

**Title: The effects of periconceptional maternal alcohol intake and a postnatal high-fat diet on obesity and liver disease in male and female rat offspring.**

**Running head: Adult obesity following periconceptional alcohol**

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## Abstract

The effects of maternal alcohol consumption around the time of conception on offspring are largely unknown and difficult to determine in a human population. This study utilized a rodent model to examine if periconceptional alcohol (PC:EtOH), alone or in combination with a postnatal high-fat diet (HFD) resulted in obesity and liver dysfunction. Sprague-Dawley rats were fed a control or ethanol-containing liquid diet (12.5% EtOH v/v) from 4 days prior to mating until 4 days of gestation (n=12/group). A subset of offspring was fed a HFD between 3-8 months of age. In males, PC:EtOH and HFD increased total body fat mass ( $P_{\text{PC:EtOH}} < 0.05$ ;  $P_{\text{HFD}} < 0.0001$ ), whereas in females, only HFD increased fat mass ( $P_{\text{HFD}} < 0.0001$ ). PC:EtOH increased microvesicular liver steatosis in male, but not female offspring. Plasma triglycerides, HDL and cholesterol were increased in PC:EtOH-exposed males ( $P_{\text{PC:EtOH}} < 0.05$ ); and LDL, cholesterol and leptin in PC:EtOH-exposed females ( $P_{\text{PC:EtOH}} < 0.05$ ). mRNA levels of *Tnf- $\alpha$*  and *Lep* in visceral adipose tissue were increased by PC:EtOH in both sexes ( $P_{\text{PC:EtOH}} < 0.05$ ) and *IL-6* mRNA was increased in males ( $P_{\text{PC:EtOH}} < 0.05$ ). This was associated with reduced miR-26a expression, a known regulator of IL6 and TNF $\alpha$ . Alcohol exposure around conception increases obesity risk, alters plasma lipid and leptin profiles and induces liver steatosis in a sex-specific manner. These programmed phenotypes were similar to those caused by a postnatal HFD, particularly in male offspring. These results have implications for the health of offspring whose mothers consumed alcohol around the time of conception.

## 38 Introduction

39 Accumulating evidence suggests that exposure to a suboptimal *in utero* environment,  
40 including maternal alcohol intake (44) can impact on fetal development and offspring health,  
41 and therefore partially explain the increasing incidence in disorders such as obesity and non-  
42 alcoholic fatty liver disease (NAFLD) (32). The timing of exposure may be important in  
43 determining the severity of the outcome (16). Most fetal programming studies have focused  
44 on maternal insults when present throughout gestation or during organogenesis but the time  
45 shortly prior to conception until embryonic implantation (the periconceptual period) is now  
46 recognized to be important (29, 31, 52). However, very little is known about how alcohol  
47 intake during this period, [when women are most likely to drink (17, 33)], impacts on the  
48 long-term health of the offspring, as previous studies have focused on the effects of alcohol  
49 when consumed throughout gestation (6, 8, 13, 30). The mechanisms through which  
50 exposure to a stressor around conception can contribute to long term disease outcomes are  
51 thought to involve epigenetic mechanisms (21) including alterations in DNA methylation,  
52 histone modifications and/or non-coding RNAs such as miRNAs (50).

53 Data from the Dutch famine have demonstrated that obesity may be programmed *in utero*  
54 (10, 40). Animal models of maternal undernutrition (24), low protein diet (2) and utero-  
55 placental insufficiency (25) in various species have confirmed this. Interestingly, in adults, a  
56 number of miRNAs have been shown to play a role in regulating obesity-induced  
57 inflammatory pathways (55). Given that many of the programmed chronic disease outcomes,  
58 including obesity, involve chronic low grade inflammatory processes, it is possibly that  
59 epigenetic regulators of key inflammatory genes are disrupted. Prenatal alcohol exposure has  
60 been investigated in relation to alterations in glucose and insulin metabolism (6, 9, 38), but  
61 despite strong links between insulin resistance, obesity and inflammation (15, 36), only one

study of high alcohol exposure during pregnancy in a guinea pig model, has examined effects of adiposity in adult offspring (11).

We have recently shown in a rat model that maternal alcohol intake, when limited to the time around conception (PC:EtOH-exposure), restricts fetal growth (20) – a strong indicator of adult onset disease– and impairs glucose tolerance and induces insulin resistance in adult offspring (19). This metabolic phenotype was associated with altered expression of regulators of hepatic gluconeogenesis. In addition, available evidence suggests that a mismatch between the *in utero* and postnatal environments can lead to disease by unmasking an *in utero*-programmed phenotype (7, 42) so we further investigated the effects of a high fat diet (HFD) during adulthood on animals exposed to alcohol around conception. Importantly, we demonstrated that although metabolic dysfunction occurred independently of offspring diet, exposure to a high fat diet (HFD) exacerbated metabolic dysfunction in male offspring. This study however did not investigate other aspects of liver function or the role of obesity in offspring metabolic dysfunction. Here, using the same model, we aimed to determine if PC:EtOH-exposure induces obesity in adult offspring and investigate mechanisms which may contribute to the metabolic dysfunction previously published. We hypothesized that PC:EtOH-exposure would program obesity in offspring, alter systemic regulators of adiposity and induce liver steatosis. As developmental programming may affect male and female offspring differently, especially in cases where a mismatch occurs between the prenatal and postnatal environment (47), we investigated all outcomes in a sex specific manner. This study has important clinical implications for women who drink around conception.

## Materials and methods

**Ethics:** All the animal experiments and procedures were approved by The University of Queensland Anatomical Bioscience Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) prior to commencement of the study.

**Treatment:** Nulliparous Sprague-Dawley rats were treated as described in detail previously (19). Briefly, dams were given a liquid diet containing either 0% or 12.5% EtOH (v/v) with similar energy composition from 4 days prior to mating until 4 days of gestation. Standard chow was offered from day 5. At postnatal day 30 (PN30) blood samples were collected from a subset of offspring. At 3 months of age, a subset of offspring had their food intake measured in metabolic cages. Male and female animals (2 of each sex per litter) were acclimatized to the metabolic cages for 2-3 hours on two occasions prior to being housed for a full 24-hour period in the metabolic cage. Food intake was monitored (n=12 litters/group) and food consumption was calculated relative to body weight (g/bw). Offspring were then randomly assigned to a HFD (21% fat, 0.15% cholesterol, 19% protein, 59.9% carbohydrates, 19.4MJ/kg) or remained on standardized chow (C; 4.0% fat, 13.6%protein 64.3% carbohydrates; 15.5 MJ/kg), generating four treatment groups: untreated (U):C; U:HFD; PC:EtOH:C and PC:EtOH:HFD for both male and female offspring. At 6 months of age, 24-h food intake measurements were repeated in the metabolic cages in all groups.

**Body composition measurements:** A dual-energy X-ray absorptiometer (DXA; model XR36, Norland) was used to determine body composition (total fat mass, total fat free mass, abdominal fat mass, bone mineral density (BMD) and bone mineral content (BMC)) at 7 months (n=10-11/group). Rats were anesthetized with an intra-peritoneal (i.p.) injection of a 50/50 mix of zoletil/xylazile (0.1mL/100g bw). Scan images were analysed using the

manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland).

**Tissue collection:** Offspring (n=12/group) were sacrificed at 8 months following an overnight fasting period via i.p. administration of a mix of 50/50 ketamine/xylazine (0.5mL/100g bw). Snout-rump length and abdominal girth were measured to the nearest mm. Liver and visceral abdominal adipose tissue were snap frozen in liquid nitrogen and stored in -80°C, and the apex of the left lateral lobe of the liver was collected in 4% paraformaldehyde for histological analyses.

**Blood sampling and plasma biochemistry:** Blood was collected via cardiac puncture on PN30 and at 8 months, immediately spun (3000 rpm, 15 minutes, 4°C) and plasma stored at -80°C. TG, HDL, LDL, total cholesterol and electrolytes were analysed with a Cobas Integra 400 Plus Chemistry Analyzer (Block Scientific, NY, USA). Leptin was analysed with a multi-species leptin radioimmunoassay kit with an assay sensitivity of 0.2ng/mL (Cat.# XL-85K, Millipore, Pty. Ltd., Kilsyth, VIC, Australia). Between and within assay CVs were both <6% for two quality controls samples of 3.3 and 15.9 ng/mL. Adiponectin concentrations were analyzed by an enzyme-linked immunosorbent assay with an assay sensitivity of 0.4ng/mL (Cat#EZRADP-62K, Millipore, Pty. Ltd., Kilsyth, VIC, Australia).

**Gene analyses:** RNA from the left lateral lobe of the liver was extracted using an RNeasy Mini-Kit and visceral adipose tissue was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen, VIC, Australia) and reversed transcribed into cDNA. Gene expression analyses were conducted via qPCR with the Taqman Assay-on-Demand primer/probe sets *Leptin* (*Lep-Rn00565158\_m1*), *Tnf-α* (*Tnf-Rn01525859\_g1*) and *Il-6* (*Il6-Rn01410330\_m1*) (Applied Biosystems, CA, USA). mRNA expression was calculated with the  $\Delta\Delta c(t)$  method, using *Rn18s* as the endogenous control (20).

## **miRNA analysis**

Potential miRNAs of interest were identified using the DIANA-microT web server v5.0 software (35). Eight miRNAs predicted to regulate at least two of the differentially expressed mRNAs investigated in the current or previous study (19) were investigated. Total miRNA was extracted from visceral adipose tissue using a miRNeasy Mini Kit (Qiagen, VIC, Australia). Total miRNA was reverse transcribed using the TaqMan® Advanced miRNA cDNA Synthesis Kit which includes a universal RT primer. miRNA expression was quantified using the following TaqMan® Advanced miRNA assays: has-miR-130b-3p (assay #477840\_mir), hsa-miR-302a-3p (assay #478006\_mir), hsa-miR-26a-5p (assay #477995\_mir), rno-miR-335 (assay # rno481065\_mir), rno-miR-192-3p (assay # rno480973\_mir), rno-miR-29b-3p (assay # rno481300\_mir), rno-miR-221-5p (assay # rno481349\_mir) and hsa-miR-181b-5p (assay # 478583\_mir). miRNA expression was calculated using the  $\Delta\Delta c(t)$  method, normalized to the geometric mean of the two highly stable housekeeper assays (45) hsa-miR-191-5p (Assay #477952\_mir) and has-mir-185 (rno481331\_mir).

**Histological analyses:** One section of the left lateral lobe of the liver was processed to paraffin and sectioned at 6 $\mu$ m. Five representative sections per liver (n=8 animals/group) were stained with hematoxylin and eosin (H&E) and scored for steatosis, microvesicular steatosis, inflammation and microgranulomas, and five were stained with Masson's trichrome for evaluation of fibrosis according to the semi-quantitative Kleiner scoring system for nonalcoholic steatohepatitis (NASH) (28). All scores were performed blinded.

**Statistical analyses:** One male and one female offspring from each litter were analysed. If more than 1 animal was used from the same litter (e.g. to measure food intake), an average value for the litter was firstly obtained. Statistical analyses were conducted with GraphPad

Prism 6 software for Windows (GraphPad Software, San Diego, CA, USA). Data for U:C, U:HFD, PC:EtOH:C and PC:EtOH:HFD comparisons were analysed by two-way ANOVAs for main effects of PC-treatment ( $P_{\text{PC:EtOH}}$ ) and postnatal diet ( $P_{\text{HFD}}$ ) with males and females being analysed in separate ANOVAs. Data were log transformed to remove heterogeneity of variance prior to performing the ANOVA analyses as appropriate (Bartlett's test). In cases where interaction and/or either treatment effect were significant, means were compared using a Tukey's multiple comparison *post hoc* test to look at statistical differences within variables.  $P < 0.05$  was considered statistically significant. All results are presented as mean  $\pm$  SEM.

## Results

**Animal model:** As previously reported, there were no differences in maternal weight, initial age, caloric intake, gestational length, weight gain or litter sizes between PC:EtOH-exposed and untreated dams (20).

**PN30 outcomes:** In order to assess the effects of PC:EtOH on offspring obesity and metabolic function across the life span, parameters were first assessed following weaning, and prior to sexual maturity (PN30). Abdominal circumference, ponderal index and liver:body weight ratio were similar between treatment groups of both male and female offspring, as was body weight for both male treatment groups. The PC:EtOH exposed female offspring tended to weigh less than their untreated counterparts ( $P_{\text{PC:EtOH}} = 0.06$ , Table 1), but the difference was not statistically significant. PC:EtOH-exposed male offspring had higher plasma leptin levels ( $P_{\text{PC:EtOH}} < 0.05$ ), and tended to have elevated plasma triglyceride levels ( $P_{\text{PC:EtOH}} = 0.09$ ) compared with untreated controls on PN30, but plasma cholesterol and HDL were similar between treatment groups (Table 1). Females had similar plasma levels of leptin,



cholesterol and HDL, but plasma triglyceride levels were lower following PC:EtOH-exposure ( $P_{\text{PC:EtOH}} < 0.05$ , Table 1).

#### **Adult offspring outcomes:**

**Food intake:** Food consumption at 3 months of age was similar between control and PC-EtOH exposed offspring albeit females ate more than males ( $P_{\text{Sex}} < 0.0001$ ) (control male;  $0.056 \pm 0.002$ , control females;  $0.072 \pm 0.002$  g/bw/24h, PC:EtOH males;  $0.053 \pm 0.002$ , PC:EtOH females  $0.069 \pm 0.004$  g/bw/24h).

At 6 months of age, PC:EtOH did not affect food consumption in either sex, though offspring on HFD consumed less than animals on a regular chow diet regardless of sex ( $P_{\text{HFD}} < 0.0001$ ) (Males: U:C;  $0.035 \pm 0.001$ , U:HFD;  $0.024 \pm 0.001$ , PC:EtOH:C;  $0.033 \pm 0.001$  and PC:EtOH:HFD;  $0.025 \pm 0.001$  g/bw/24h. Females: U:C;  $0.045 \pm 0.003$ , U:HFD;  $0.031 \pm 0.004$ , PC:EtOH:C;  $0.047 \pm 0.003$  and PC:EtOH:HFD;  $0.034 \pm 0.002$  g/bw/24h).

**Offspring body weight and composition:** HFD-fed offspring weighed more than control-fed offspring for both sexes at 7 months ( $P_{\text{HFD}} < 0.001$ , Fig. 1A & B), but PC:EtOH-exposure did not affect body weight. At the time of tissue collection at 8 months, similar differences in body weight were observed ( $P_{\text{HFD}} < 0.05$ , Table. 2). Given the fact that absolute body weight was not affected in PC:EtOH-exposed offspring, we investigated the impact of PC:EtOH on body composition. PC:EtOH-exposed males had lower fat-free mass and higher total and abdominal fat mass compared to untreated groups ( $P_{\text{PC:EtOH}} < 0.05$ , Fig. 1C, E, G). The relative weight of intra-abdominal fat pads to total body weight at the time of tissue collection also tended to be higher, following PC:EtOH-exposure although not significantly ( $P_{\text{PC:EtOH}} = 0.06$ , Table. 2). PC:EtOH-exposed males had a larger abdominal circumference compared with untreated males at 8 months ( $P_{\text{HFD}} < 0.05$ , Table. 2), despite a similar

202 ponderal index (Table. 2). Both PC:EtOH-exposed and untreated males that were fed a  
203 postnatal HFD had a lower percentage (%) fat-free mass, and a higher total- and abdominal  
204 fat mass ( $P_{\text{HFD}} < 0.0001$ , Fig. 1C, E, G) and a larger amount of intra-abdominal fat per gram  
205 body weight ( $P_{\text{HFD}} < 0.0001$ , Table. 2) compared with those fed a control diet. HFD-fed male  
206 offspring also had a larger abdominal circumference ( $P_{\text{HFD}} < 0.001$ ) and ponderal index ( $P_{\text{HFD}}$   
207  $< 0.01$ , Table 2) but a HFD did not potentiate the effects of PC:EtOH-exposure. These results  
208 highlight that PC:EtOH- exposure predisposes male offspring to visceral adiposity.

209 The body composition of female offspring was unaffected by PC:EtOH, but HFD-fed females  
210 had a lower % of fat-free mass, higher total- and abdominal fat mass  $P_{\text{HFD}} < 0.0001$ , Fig. 1D,  
211 F, H), and a larger amount of intra-abdominal fat per gram body weight ( $P_{\text{HFD}} < 0.001$ , Table.  
212 2). Both abdominal circumference ( $P_{\text{HFD}} < 0.001$ ) and ponderal index ( $P_{\text{HFD}} < 0.01$ ) (Table.  
213 2) were higher in HFD-fed females. Similar to male offspring, % BMC and BMD were  
214 unaffected by both PC:EtOH-exposure and HFD.

215 **Plasma biochemistry at 8 months:** Given the fact the male PC:EtOH-exposed offspring had  
216 increased adiposity without any changes in weight gain, plasma biochemistry was assessed to  
217 attempt to ascertain the mechanisms involved. PC:EtOH-exposed male offspring aged 8  
218 months – regardless of postnatal diet – had increased plasma levels of triglycerides ( $P_{\text{PC:EtOH}}$   
219  $< 0.05$ ), HDL ( $P_{\text{PC:EtOH}} < 0.05$ ), and cholesterol ( $P_{\text{PC:EtOH}} < 0.01$ ) (Table 3). HFD-fed males  
220 had lower HDL ( $P_{\text{HFD}} < 0.05$ , Table. 3) and higher leptin levels ( $P_{\text{HFD}} < 0.01$ , Fig. 2A)  
221 compared to control-fed males. PC:EtOH-exposed female offspring aged 8 months had  
222 higher plasma levels of LDL and cholesterol ( $P_{\text{PC:EtOH}} < 0.05$ , Table 2). PC:EtOH-exposed  
223 females also had increased leptin ( $P_{\text{PC:EtOH}} < 0.05$ , Fig. 2B; and leptin ( $P_{\text{HFD}} < 0.01$ , Fig. 2B)  
224 and triglycerides ( $P_{\text{HFD}} < 0.01$ , Table. 3) were also increased in females fed a HFD, whereas

225 HDL levels were decreased ( $P_{\text{HFD}} < 0.05$ , Table. 3). Adiponectin (Fig. 2C-D) and electrolytes  
226 (not shown) were not affected by either PC:EtOH-exposure or a HFD in either sex.

227 **Adipose and hepatic gene expression:** Adipocytes are now understood to play a major  
228 endocrine role, secreting adipokines and as well as inflammatory cytokines, many of which  
229 have been implicated in the development of insulin resistance and obesity (15, 22). As such,  
230 expression of key inflammatory genes were measured in adipose and liver tissue. PC:EtOH  
231 significantly increased mRNA-levels of *Tnf- $\alpha$* , *IL-6*, and *Lep* in abdominal adipose tissue in  
232 male offspring at 8 months ( $P_{\text{PC:EtOH}} < 0.01$ , 0.05 and 0.05, Fig. 3A, C & E respectively). In  
233 females, PC:EtOH-exposure increased mRNA-levels of *Tnf- $\alpha$*  and *Lep* in abdominal adipose  
234 tissue ( $P_{\text{PC:EtOH}} < 0.05$ , Fig. 3B & F) whereas *IL-6* was not different (Fig. 3D). A HFD had no  
235 effect on mRNA-levels of *Tnf- $\alpha$* , *IL-6* or *Lep* in abdominal adipose tissue. PC:EtOH did not  
236 affect the hepatic gene expression of *Tnf- $\alpha$*  in either sex, however the HFD increased *Tnf- $\alpha$*   
237 mRNA-levels in male (U:C  $1.23 \pm 0.13$ ; EtOH:C  $1.08 \pm 0.13$ ; U:HFD  $3.22 \pm 0.53$ ; EtOH:HFD  
238  $2.28 \pm 0.46$ ,  $P_{\text{HFD}} < 0.001$ ), but not female liver (data not shown). Hepatic mRNA-levels of *IL-*  
239 *6* was unaffected by both PC:EtOH and a HFD (data not shown).

240 **miRNA expression analysis in abdominal adipose tissue:** Based on the above changes in  
241 gene expression, miRNAs predicted to regulate their expression were measured in adipose  
242 tissue. PC:EtOH-exposure decreased miR-26a expression in abdominal adipose tissue  
243 ( $P_{\text{PC:EtOH}} < 0.05$ , Fig. 4A). PC:EtOH-exposed offspring also had increased miR-29b  
244 expression, but post-hoc analysis demonstrated this was significant only in male offspring  
245 (Fig 4B). PC:EtOH exposure had no effect on expression of miR-130b, miR-181b, miR-192,  
246 miR-221, miR-302a or miR-335 (data not shown). Male offspring had higher expression of  
247 miR-181b (Fig. 4D) but lower expression of miR-335 (Fig. 4C).

**Assessment of non-alcoholic fatty liver disease:** PC:EtOH-exposure did not grossly affect hepatic morphology (Fig. 5). Signs of macrovesicular steatosis were absent in control-fed offspring of both sexes. The HFD increased the prevalence of macrovesicular steatosis in both sexes, although male offspring were more affected than females. The macrovesicular steatosis was more likely to be located around the central vein (zone 3) in untreated males fed a HFD, whereas PC:EtOH-exposed males consuming a HFD had a greater % with panacinar steatosis compared to any other treatment group. Signs of microvesicular steatosis were present in livers of both male and female offspring following both PC:EtOH and a HFD, whereas this was not seen in control-fed untreated offspring (Fig. 5). All PC:EtOH-exposed males had lobular inflammation when fed a control diet compared to 50% of U:C males. Although not all HFD-fed animals had lobular inflammation, in male offspring, the severity of this condition was worsened regardless of PC-treatment. Microgranulomas were present in all groups, however this was more frequent following PC:EtOH and/or a HFD. There were no signs of hepatic fibrosis in liver sections from any treatment group.

## **Discussion**

Numerous studies have demonstrated prenatal perturbations can result in increased fat deposition in offspring [for review see (34)]. Although total fat mass is strongly associated with morbidity and correlates with coronary artery disease and diabetes, visceral distribution of fat is an even stronger predictor of adverse health outcomes. This current study demonstrates that alcohol, when consumed only around conception, predisposes male offspring to visceral adiposity, associated with altered plasma hormones and biochemistry, along with increased mRNA expression levels of key cytokines. PC:EtOH-exposed female offspring also had altered plasma biochemistry and increased gene expression of inflammatory markers, despite no changes in body composition. PC:EtOH offspring of both

sexes were more likely to exhibit signs of microvesicular steatosis. Taken together with our previous results demonstrating that PC:EtOH can result in glucose intolerance and insulin resistance in older age, our results highlight that alcohol consumption, even prior to implantation, can have lifelong adverse consequences for the metabolic health of offspring.

We have previously reported that exposure to PC:EtOH results in fetal growth restriction (20) but catch up growth occurs by PN30 (19). Here we report that although there was no effect of PC:EtOH on body weight in the PN30 male offspring, they had significantly increased plasma leptin. We hypothesized the elevated leptin may dysregulate appetite and stimulate increased food intake as maternal undernutrition has been shown to increase food intake and leptin concentrations in offspring (48). However, we measured food intake at 3 and 6 months of age and found there were no differences between the treatment groups. It is possible that subtle differences in food intake exist, that were not detected in the metabolic cage experiment, but considering PC:EtOH did not affect body weight, the data suggests no overt hyperphagia in adult males. Of note, we have recently reported that PC:EtOH may alter food preference with females preferring a high fat diet when offered a choice (12). Interestingly, female offspring exposed to PC:EtOH had normal plasma leptin concentrations at PN30 and did not develop altered body composition later in life. However, at 8 months of age, leptin concentrations were increased in female offspring exposed to PC:EtOH suggesting that increased fat mass may be developing at a later stage than males. Furthermore leptin gene expression was increased in abdominal adipose tissue in both male and female offspring at 8 months as a result of PC:EtOH exposure.

Increased body fat in male offspring exposed to PC:EtOH is consistent with studies using different periconceptual stressors. In ewes which were periconceptionally undernourished and offspring studied at 3-4 years of age, males, but not females exhibited increased

percentage fat mass despite similar body weight (24). Similarly, mice fed a low-protein diet during gestation produced offspring with greater fat mass and percentage body fat at 9 months of age. In that study, adiposity was exacerbated in males fed a hypercaloric diet from weaning, similar to the high fat exposed males in our study (2). Human studies have also demonstrated increased % body fat in intrauterine growth restricted males (26). An increase in body fat despite normal body mass index (BMI) (or ponderal index) is referred to as ‘normal weight obesity’ and is associated with cardiometabolic dysregulation and metabolic syndrome in humans (41).

Recent evidence suggests that inflammatory pathways, are affected in offspring following maternal perturbations (1, 39) and may underlie the insulin resistance and adiposity (46, 49). TNF- $\alpha$ , overexpression in obese rodents was the first molecular link between obesity and inflammation (23, 43). Here we report increased TNF- $\alpha$  mRNA expression in abdominal adipose tissue following PC:EtOH in both sexes independent of postnatal diet. TNF- $\alpha$  expression which is increased in adiposity (15), increases plasma levels of TG and VLDL (36) and thus the increased TNF- $\alpha$  expression may have contributed to the increased levels of triglycerides in males and LDL in females exposed to alcohol. The elevated expression of TNF- $\alpha$  is also a likely mediator of the insulin resistance in the PC:EtOH offspring. Mice lacking TNF- $\alpha$  or its receptors exhibit improved insulin sensitivity compared to wild-type mice (23, 46). We also demonstrated increased gene expression of IL-6 in the male PC:EtOH offspring. Increased levels of IL-6, which can be induced by TNF- $\alpha$  (27), correlates with risk of type 2 diabetes irrespective of body fat content(37). Furthermore, adipose IL-6 expression is increased in obese or diabetic subjects compared to lean subjects (18). It has also been shown that inflammation can predict future adiposity (14) and hence the increased levels of *Tnf- $\alpha$*  transcripts in females exposed to PC:EtOH may suggest that they are likely to gain adipose tissue in later life.

Having established changes in plasma leptin along with altered expression of *Tnf- $\alpha$*  and *Il-6* transcripts following PC:EtOH, we investigated the expression of key miRNAs which are predicted to regulate the expression of these factors (35). Expression of miR-26a was reduced in adipose tissue of PC:EtOH offspring. This is consistent with a recent finding that decreased miR-26a results in increased IL-6 concentrations in an animal model of multiple sclerosis (51). In addition to its potential role in regulating IL-6, it has previously been demonstrated that miR-26 prevents *Tnf- $\alpha$*  induced transcription through silencing of key regulatory factors (5). A recent study demonstrated a direct link between miR-29b and regulation of *Tnf- $\alpha$*  transcription in human adipose cells highlighting that oxidative stress regulates *Tnf- $\alpha$*  through a miR-29b-regulated pathway (53). In this study, we identified increased miR-29b expression in male but not female offspring exposed to PC:EtOH. Together, these data suggest that changes in miRNA expression may be associated with some of the gene expression changes identified in this model.

Finally, we examined the livers of offspring exposed to alcohol for signs of steatosis. Steatosis, or accumulation of triglycerides in hepatocytes, is considered the “first hit” in NAFLD, which increases the susceptibility to “second hits” of cell injury, fibrosis and inflammation (3). Maternal obesity is known to cause NAFLD in offspring, despite no differences in bodyweight or adiposity (1). Shen *et al* (44), demonstrated increased hepatic steatosis in female rat offspring when exposed to high doses of alcohol prenatally during the second half of pregnancy. The periconceptional alcohol exposure used in this study did not severely affect liver histology, however the combination of PC:EtOH and a HFD was more likely to cause a panacinar distribution of steatosis, whereas a HFD alone were more likely to cause steatosis around the central vein. Panacinar steatosis has been associated with a greater likelihood of developing ballooning injury and fibrosis, compared to steatosis around the central and/or portal vein, which show no correlation with an early degree of fibrosis (4). We

therefore suggest that the combination of PC:EtOH and a postnatal HFD has a more profound effect on the development of NAFLD.

In conclusion, alcohol consumption by the dam around the time of conception resulted in increased adiposity in male offspring at 8 months of age and this was associated with increased triglycerides. This phenotype displayed by male offspring following PC:EtOH was comparable to that of control animals that consumed a HFD for the major part of adult life and was, in some measures, further exacerbated by a HFD. In contrast, body composition of female offspring was significantly altered by a postnatal HFD, but not by PC:EtOH highlighting distinct sexual dimorphism in the development of disease. Changes in expression of miRNAs known or predicted to regulate factors secreted by adipocytes suggests that alcohol exposure around conception has resulted in long term changes in the epigenetic regulation of gene expression which may contribute to the observed phenotypic outcomes. These findings from an animal model have implications for the many women who drink around conception.

## **Disclosures**

The authors declare no conflicts of interest.

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**Figure 1:** The effect of PC:EtOH-exposure (black bars) and/or a high-fat diet (HFD) on body weight and body composition as analysed by a dual X-ray absorptiometry scan, at 7 months compared to untreated offspring (white bars) on a control diet; n=10-11 per group. PC:EtOH did not affect body weight in neither sex but HFD-fed offspring weighed more compared with those consuming a control diet (A & B). Male offspring periconceptionally exposed to EtOH, and/or following a postnatal HFD had a smaller % of fat-free mass (C), and a bigger % of total body fat mass (E) and abdominal fat mass (G) compared to the male untreated control group. Only females that consumed a HFD, regardless of periconceptional exposure, had a smaller % of fat-free mass (D), and a bigger % of total body fat mass (F) and abdominal fat mass (H) when compared to the female untreated control group. Data were analysed with two-way ANOVAs and represented as mean  $\pm$  SEM.

**Figure 2:** Circulating levels of fasting plasma adipokines following PC:EtOH-exposure (black bars) or untreated (white bars) offspring on a control or high-fat diet (HFD) at 8 months; n=8-10 per group. Both PC:EtOH-exposed and untreated male offspring fed a HFD had elevated plasma leptin levels (A) but there were no differences in adiponectin levels between males of different treatment groups (C). Leptin levels were higher in female offspring following both PC:EtOH and a postnatal HFD (B), but similar to males, there was no difference in adiponectin levels between females of different treatment groups (D). Data were analysed with two-way ANOVAs and represented as mean  $\pm$  SEM.

**Figure 3:** The effect of PC:EtOH-exposure (black bars) and/or a high-fat diet (HFD) on gene expression in visceral abdominal adipose tissue at 8 months, compared with untreated (white bars) offspring on a control diet; n=7-8 per group. Relative gene expression of *Tnf- $\alpha$*  was increased following PC:EtOH-exposed in both male (A) and female (B) offspring. PC:EtOH-exposed male offspring also had increased gene expression of *IL-6* (C), but this was not seen in females (D). PC:EtOH-exposure also increased *Lep* gene expression in both males (E) and

females (F). The HFD did not affect gene expression of neither *Tnf- $\alpha$* , *IL-6* or *Lep*. Data were analysed with two-way ANOVAs and represented as mean  $\pm$  SEM.

**Figure 4:** The effect of PC:EtOH-exposure (black bars) on miRNA expression in visceral abdominal adipose tissue at 8 months, compared with untreated (white bars) offspring; n=7-8 per group. Relative miR-26a was decreased in offspring following PC:EtOH exposure (A). PC:EtOH -exposed offspring also had increased miR-29b expression, but only in male offspring (B). PC:EtOH exposure had no effect on miRNA expression of miR-335 (C) or miR-181b (D). Female offspring had higher expression of miR-335 (C) compared to males and male offspring had higher miRNA expression of miR-181b (D). Data were analysed with two-way ANOVAs and represented as mean  $\pm$  SEM.

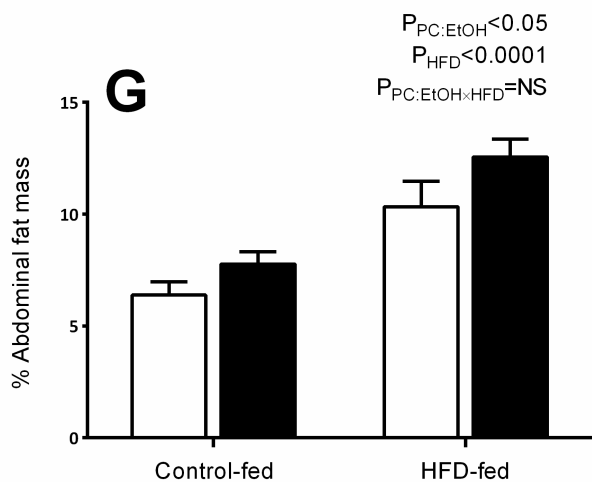
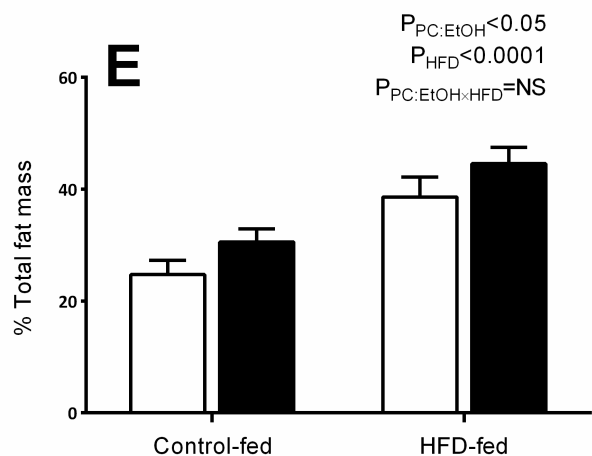
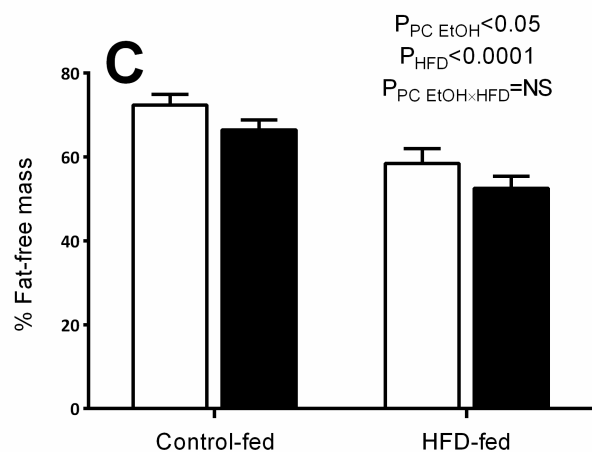
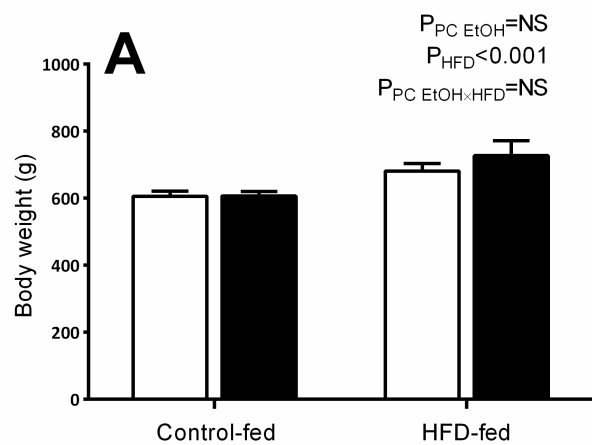
**Figure 5:** Representative H&E liver histology images from male untreated (A) and PC:EtOH-exposed (B) on a control diet, and for male untreated (C) and PC:EtOH-exposed (D) on a high-fat diet (HFD) at 8 months. Scale bars are 100 $\mu$ m for inserts and 200 $\mu$ m for large images, n=8 per treatment group. Panel (E) Quantification of non-alcoholic fatty liver disease according to the Kleiner-scoring system.

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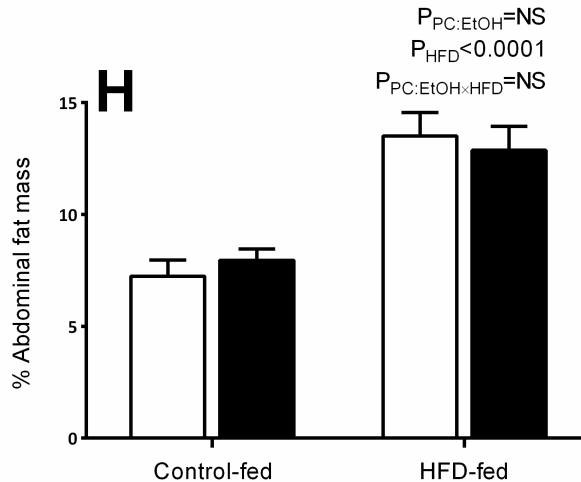
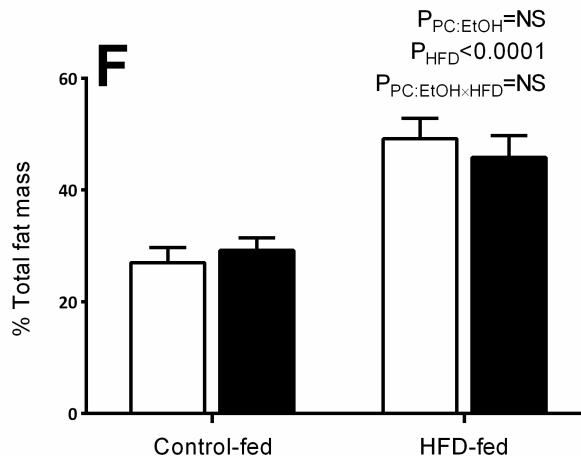
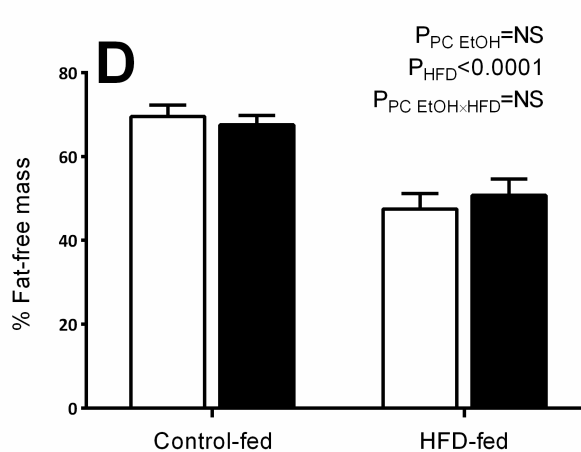
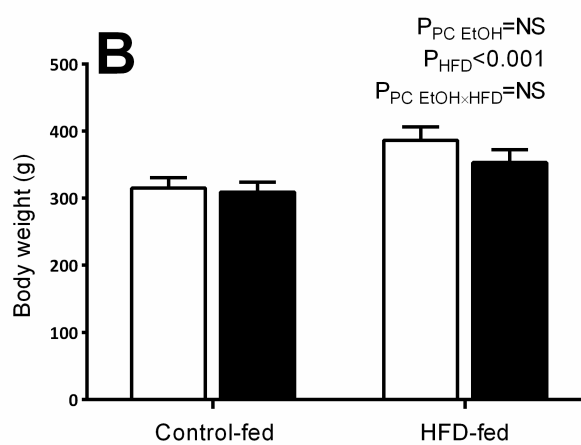
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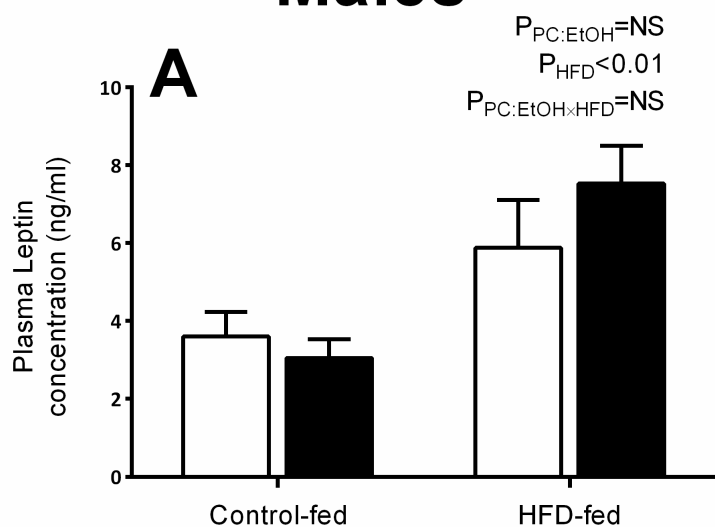
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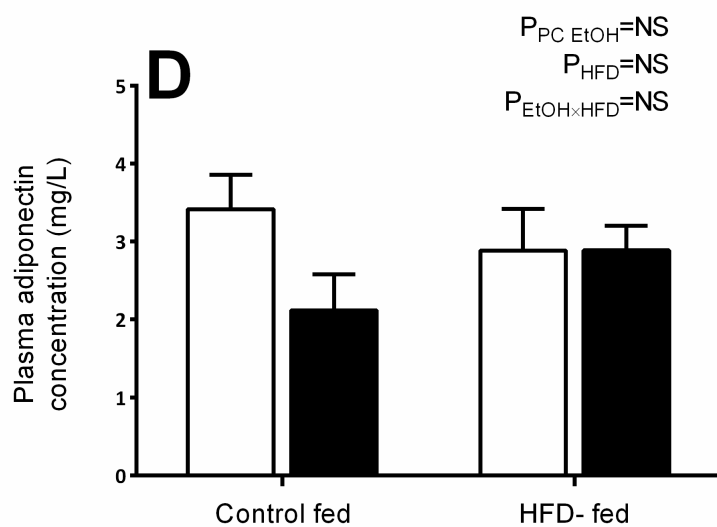
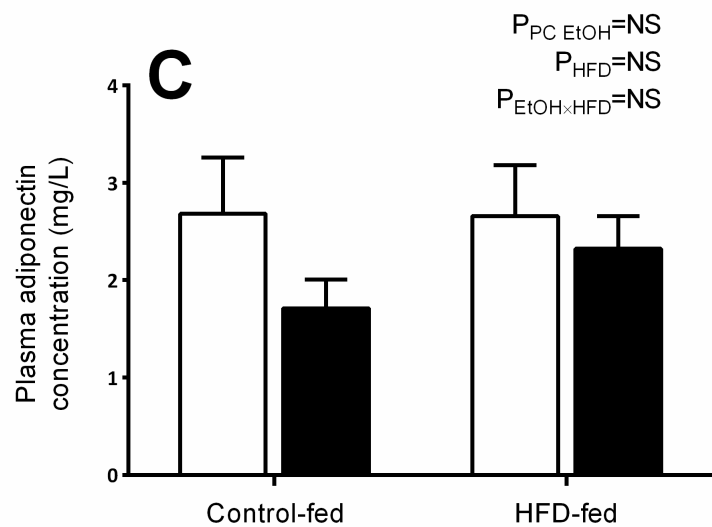
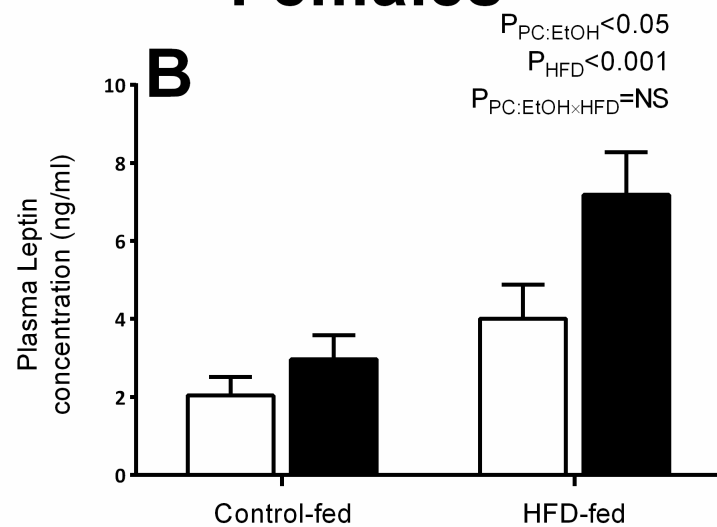
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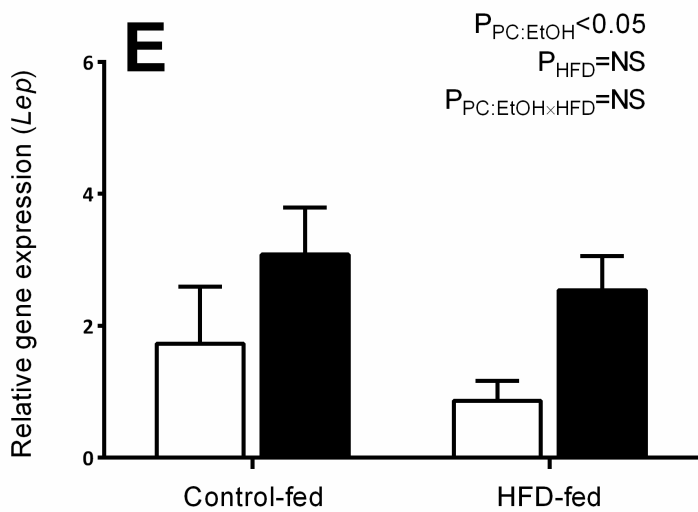
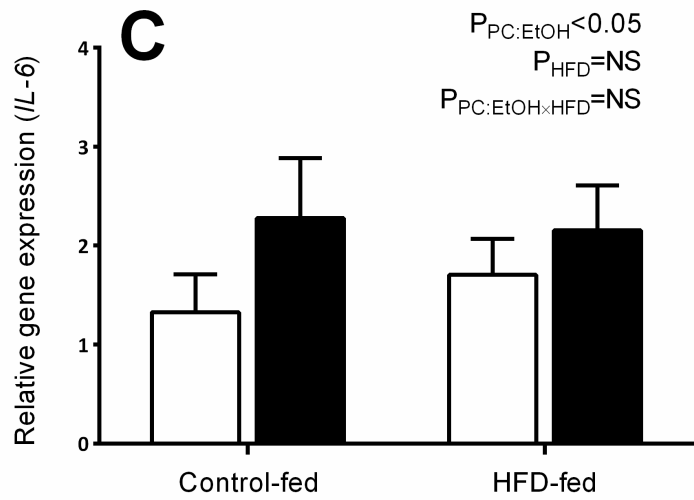
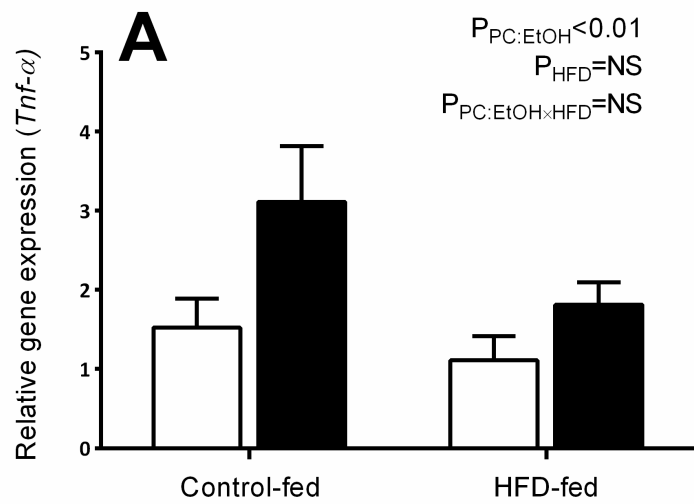
## Males



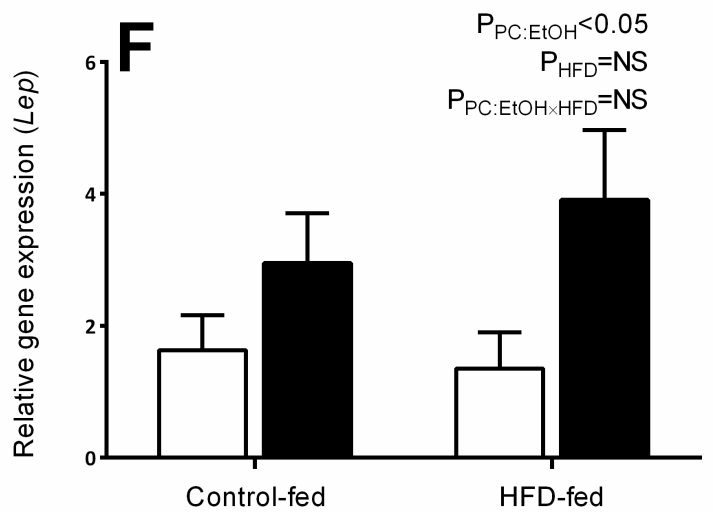
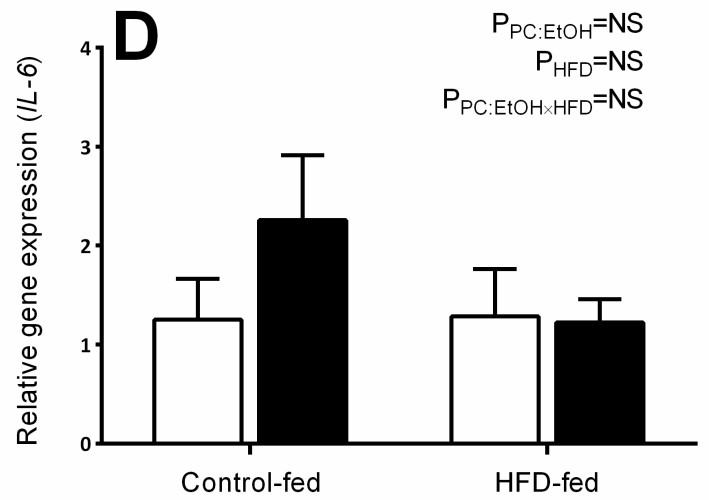
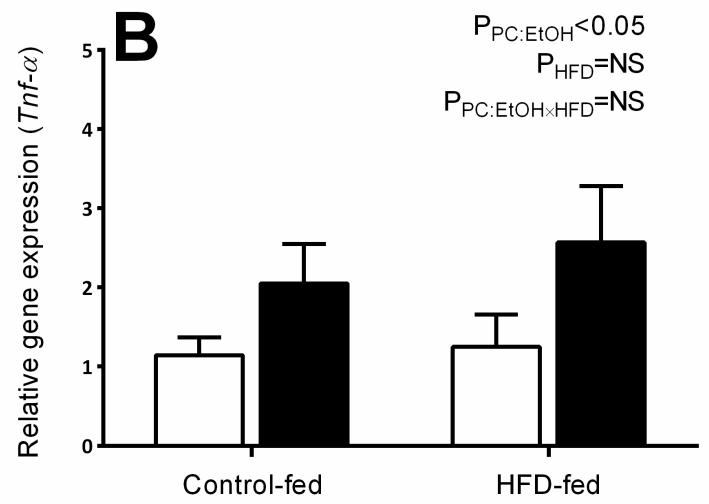
## Females



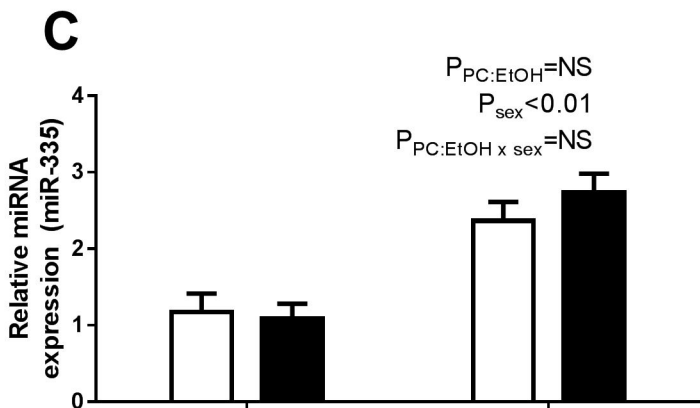
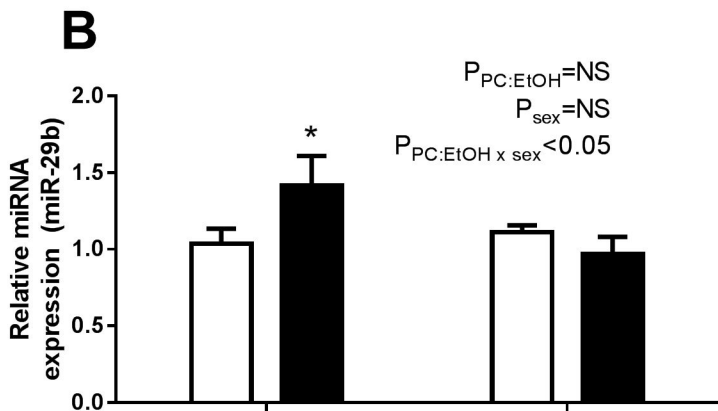
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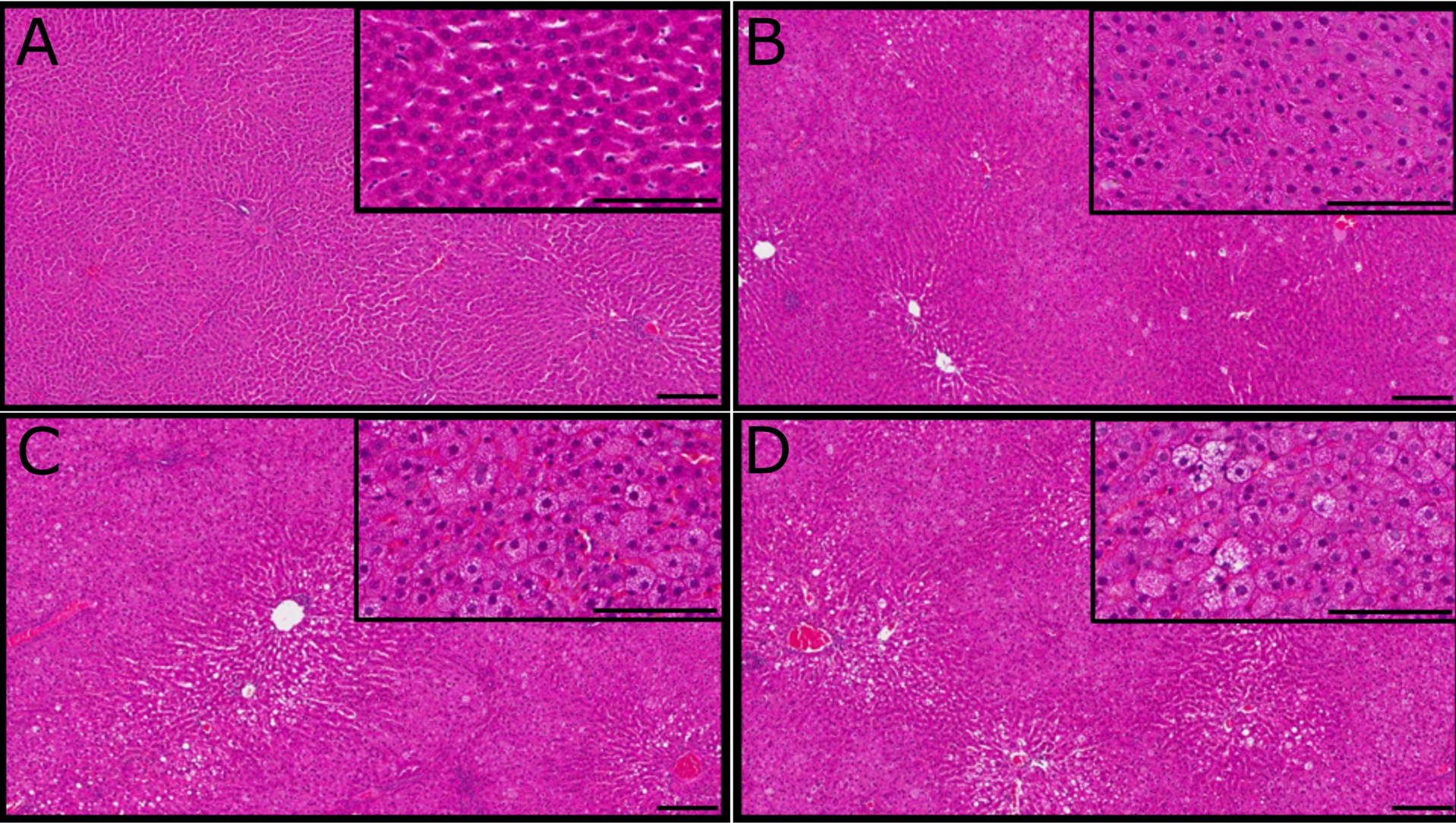


# Females









E		Quantification of non-alcoholic fatty liver disease according to the Kleiner-scoring system							
		Male				Female			
		U:C	EtOH:C	U:HFD	EtOH:HFD	U:C	EtOH:C	U:HFD	EtOH:HFD
Steatosis grade	<5 %	100	100	10	0	100	100	71	86
	5-33 %	0	0	50	60	0	0	29	0
	>33-66 %	0	0	20	20	0	0	0	0
	>66 %	0	0	20	20	0	0	0	14
Location of steatosis	Absent	100	100	10	0	100	100	71	86
	Central vein	0	0	40	40	0	0	14.5	0
	Portal vein	0	0	10	0	0	0	0	0
	Azonal	0	0	20	10	0	0	0	0
	Panacinar	0	0	20	50	0	0	14.5	14
Micro-vesicular steatosis	Absent	100	67	20	0	100	71	0	29
	Present	0	33	80	100	0	29	100	71
Lobular inflammation	Absent	50	0	30	20	14	43	14	0
	Grade 1	50	100	50	60	86	43	86	100
	Grade 2	0	0	20	20	0	14	0	0
Micro-granulomas	Absent	50	17	30	30	57	29	43	14
	Present	50	83	70	70	43	71	57	86
Stage of fibrosis	None	100	100	100	100	100	100	100	100

Abbreviations: C, control; HFD, high-fat diet; PC:EtOH, periconceptional alcohol; U, untreated.

Table 1. Body weight and plasma parameters at postnatal day 30

	Treatment group	
	U:C	PC:EtOH:C
Male		
Parameter		
Body weight (g)	76.5 ± 3.5	73.8 ± 2.2
Abdominal circumference (cm)	11.2 ± 0.2	10.8 ± 0.2
Ponderal index (g/(100xcm <sup>3</sup> ))	30.4 ± 1.4	28.9 ± 2.6
Liver weight (g/gbw)	0.04 ± 0.01	0.04 ± 0.01
Triglycerides (mmol/L)	0.7 ± 0.1	0.9 ± 0.1
HDL (mmol/L)	1.49 ± 0.05	1.53 ± 0.07
Cholesterol (mmol/L)	2.4 ± 0.1	2.5 ± 0.1
Leptin (ng/ml)	1.68 ± 0.23	2.47 ± 0.31*
Female		
Parameter		
Body weight (g)	71.8 ± 2.3	65.4 ± 2.2
Abdominal circumference (cm)	10.6 ± 0.4	10.6 ± 0.2
Ponderal index (g/(100xcm <sup>3</sup> ))	31.0 ± 1.8	30.3 ± 1.5
Liver weight (g/gbw)	0.04 ± 0.01	0.04 ± 0.01
Triglycerides (mmol/L)	1.0 ± 0.1	0.6 ± 0.1*
HDL (mmol/L)	1.57 ± 0.08	1.58 ± 0.14
Cholesterol (mmol/L)	2.6 ± 0.1	2.5 ± 0.2
Leptin (ng/ml)	1.75 ± 0.18	1.70 ± 0.21



**Table 2.** Body and organ measurements at tissue collection and during Dual X-ray absorptiometry scan of body composition in male and female offspring aged 7-8 months

	Treatment group			
	U:C	PC:EtOH:C	U:HFD	PC:EtOH:HFD
Male				
Parameter				
Body weight at PM (g)	606 ± 16	607 ± 13	681 ± 22 <sup>†</sup>	727 ± 45 <sup>†</sup>
Abdominal circumference (cm)	22.0 ± 0.5	22.4 ± 0.3 <sup>*</sup>	23.4 ± 0.6 <sup>†</sup>	25.9 ± 1.1 <sup>*†</sup>
Ponderal index (g/(100xcm <sup>3</sup> ))	2.6 ± 0.1	2.7 ± 0.1	2.8 ± 0.1 <sup>†</sup>	3.0 ± 0.1 <sup>†</sup>
Liver weight (g/gbw)	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Intra-abdominal fat weight (g/gbw)	0.04 ± 0.01	0.05 ± 0.01 <sup>*</sup>	0.06 ± 0.01 <sup>†</sup>	0.08 ± 0.01 <sup>*†</sup>
Tibial length (mm)	51.1 ± 1.0	52.0 ± 1.2	50.6 ± 1.1	51.1 ± 1.2
BMC (%)	2.85 ± 0.05	2.93 ± 0.07	2.88 ± 0.05	2.85 ± 0.05
BMD (g/cm <sup>2</sup> )	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
Female				
Parameter				
Body weight at PM (g)	315 ± 16	309 ± 14	387 ± 20 <sup>†</sup>	354 ± 18 <sup>†</sup>
Abdominal circumference (cm)	17.3 ± 0.6	17.1 ± 0.3	19.1 ± 0.7 <sup>†</sup>	19.3 ± 0.4 <sup>†</sup>
Ponderal index (g/(100xcm <sup>3</sup> ))	2.3 ± 0.1	2.5 ± 0.08	2.7 ± 0.07 <sup>†</sup>	2.7 ± 0.1 <sup>†</sup>
Liver weight (g/gbw)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Intra-abdominal fat weight (g/gbw)	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01 <sup>†</sup>	0.09 ± 0.01 <sup>†</sup>
Tibial length (mm)	46.4 ± 1.1	43.9 ± 1.1	45.9 ± 1.2	45.7 ± 1.0
BMC (%)	3.42 ± 0.09	3.25 ± 0.08	3.27 ± 0.12	3.38 ± 0.08
BMD (g/cm <sup>2</sup> )	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01

**Table 3.** Fasting plasma profile of 8 months old male and female offspring

	Treatment group			
	U:C	PC:EtOH:C	U:HFD	PC:EtOH:HFD
Male				
Parameter (mmol/L)				
Triglycerides	0.9 ± 0.2	1.1 ± 0.1*	0.9 ± 0.1	1.3 ± 0.1*
HDL	1.68 ± 0.12	1.81 ± 0.07*	1.21 ± 0.14†	1.6 ± 0.1*†
LDL	0.09 ± 0.03	0.10 ± 0.02	0.13 ± 0.04	0.16 ± 0.05
Cholesterol	1.7 ± 0.1	1.9 ± 0.1*	1.5 ± 0.2	2.00 ± 0.1*
Female				
Parameter (mmol/L)				
Triglycerides	0.5 ± 0.1	0.6 ± 0.1	1.2 ± 0.3†	1.5 ± 0.3†
HDL	1.94 ± 0.15	2.01 ± 0.14	1.53 ± 0.16†	1.72 ± 0.09†
LDL	0.10 ± 0.03	0.17 ± 0.05*	0.09 ± 0.02	0.19 ± 0.03*
Cholesterol	2.0 ± 0.2	2.2 ± 0.2*	1.9 ± 0.2	2.4 ± 0.4*