A high unsaturated fat, high protein and low carbohydrate diet during pregnancy and lactation modulates hepatic lipid metabolism in female adult offspring

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Running title: Maternal diet modulates offspring hepatic lipid metabolism

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Abbreviations

CHO: carbohydrate; CPT: carnitine palmitoyltransferase; NEFA: non-esterified fatty acids; PPAR: peroxisomal proliferator activated receptor; SREBP: sterol response element binding protein; TG: triglyceride.
Abstract

Whether a high unsaturated fat, high protein (HFP) and low carbohydrate (CHO) diet during gestation has long-lasting beneficial effects on lipid metabolism in the offspring was investigated using a mouse model. Female mice were fed either a standard (CHO-rich) chow diet or a low carbohydrate HFP diet, prior to and during gestation and lactation. All offspring were weaned onto the same chow until adulthood. Although liver cholesterol concentration and fasting plasma TG, cholesterol and free fatty acid concentrations were not affected in either male or female HFP offspring, hepatic triglyceride (TG) concentration was reduced by ~51% (p < 0.05) in the female adult offspring from dams on the HFP diet, compared to females from dams on the chow diet (a trend toward reduced TG concentration was also observed in the male). Furthermore, hepatic protein levels for CD36, carnitine palmitoyltransferase-1 (CPT-1) and peroxisomal proliferator activated receptor-α (PPARα) were increased by ~46% (p < 0.001), ~52% (p < 0.001) and ~14% (p = 0.035) respectively in the female HFP offspring. Liver TG levels were negatively correlated with protein levels of CD 36 (r = -0.69, p = 0.007), CTP-1 (r = -0.55, p = 0.033) and PPARα (r = -0.57, p = 0.025) in these offspring. In conclusion, a maternal HFP diet during gestation and lactation reduces hepatic TG concentration in female offspring, which is linked with increased protein levels in fatty acid oxidation.
INTRODUCTION

Epidemiological and animal studies have supported the “fetal programming” hypothesis linking adult diseases such as the metabolic syndrome, type 2 diabetes and cardiovascular disease with events occurring during early development (8, 17, 18, 25). The fetal programming hypothesis is supported by data from animal models that have manipulated maternal diet (23, 26, 35). Whereas these studies provide data showing a detrimental effect on the offspring (i.e. linking increased risk of development of the metabolic syndrome and cardiovascular disease to early development), emerging evidence also suggest a long-lasting beneficial effect on the offspring may occur if a “good” manipulation or stimulus is applied to the mother during gestation and lactation (we suggest the term of “beneficial fetal programming). For example, adding vitamin E or cholestyramine to the maternal diet during gestation markedly reduces the progression of atherogenic lesions in the aorta of offspring in rabbits (28).

There is an increasing interest in low carbohydrate (CHO) diets among obese individuals who are trying to lose weight (6). The Atkins diet is a low CHO, high lipid and high protein diet (6). In randomised controlled studies, this diet generated comparable or slightly better results, in terms of weight loss and improvement in lipid profile, than a conventional low fat, high CHO, low calorie diet after 3 and 6 months of consumption (13, 32). As more people are interested in losing weight using these low CHO diets, their use will increase, and this may result in their consumption during pregnancy. There is therefore a need to determine whether there are long-term benefits to the offspring of maternal consumption of these diets during pregnancy and lactation.
We have used a murine model to assess whether a low CHO, high unsaturated fat diet (as unsaturated fat produces favourable effects on lipid metabolism) during pregnancy has any long-term beneficial effect on the offspring with a focus on the metabolic parameters and hepatic lipid metabolism. We have examined whether the low CHO, high unsaturated fat and protein (HFP) diet affected body weights of the dams, and those of her offspring. We have also measured hepatic fat concentrations and protein levels of key molecules involved in hepatic fat metabolism, as well as fasting plasma lipid (triglyceride, cholesterol, non-esterified fatty acids) concentrations in the adult offspring.
MATERIALS AND METHODS

Animals and experimental protocols

All animal procedures carried out in this study were in accordance with the British Home Office Animals (Scientific Procedures) Act, 1986. This study was approved by the University ethics committee. Virgin female Balb/C mice (3 weeks old) were randomly assigned to two dietary groups. They were fed ad libitum with either a low CHO, high fat and high protein (HFP, n = 9) diet (Table 1a & 1b) containing 16.5% CHO, 26.9% protein and 52.6% lipid in energy respectively, or a standard laboratory chow diet (n = 6) for 6 weeks prior to conception (Fig. 1). The ingredients for both diets were purchased from commercial sources and prepared in-house. At 9 weeks old, all animals were time-mated and pregnancy was determined by the presence of vaginal plug (defined as Day 0), and there were 4 or 6 pregnancies from mice on the chow or HFP diets respectively. The diet assigned to an animal prior to conception was also given throughout the gestation and lactation period. Food intake and body weights of the dams were measured every three days until their offspring had been weaned. All offspring were weaned at 3 weeks of age onto the standard chow diet and maintained on this diet ad libitum until they reached adulthood (Fig. 1). All mice had free access to water throughout the study. We refer to the adult offspring born to dams fed the HFP diet during gestation and lactation as ‘HFP offspring’, and to the adult offspring born to dams fed the chow diet as ‘control offspring’. Offspring were examined at 8 weeks old, as the onset of puberty of Balb/c mice start at ~ 4 weeks of age (7) and sexual maturity at ~ 5 weeks of age (4). The offspring (5 males and 6 females from the dams on the chow diet and 6 males and females randomly selected from each litter born to dams on the HFP diet) were fasted for ~12 hours and sacrificed the following day by CO₂ inhalation and cervical dislocation. Blood was collected
by cardiac puncture, and liver tissue was dissected, snaps frozen in liquid nitrogen and stored at -80°C for later analysis. Plasma glucose, non-esterified fatty acid (NEFA), total cholesterol and triglyceride concentrations were determined using an autoanalyzer (Konelab 20, Thermo Electron Corporation).

Liver lipid extraction and analysis

Livers (~ 60 mg) were homogenized in phosphate buffered saline and protein concentration determined (24). Homogenate (300 µl) was extracted with 5 ml of chloroform/methanol (2:1) and 0.5 ml of 0.1% sulfuric acid (12). The organic phase was dried under nitrogen, and re-suspended in ethanol. Hepatic triglyceride (TG) and cholesterol content were determined using commercially available kits (Randox Laboratories Ltd, UK). Data were normalized for differences in protein concentration.

Western blotting

Liver tissues (~ 100 mg) were added to a homogenising buffer containing 50 mM Tris.HCl (pH7.6), 0.25% Triton X-100, 0.15M NaCl, 10mM CaCl₂, 0.1mM phenylmethylsulfonylfluoride, 10 µM leupeptin, 10 µM pepstatin A, 0.1 mM iodoacetamide, 25 µg/ml aprotinin and 0.1 mM Phenylmethylsulfonylfluoride. The cell homogenates were spun at 15000 × g for 10 min at 4 °C. The supernatants were transferred to Eppendorf tubes and stored at –70°C. Protein concentrations were measured (24). Equal amounts (60 µg) proteins for each sample were mixed with 5 × sample buffer (5 ml of sample buffer contains 2.5 ml of glycerol, 1.25 ml of 2-mercaptoethanol, 0.5 g of SDS, 1.04 ml of 1.5 M Tris, pH 6.8, and 1.25 mg of bromphenol blue), boiled for 4 min and loaded on 7% SDS-polyacrylamide mini-gels. The gels were run at 200 volts for 1 h, and transferred on an Immobilon-P transfer
membrane (Millipore, Bedford, MA) in 25 mM Tris, 150 mM glycine and 20% methanol. After transfer the membranes were blocked in 10 ml of TBST (20 mM Tris, 0.15 M NaCl, and 0.1% Tween) and 5% dry milk for 1 h and then incubated for 16-18 h at 4 °C in 10 ml of TBST and 5% dry milk containing primary antibody (see results section). At the end of the incubation period the membranes were washed in TBST for 3 × 10 min at room temperature and then incubated for 1 h in TBST and 5% dry milk containing secondary antibodies conjugated to horseradish peroxidase (Santa Cruz, 1: 4000). The membranes were washed in TBST for 3 × 10 min at room temperature and processed with the ECL (Perbio Science UK Ltd) detection system. Specific protein bands were then detected by exposing the membrane on a Kodak BioMax Light film (Sigma). To quantify the protein bands, the image of the membrane on the film was analysed using a Phoretix 1 D advanced v.4.01 (Newcastle upon Tyne, UK) software. Values are presented as the relative intensity of the protein bands (calculated as volume).

**Statistical analysis.** All statistical calculations were performed using SPSS (10.1) software. Differences in mean values were examined by unpaired Students t test. Results were presented as means ± SD. Non normally distributed data was normalised by transformation and parametric statistical analyses undertaken (t tests and Pearson univariate regression).
Results

1. Maternal growth. Female mice were randomly assigned to be fed either a chow (n = 6) or HFP diet (n = 9) and their body weights measured every three days. There was no difference in body weights between female mice on the HFP diet and those on the chow diet on all measurements prior to mating. Interestingly, mice on the HFP diet ate on average ~1.58 gm of diet per day (1.58 ± 0.25), which was ~ 21% less by weight than those on the chow diet (2.00 ± 0.23 gm/day, p = 0.002). However, the average daily energy intake was similar for mice on the two types of diets (chow v HFP: 8.74 ± 0.95 v 8.65 ± 1.36 kcal/day, p = 0.85), as the energy density of the HFP diet was ~25% greater than that of the chow diet (Table 1b). Thus, mice on the HFP diet ate on average 57.5% less CHO, 23% more protein and 153% more lipid (by calorie) without increasing their total calorie intake, compared to mice on the chow diet (Table 1b).

2. Offspring. Of the total pups born, 6 males and 17 females were born to 4 dams on the chow diet (average litter size was 5.75), and 13 males and 20 females were born to 6 dams on the HFP diet (average litter size was 5.5, Table 2). The mean birth weight of the HFP offspring was ~10.8% greater than that of the control offspring (p < 0.01, Table 2), although the litter size was similar between the two groups (Table 2). However, the mean body weight of HFP offspring (nursed by dams on the HFP diet) and that of the control offspring (nursed by dams on the chow diet) was not significantly different at the time of weaning for both male and female offspring (Table 2). From post-weaning, all offspring were fed the same chow diet for 5 weeks (i.e. to 8 weeks old). There was no difference in body weights of adult offspring between experimental groups (Table 2).
3. Plasma lipid concentrations. All offspring were sacrificed 55-56 days after birth and plasma samples were analysed. Fasting plasma triglyceride, total cholesterol and non-esterified fatty acids (NEFA) concentrations were similar between the HFP and control groups for both male and female offspring (Table 3).

4. Liver lipid analysis. Analysis of liver lipids and protein levels were undertaken using the same piece of liver tissue divided in half from adult offspring. There was a trend toward reduced liver TG concentration in male HFP (n = 6) compared to male control offspring (n = 5, p = 0.22, Fig. 2A). Interestingly, liver TG concentrations in the female HFP offspring (n = 6) was markedly lower compared to female control offspring (n = 6, p < 0.05, Fig. 2B). Liver cholesterol concentrations were similar between the HFP and control groups for both male and female offspring (Fig. 2C, 2D).

5. Level of protein expression key to lipid metabolism. We then determined if protein levels of key genes in hepatic lipid metabolism were altered in the female offspring (as no significant change in hepatic TG concentration was observed in the male offspring), in association with altered hepatic TG concentration.

Protein levels of CD36, a key long-chain fatty acid transporter (1, 19) were markedly increased in the HFP offspring (n = 6) compared to the controls (n = 6, p < 0.001, Fig. 3A). Protein levels of CPT-1, the rate-limiting enzyme in fatty acid β-oxidation (10), were increased in the female HFP offspring (p < 0.001, n = 6 for both groups, Fig. 3B). Protein levels of PPARα, a transcription factor regulating genes in hepatic fatty acid oxidation (5, 20), were also increased in the HFP offspring (p < 0.05, n = 6 for both groups, Fig. 3C).
6. Correlation between liver TG concentrations and protein levels of CD36, CPT-1, PPARα. We then examined whether there was any association between observed changes in hepatic TG concentrations and levels of key metabolic proteins. Data of protein levels from female offspring born to dams on both the chow and HFP diets (n = 12) were pooled and analysed against hepatic lipid concentrations obtained from these mice. Liver TG concentrations were negatively correlated with protein levels of CD36 (r = -0.69, p = 0.007, Fig 4A), CPT-1 (r = -0.547, p = 0.033, Fig 4B) and PPARα (r = -0.574, p = 0.025, Fig 4C).
DISCUSSION

We have presented novel data showing that feeding female mice a low carbohydrate high protein, high fat diet for 6 weeks prior to conception and during gestation and lactation leads to a marked reduction in hepatic TG concentration in the female offspring. In parallel to reduced hepatic TG concentration, hepatic protein levels of CD36, CPT-1 and PPARα were increased in these offspring. Furthermore, hepatic TG concentrations were negatively correlated with hepatic protein levels of CD36, CPT-1 and PPARα in these offspring. As reduced hepatic TG concentration is linked with increased hepatic insulin sensitivity (36), our data favour a beneficial long-lasting effect on the female offspring born to dams fed the HFP diet during gestation and lactation.

The most striking effect in giving the HFP diet to the dam during pregnancy and lactation is the long-term reduction in hepatic TG concentration in the adult female offspring, the magnitude of this reduction is over 2-fold. Importantly, this reduction in liver TG concentration occurs despite the HFP offspring being fed the standard laboratory chow diet from weaning until adulthood.

Hepatic TG concentration is affected by de novo lipogenesis and fatty acid oxidation in the liver (15). As only small quantities of hepatic TG are derived from de novo lipogenesis (2, 14), we focused our study on molecules relevant to fatty acid oxidation. PPARα is a master transcription factor regulating a number of proteins involved in fatty acid oxidation (3, 16, 27, 30, 34, 38). CPT-1 is the rate-limiting enzyme in fatty acid β-oxidation (10) and CD36 is an 88-kDa membrane glycoprotein that belongs to the class B scavenger receptor family (21). CD36 binds with many ligands including native and oxidized lipoproteins (21) and long-chain
fatty acids, transports long-chain fatty acids across cell membrane (1, 19) Both CD36 and CPT-1 are responsive to PPARα activation because the promoter of CD36 contains a PPAR response element that binds to PPARα (33), and PPARα activation or over expression stimulates CPT-1 mRNA in human hepatocytes (22). Previous data support a link between increased PPARα expression and reduced hepatic lipid content. For example, PPARα agonists prevented fatty liver in ethanol-fed mice by stimulating fatty acid β-oxidation via up-regulating mRNA levels of PPARα target genes (11). In contrast, in mice with fatty liver dystrophy, hepatic fatty acid oxidation is markedly reduced with altered expression of a number of peroxisome proliferator regulated proteins (29). Furthermore, in PPARα deficient mice, etomoxir (a CPT-1 inhibitor) induced a much greater increase in hepatic lipid concentration compared to the effect of etomoxir in the wild type mice (9). These data are consistent with our data showing a reduced hepatic TG concentration with increased hepatic protein levels of PPARα, CD36 and CTP-1 in the HFP offspring, suggesting that increased protein levels of PPARα may be relevant to reduced hepatic TG concentration in the HFP offspring. Indeed, our data show that hepatic TG concentrations were negatively correlated with hepatic protein levels of CD36, CPT-1 and PPARα in female offspring. Taken together, these data suggest that feeding the HFP diet during gestation and lactation programs reduced hepatic TG concentration, which is associated with increased hepatic protein levels of PPARα and its response genes including CD36 and CPT-1.

The Atkins diet is gaining popularity in reducing body weight among people with obesity or diabetes (6). Interestingly, our study has shown that feeding female mice with a HFP diet 6 weeks prior to conception did not affect their body weights, despite dietary fat intake being ~53% of the total energy intake (~2-fold more than the amount of lipid consumed by the control mice). The actual calorie intake in mice on the HPF diet was similar to that on the
chow diet, although the energy density of the HFP diet was markedly greater. Our data also show that the plasma lipid profile in the male and female adults are similar between those born to dams on the HFP diet and the dams on the chow diet. Thus, these data suggest that the HFP diet during gestation and lactation does not have any adverse effect in terms of body weight and plasma lipid profiles in the adult offspring. Further investigation is required to assess what happens to the plasma lipid profile and hepatic lipid metabolism in these mice with the effects of ageing.

It is worth noting that the HFP diet we used was enriched in mono- and polyunsaturated fats. The HFP diet contained a much higher proportion of unsaturated fatty acids compared to the chow diet. This makes our HFP diet distinct from most other high fat diets used in previous animal studies that contained predominantly saturated fatty acids. It is well established that treatment with unsaturated fatty acids produces a favourable effect on triglyceride metabolism in adult human studies. It is worth noting that the HFP diet contains ~77.5% less sucrose than the chow diet. Sucrose rich diet increases hepatic TG deposition (37) and plasma TG concentration in rats (31). Thus, a low sucrose in our HFP diet may also contribute to reduced hepatic TG levels in the HFP offspring. Although we have not tested the effects of unsaturated fatty acids and low sucrose per se, our data suggests that consumption of a diet that is enriched with unsaturated fat and is low in sucrose during pregnancy and weaning, induces a lasting reduction in liver TG in the offspring. Furthermore, the changes we observed were purely due to dietary modification in the mother, as we have used a genetically homogeneous strain to eliminate any potential confounding effect due to genetic background.

Our data showing that reduced hepatic TG concentrations occurred in the female HFP offspring (although there was a trend towards reduced TG concentrations in the male HFP
offspring), suggests that there maybe a gender-specific effect in the reduction of hepatic TG in female offspring, as current evidence suggests that sex hormone affects lipid metabolism in skeletal muscle and the liver. For example, \(17\beta\)-estradiol increases maximum activity of CPT-1 (1) and upregulates the expression of PPAR\(\alpha\) and CPT-1 genes in skeletal muscle (2), but suppresses expression of CPT-1 in the liver (4). Hepatic expression of PPAR\(\alpha\) is regulated in a gender-specific manner in liver (3, 6, 10). The mechanism underlying the interaction between gender and fetal programming requires further investigation. However, these data suggest that liver TG in the female HFP offspring may be affected by sex hormone and not entirely due to maternal dietary modification.

In conclusion, we have presented novel data showing that a maternal diet enriched with unsaturated fat and low in sucrose during gestation and lactation reduces hepatic TG concentration in adult female offspring. Reduced hepatic triglyceride content is associated with up-regulation of levels of key proteins regulating fat oxidation.
ACKNOWLEDGEMENTS

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Refs:


Figure legends

**Table 1. Dietary composition (Table 1a) and percentage of macronutrients (Table 1b).**
Corn oil contains 13% saturated fatty acids, 25% monounsaturated fatty acids and 62% polyunsaturated fatty acids. *Lard contains 45% saturated fatty acids, 45% monounsaturated fatty acids and 10% polyunsaturated fatty acids, and thus, the lipid in the HFP diet contains 35% saturated fatty acids, 38.8% monounsaturated fatty acids and 26.2% polyunsaturated fatty acids. Data in the HFP/Chow column were calculated by dividing either the weight or the energy of macronutrients from the HFP diet by those from the chow diet ×100.

**Table 2. Birth and body weights of offspring.** *One male offspring died prior to weaning.

**Table 3. Fasting plasma lipid concentrations.**

**Fig. 1. Study design showing maternal dietary modification** (see text for detailed description of the study design).

**Fig. 2. Hepatic lipid concentrations.** Liver lipids were extracted and triglyceride and cholesterol (TC) concentrations determined as described in the methods section. Male, n = 5 or 6, for the chow or HFP offspring. Female, n = 6 for both groups. *p < 0.05.

**Fig. 3. Hepatic protein levels of CD36, CPT-1 and PPARα in female offspring.** Equal amount (60 µg) of total protein was loaded onto each lane for SDS-PAGE and analysed by western blotting with polyclonal antibodies against CD36 (Fig 3A), CTP-1 (Fig 3B) and PPARα (Fig 3C). Each blot is a representative of 2 independent experiments. Data are
presented as mean ± SD. N = 6 for both groups. *p < 0.05, **p < 0.001.

**Fig. 4. Correlation between hepatic TG concentrations and protein levels of CD36, CTP-1, and PPARα in female offspring.** Protein levels (arbitrary units) of CD 36 (Fig. 4A), CPT-1 (Fig. 4B) and PPARα (Fig. 4C) were subjected to Pearson correlation analysis against liver TG concentrations. Open triangles represented data from offspring of dams fed a chow diet, and closed circles represented data from offspring of dams fed a HFP diet. P value < 0.05 was considered significant (n = 12). CD36 protein data were normalised by logarithmic transformation.
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<th>HFP diet</th>
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<td>Casein</td>
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<tr>
<td>Corn oil*</td>
<td>10</td>
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</tr>
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<td>Lard</td>
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<tr>
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<td>Cellulose</td>
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<tr>
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**Table 1a**
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<tr>
<td>Carbohydrate</td>
<td>68.8%</td>
<td>36.8%</td>
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<tr>
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<td>Lipids</td>
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<tr>
<td><strong>Percentage in energy (kcal)</strong></td>
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Table 1b
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<td>Birth weight (g)</td>
<td>$1.29 \pm 0.18$ (n = 23)</td>
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<td>Litter size</td>
<td>$5.75 \pm 1.26$</td>
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**Males**

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<td>Body weight at weaning (g)</td>
<td>$9.70 \pm 1.30$ (n* = 5)</td>
<td>$11.27 \pm 2.19$ (n = 13)</td>
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<td>Body weight at adults (g)</td>
<td>$23.00 \pm 1.62$ (n = 5)</td>
<td>$24.04 \pm 1.98$ (n = 13)</td>
<td>$0.28$</td>
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**Females**

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<td>Body weight at weaning (g)</td>
<td>$10.71 \pm 1.45$ (n = 17)</td>
<td>$10.83 \pm 1.96$ (n = 20)</td>
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<td>Body weight at adults (g)</td>
<td>$20.32 \pm 1.72$ (n = 14)</td>
<td>$20.47 \pm 0.83$ (n = 15)</td>
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Table 2.
### Table 3.

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<td>N</td>
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<td>Triglyceride (mM)</td>
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<td>4.41 ± 0.61</td>
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<td>NEFA (mM)</td>
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<td>1.35 ± 0.51</td>
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<td>9</td>
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<tr>
<td>Triglyceride (mM)</td>
<td>1.01 ± 0.21</td>
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<tr>
<td>NEFA (mM)</td>
<td>1.55 ± 0.16</td>
<td>1.55 ± 0.29</td>
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Fig 1
Fig 2

**Male liver TG**

![Graph A](image)

**Female liver TG**

![Graph B](image)

**Male liver TC**

![Graph C](image)

**Female liver TC**

![Graph D](image)
Fig 3
Fig 4

(A) Liver TG (mM/mg protein) vs. LnCD36 protein levels (AU)

(B) Liver TG (mM/mg protein) vs. CPT-1 protein levels (AU)

(C) Liver TG (mM/mg protein) vs. PPARα protein levels (AU)

- **A**
  - $r = -0.69$
  - $p = 0.007$

- **B**
  - $r = -0.547$
  - $p = 0.033$

- **C**
  - $r = -0.574$
  - $p = 0.025$