reversed, and OCA did not impact  
41 maternal bile acid levels or dyslipidemia. In conclusion, OCA administration during gestation had no  
42 apparent detrimental impact on maternal or fetal morphometry and improved fetal  
43 hypercholanemia. As high serum bile acid concentrations in ICP are associated with increased rates  
44 of adverse fetal outcomes, further investigations into the potential use of OCA during cholestatic  
45 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

## 55 **Introduction**

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

64 The adverse fetal outcomes that occur in ICP include preterm birth, fetal hypoxia, meconium-stained  
65 amniotic fluid, stillbirth and prolonged admission to the neonatal unit (19). Maternal bile acid levels  
66 have been reported to be positively correlated to fetal bile acid levels, and incremental rises in  
67 maternal serum bile acids above 40  $\mu\text{mol/l}$  are associated with higher risk of adverse fetal outcomes  
68 (7, 19, 21). The fetal lipid profile has also been shown to be affected by maternal cholestasis, with  
69 increased cholesterol accumulation in the fetal liver and placenta in a mouse model of gestational  
70 cholestasis and in the umbilical cord of neonates exposed to maternal ICP (41).

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

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

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

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

86 **Materials and methods**

87 *Animal experiments*

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

93 We have previously shown that cholic acid (CA) feeding can induce maternal hypercholanemia in  
94 mice (32, 41). Female mice were assigned to either standard maintenance and breeding diet (CRM),  
95 referred to as normal chow diet (NC), a 0.5% CA-supplemented CRM diet, a 0.03% obeticholic acid  
96 (Intercept Pharmaceuticals, USA) (OCA)-supplemented CRM diet, or a 0.5% CA + 0.03% OCA  
97 (CA+OCA)-supplemented CRM diet one week prior to mating, and maintained on their assigned diet  
98 for the duration of the experimental procedures. The dose of OCA was selected based on previously  
99 published literature (5), and was equivalent to approximately 42 mg/kg/day. Females were mated to  
100 control males and checked daily for the presence of a copulatory plug. The day of identification of  
101 the copulatory plug was considered day 1 of pregnancy (D1). Body weight of pregnant females was  
102 measured on days 7, 14 and 18 of pregnancy (D7, D14, D18). On D18, females were fasted for 4  
103 hours and euthanized by CO<sub>2</sub> inhalation. Maternal and fetal sera were collected and pup number per  
104 litter was assessed. Maternal liver, subcutaneous white adipose tissue (sWAT), gonadal white  
105 adipose tissue (gWAT), brown adipose tissue (BAT), fetal and placental weight were measured.  
106 Maternal liver, terminal ileum, fetal liver and placenta were collected and snap-frozen. Non-  
107 pregnant control female mice were maintained on the same diets as pregnant females for an  
108 equivalent length of time and were assessed for the same parameters.

109

110 *Gene expression studies*

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

122

123 *Serum and tissue lipid quantification*

124 Serum and tissue lipid content were extracted and measured as previously described (38). In brief,  
125 frozen tissues of interest were homogenized in Hank's Balanced Salt Solution using a TissueLyser II  
126 (Qiagen, UK) system. Samples were then centrifuged at 12000 rpm for 15 minutes at 4°C (Rotina  
127 420R Benchtop Centrifuge, Hettich, Germany). The supernatant was discarded. The pellet was re-  
128 suspended in 500 µL of lysis buffer containing 0.125 M potassium phosphate, 1 mM EDTA and 0.1%  
129 Triton-X 100 at pH 7.4. Samples were sonicated at 4°C for 8 minutes in a Bioruptor Plus (4 cycles of  
130 sonication for 30 seconds followed by 4 cycles of resting for 30 seconds). Samples were  
131 subsequently centrifuged at 10000 rpm for 15 minutes at 4°C. Total cholesterol, LDL-cholesterol,  
132 HDL-cholesterol, triglycerides (TGs), free fatty acids (FFAs) and total protein were measured in  
133 plasma and tissue extracts with an Unicel DxC 800 autoanalyzer (Beckman-Coulter, the Netherlands)  
134 using dedicated kits, with the exception of FFAs which were measured using a kit from Wako

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

137

#### 138 *Serum and cecal bile acid quantification*

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

145

#### 146 *16S rRNA gene sequencing analysis*

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

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

163

#### 164 *Statistical analysis*

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

169 **Results**

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

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

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

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

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

187

188 ***OCA administration during hypercholanemic pregnancy reduces fetal hypercholanemia***

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

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

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

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

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

207

#### 208 ***OCA administration alone reduces cecal bile acid levels***

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

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

218

### 219 ***Bile acid supplementation impacts the cecal microbiome's microbiota composition***

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

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

243

#### 244 ***OCA administration represses maternal hepatic Cyp7a1 expression via intestinal FXR***

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

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

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

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

265

266 ***Maternal OCA administration represses fetal hepatic Cyp7a1 expression***

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

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

283

284 ***OCA administration during hypercholanemic pregnancy does not reverse maternal dyslipidemia***

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

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

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

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

303

#### 304 ***OCA administration during hypercholanemic pregnancy does not reverse fetal dyslipidemia***

305 As maternal dyslipidemia is commonly associated with fetal dyslipidemia, we next investigated the  
306 fetal lipid profile. Maternal CA feeding significantly increased fetal serum cholesterol levels,  
307 including LDL-cholesterol, and this was not altered by maternal CA+OCA supplementation (Figure  
308 8A). In parallel, fetal serum HDL-cholesterol concentrations were reduced in maternal CA and  
309 CA+OCA supplementation groups. Fetal circulating triglycerides were increased in fetuses from CA-  
310 fed mothers and were not improved by maternal CA+OCA feeding (Figure 8A). Of note, maternal  
311 OCA-feeding alone had no effect on fetal total and LDL- or HDL-cholesterol levels or triglyceride and  
312 FFA concentrations (Figure 8A).

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

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

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

## 330 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

446

#### 447 *Author contributions*

448 VP, GP and CW were responsible for study conception and design. VP, JAKM, AW, EJ and HUM  
449 generated experimental data. VP, SM and JAKM performed data analysis. CW supervised the  
450 research and acquired funding. VP, SM and CW drafted the article. GP, CO, JAKM, AW, EJ, LA, DS,  
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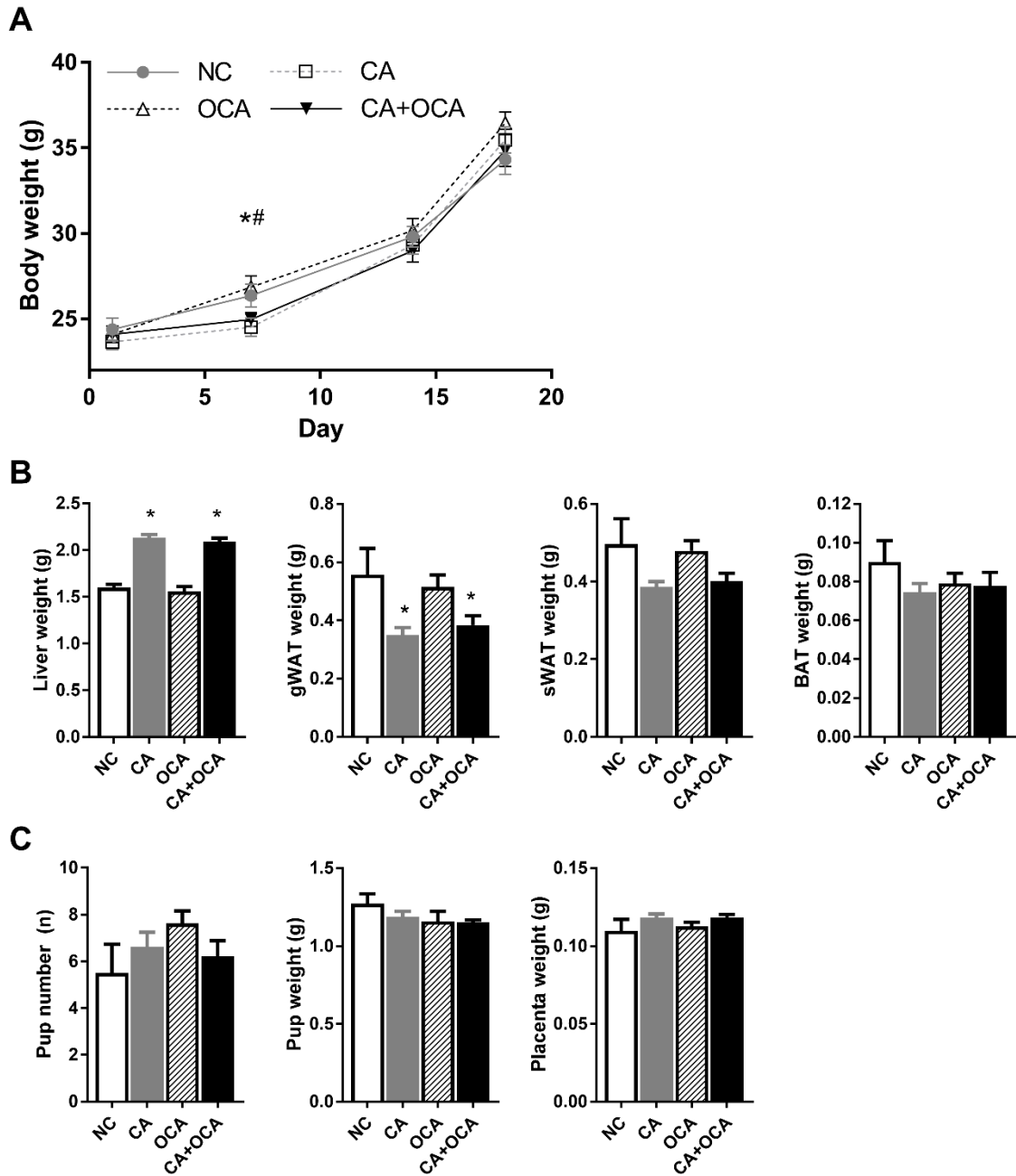
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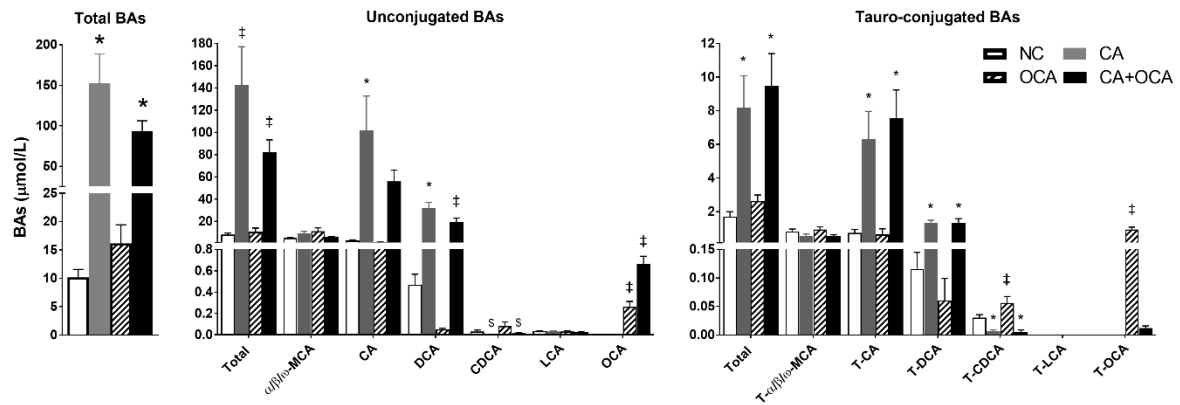
640 52. **Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, and Edwards PA.**  
641 Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice.  
642 *Proc Natl Acad Sci USA* 103: 1006-1011, 2006.

643

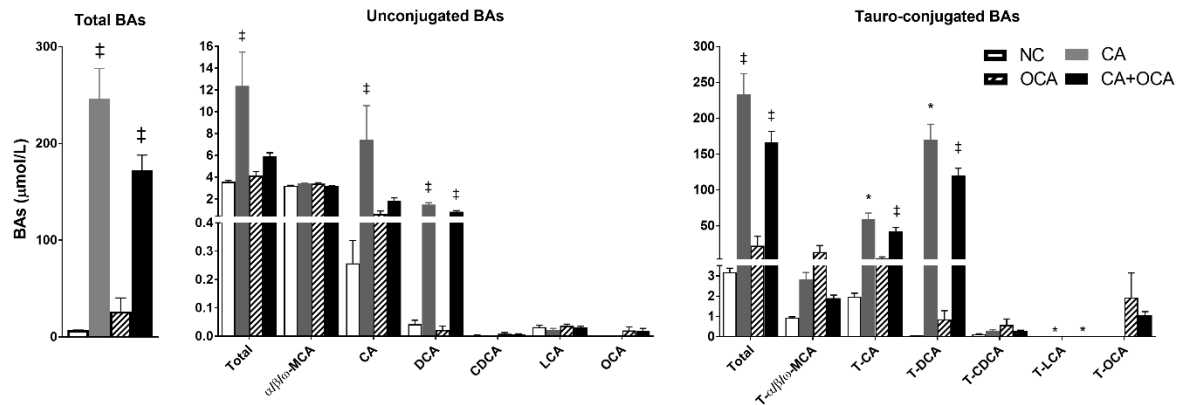


645  
 646 **Figure 1 – Effects of hypercholanemia and OCA treatment during pregnancy on body and organ**  
 647 **morphometry.** (A) Body weight of pregnant females on D1, D7, D14 and D18. #  $P \leq 0.05$  for CA vs  
 648 OCA, \*  $P \leq 0.05$  for CA+OCA vs OCA groups. (B) Weight of liver, gonadal 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. \*  $P \leq 0.05$  in  
 651 comparisons vs NC and OCA groups. Data are presented as mean  $\pm$  SEM. n = 6-9

**A D18 Maternal serum**

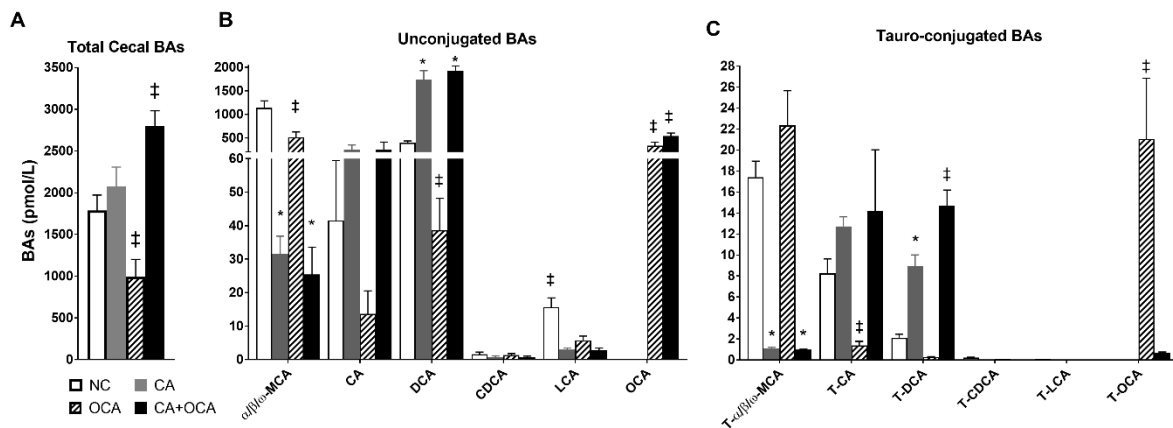


**B D18 Fetal serum**



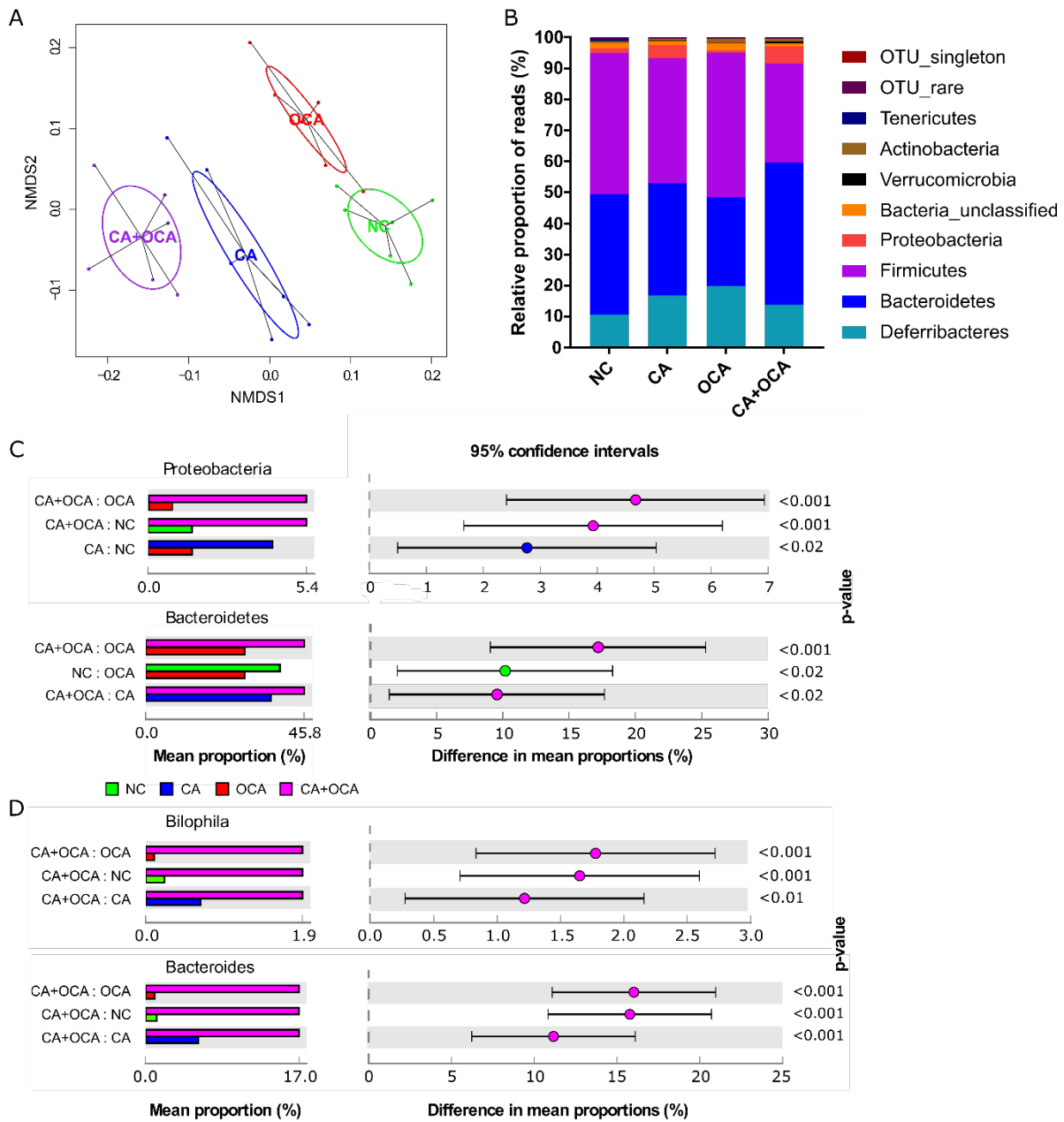
652

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.



659

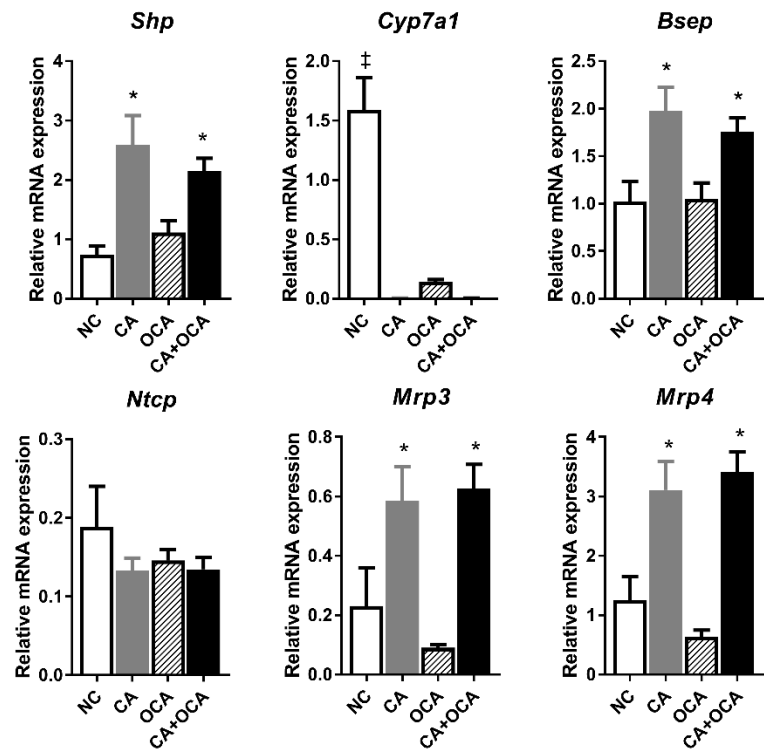
660 **Figure 3 - Effects of hypercholanemia and OCA treatment during pregnancy on cecal bile acid**  
 661 **profile.** Bile acid levels in cecum of D18 pregnant females. (A) Total cecal bile acids (BAs). (B)  
 662 Unconjugated bile acids. (C) Taurine-conjugated bile acids. Data are presented as mean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



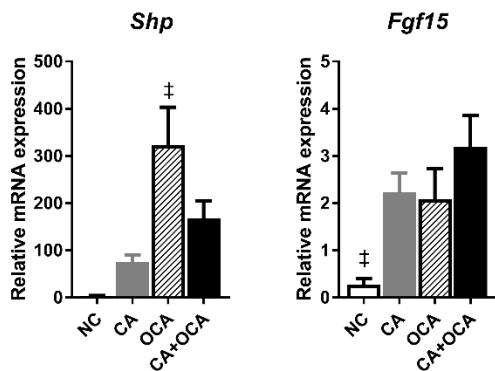
664 **Figure 4 – Changes in cecal microbiota in pregnant mice measured by 16S rRNA gene sequencing.**  
 665 (A) Nonmetric multidimensional scaling (NMDS) plot showing differences in bacterial community  
 666 structure based on the weighted UniFrac distance metric. For p-values see Supplementary Table S2.  
 667 (B) Changes in relative proportion of reads at phylum level. (C and D) Significant changes in the  
 668 average relative proportion of sequences assigned to each taxa for each dietary group, at phylum (C)  
 669 and genus level (D). Data presented as extended error bar plots showing p-value, effect size and  
 670

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

**A D18 Maternal liver**



**B D18 Maternal terminal ileum**



673  
 674 **Figure 5 – Expression of key bile acid homeostasis genes in pregnant females.** (A) mRNA expression  
 675 of genes regulating bile acid synthesis and transport in the liver. (B) mRNA expression of genes  
 676 regulating bile acid synthesis and transport in the terminal ileum. Data are presented as mean ±  
 677 SEM. n = 4-6. \* P ≤ 0.05 in comparisons vs NC and OCA groups. ‡ P ≤ 0.05 in comparisons vs all  
 678 groups.

679

**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
<i>Shp</i>	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*
<i>Cyp7a1</i>	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
<i>Ntcp</i>	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*
<i>Bsep</i>	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*
<i>Mrp3</i>	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*
<i>Mrp4</i>	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.

681

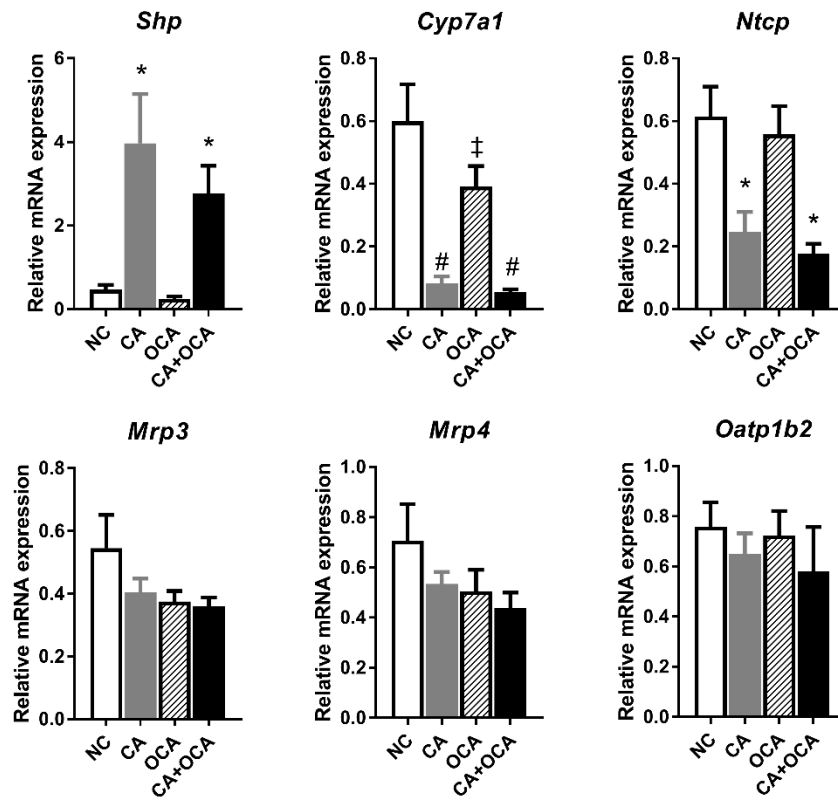
682

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

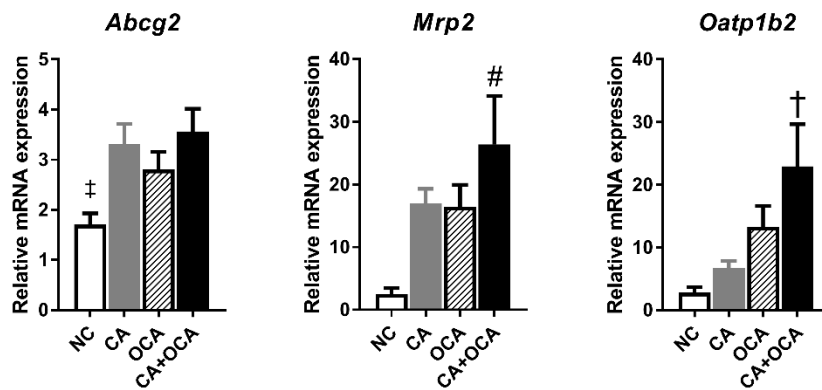
	NC		CA		OCA		CA+OCA	
	NP	P	NP	P	NP	P	NP	P
<i>Shp</i>	3.17 ± 1.96	3.93 ± 0.36	229.70 ± 57.30	76.18 ± 14.53*	670.4 ± 211.6	322.50 ± 80.76	398.90 ± 73.87	167.70 ± 36.85*
<i>Fgfr15</i>	1.47 ± 0.57	0.27 ± 0.14	4.29 ± 0.27	2.23 ± 0.40*	2.88 ± 0.55	2.08 ± 0.64	4.10 ± 0.62	3.20 ± 0.66

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.

**A D18 Fetal liver**



**B D18 Placenta**

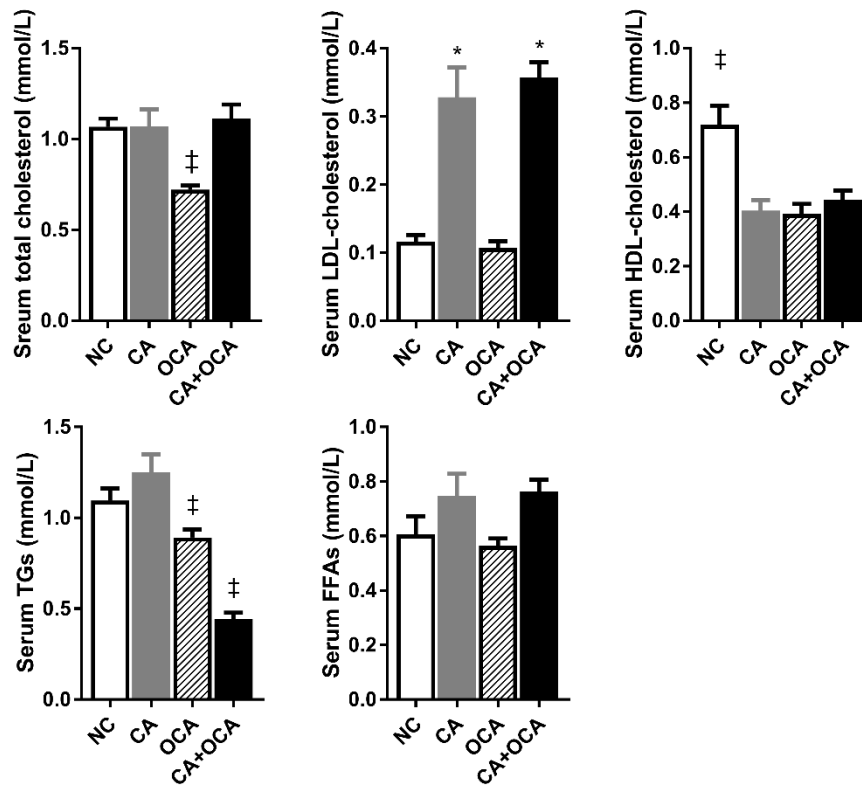


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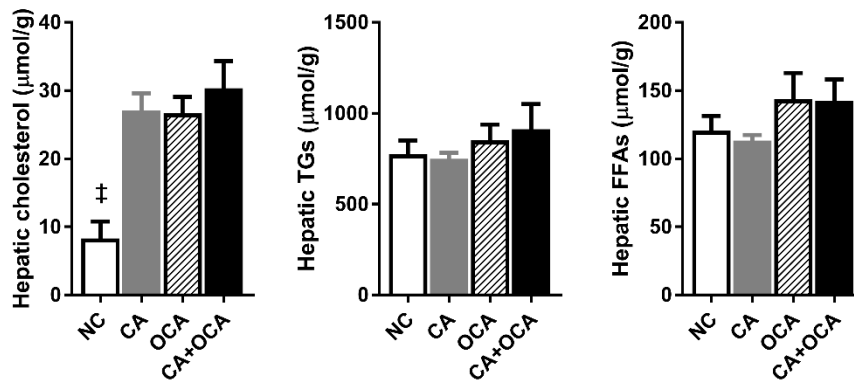
684 **Figure 6 - Expression of key bile acid homeostasis genes in the fetoplacental unit.** (A) mRNA  
 685 expression of genes regulating bile acid synthesis and transport in the fetal liver. (B) mRNA  
 686 expression of genes regulating bile acid transport in the placenta. Data are presented as mean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

689

### A D18 Maternal serum



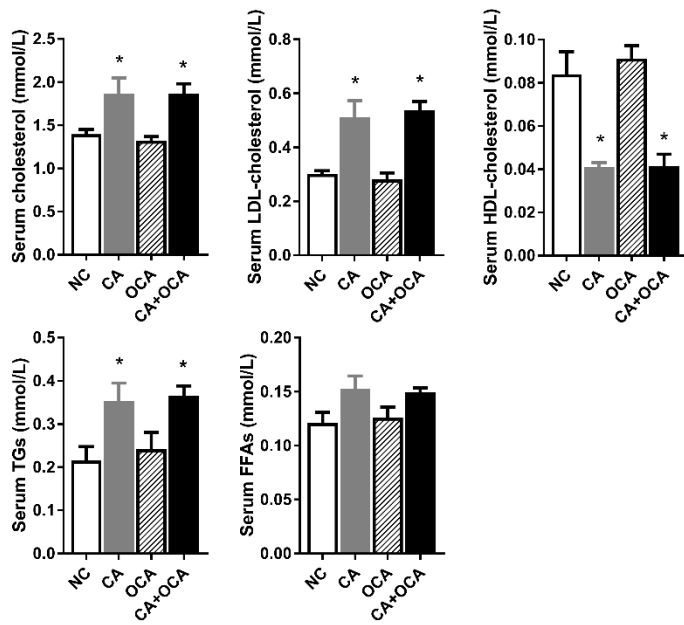
### B D18 Maternal liver



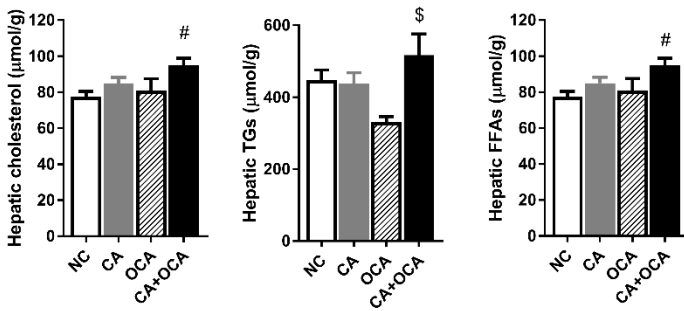
690

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.

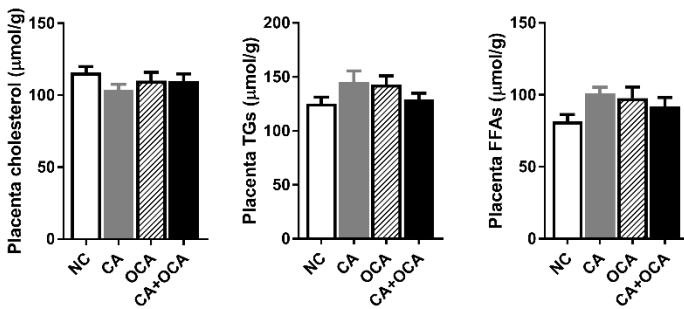
**A D18 Fetal serum**



**B D18 Fetal liver**



**C D18 Placenta**



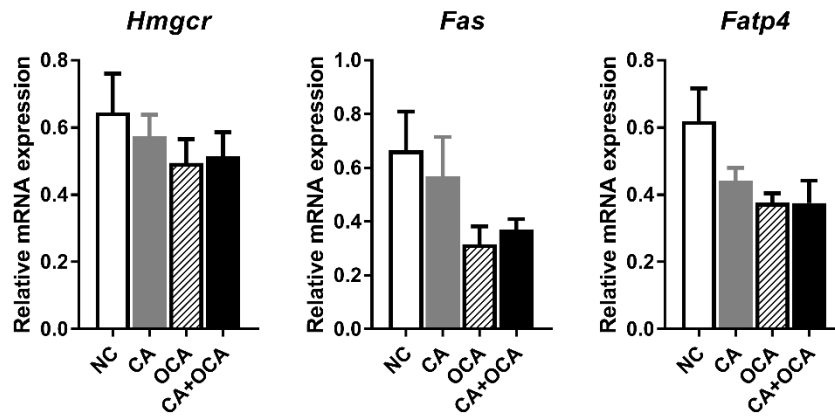
695

696 **Figure 8 – Effects of hypercholanemia and OCA treatment on lipid levels in the fetoplacental unit.**

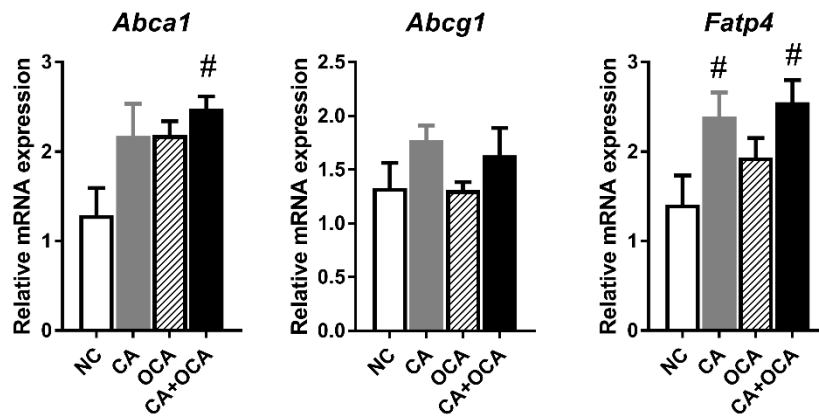
697 (A) Fetal serum lipid levels. (B) Fetal hepatic lipid levels. (C) Placental lipid levels. Data are presented  
 698 as mean ± SEM. n = 4-6. \* P ≤ 0.05 in comparisons vs NC and OCA groups. # P ≤ 0.05 in comparisons  
 699 vs NC. \$ P ≤ 0.05 in comparisons vs OCA group. TGs, triglycerides; FFAs, free fatty acids.

700

**A D18 Fetal liver**



**B D18 Placenta**



701

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