Maternal L-carnitine supplementation improves glucose and lipid profiles in female offspring of dams exposed to cigarette smoke

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Short title: protection of maternal L-carnitine intake

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Abstract

Sex differences in disease susceptibility due to maternal programming have been reported. We previously observed that maternal smoking induced renal disease and neurological changes are restricted to males, whilst both male and female offspring develop metabolic disorders. We have also found that maternal L-carnitine supplementation during gestation and lactation can significantly improve glucose intolerance and hyperlipidaemia in male offspring. This study aimed to determine whether such beneficial effects can also occur in female offspring. Balb/c female mice were exposed to cigarette smoke (SE) 6 weeks prior to gestation, during gestation and lactation. A subgroup of the SE dams was given L-carnitine (1.5mM in drinking water) during gestation and lactation. Female offspring were studied at 20 days (weaning) and 13 weeks (adulthood). Maternal smoking increased liver weight (%) and blood glucose levels at 20 days, as well as glucose intolerance and plasma triglycerides levels at adulthood ($P<0.05$). The hepatic lipid metabolic marker adipose triglyceride lipase was downregulated in the SE offspring at 20 days ($P<0.05$). At 13 weeks, the hepatic pro-inflammatory markers IL-1β and TNF-α mRNA expression were upregulated, whilst the anti-inflammatory marker IL-10 mRNA expression was downregulated in the SE offspring ($P<0.05$). Liver fibrosis was apparent at 20 days and 13 weeks. Maternal L-carnitine supplementation either normalised or suppressed all detrimental effects induced by smoke exposure ($P<0.05$). We conclude that maternal L-carnitine supplementation improves metabolic parameters in the female offspring of SE dams.

Key words: maternal smoking, glucose intolerance, lipid metabolism, liver fibrosis.

Introduction

Cigarette smoking is associated with increased systemic inflammation, insulin resistance, glucose intolerance and dyslipidemia in humans (1). The association between maternal cigarette smoke exposure (SE) and the risk of metabolic disorders in the offspring has also been reported in a mouse model of maternal SE (2). Interaction between pro-inflammatory cells and metabolic regulation maintains energy homeostasis (3). It has been found that liver lipid accumulation leads to hepatic inflammation, which in turn leads to insulin resistance, glucose intolerance and even fibrotic changes (4). Maternal SE causes liver oxidative stress damage in the offspring, which may be linked to liver microsteatosis (5). As the liver plays a key role in both glucose and lipid metabolism, the above evidence suggests that the liver may be the key orchestrator of maternal smoking related substrate metabolic disorders in the offspring.

Previous studies suggest that female offspring are relatively protected from adverse in-utero exposures. We have demonstrated that female offspring are protected from renal and neurological pathology induced by maternal SE in mice (6-8). However, they still developed glucose intolerance at adulthood (2), which is consistent with human studies (1). Mattsson and colleagues demonstrated that women smoking during pregnancy increases their daughters' risk of developing obesity and gestational diabetes (9). In addition, maternal smoking during the second trimester has been shown to change foetal liver markers involved in glucose metabolism in female children, which is clearly sex-specific (10). Such changes may contribute to dysregulated glucose and lipid metabolism, and ultimately cardiovascular disease in adult females. As the critical organ for substrate metabolism, the influx of both dietary glucose and lipids in the liver can induce inflammatory responses resulting in insulin resistance and dysregulated lipid metabolism (11). Chronic liver inflammation can also lead to fibrosis which can in turn affect liver function (12). To date, the impact of maternal SE on liver makers of inflammation, substrate metabolism and fibrosis is unclear.

L-carnitine is an essential nutrient that serves as a biological co-factor involving in transporting long-chain fatty acid across the inner mitochondrial membrane for β-oxidation and ketones metabolism for energy production (13). L-carnitine has also been shown to have an anti-inflammatory effect in the liver of mice fed a high-fat diet (14). We have previously shown that L-carnitine supplementation during gestation and lactation in the SE dams can improve in-utero cigarette smoke exposure-induced brain and renal pathology, as well as glucose intolerance at adulthood in the male offspring (15, 16). Although female offspring from the SE dams are resistant to the development of neurological and renal pathologies (7), they can still develop metabolic disorders such as glucose intolerance at

adulthood (2). Thus, this study aimed to evaluate the effect of maternal L-carnitine supplementation during gestation and lactation on glucose tolerance and liver markers involved in inflammation, substrate metabolism and fibrosis in the female offspring.

Results

1. Effect of cigarette smoke exposure on the mothers

The SE dams weighed least among the 3 groups prior to mating (P=0.05 vs Sham) and had a trend to a lower weight when the pups were weaned (P=0.08 vs Sham, Supplementary Table 1). No difference was found in weight gain after gestation, litter size, or male and female numbers within the litter among the three dam groups (Supplementary Table 1).

2. Body and blood parameters in the female mice offspring

At day 1, female SE offspring (1.28 ± 0.04g) were 8.5% lighter than the Sham offspring (1.40 ± 0.07g), whereas SE+LC offspring were the heaviest of the 3 groups (1.63 ± 0.05g, P=0.007 vs Sham, P=0.0001 vs SE). At 20 days (weaning age), the body weight and liver weight of the female offspring were not significantly affected by maternal SE; however, the body weight of the SE groups was 7.8% lighter than the Sham group (Table 1). Maternal SE increased the liver weight, expressed as a percentage of bodyweight, and random blood glucose levels (P=0.04, Table 1). These parameters were normalised in the female SE+LC. At 13 weeks (mature age), body weight, liver weight, percentage of liver weight against body weight and non-fasting blood glucose level were similar between the SE offspring and Sham offspring (Table 1). Maternal L-carnitine supplementation decreased the percentage of liver weight/body weight in the female SE+LC offspring (P=0.03, Table 1).

Plasma triglycerides (TG) levels were significantly increased in the female SE offspring compared to the Sham offspring at both 20 days (P=0.004) and 13 weeks (P=0.0007, Table 1). Plasma non-esterified fatty acids (NEFA) level were not different among the three groups at any age. There was no difference in plasma insulin levels between the SE and Sham groups at 20 days, which showed a nearly 20% increase in the SE offspring at 13 weeks, although this was not statistically significant (Table 1). The intraperitoneal glucose tolerance test (IPGTT) demonstrated an Area Under the Curve (AUC) value in the SE offspring to be significantly higher than the Sham offspring at 12 weeks suggesting that the SE offspring are glucose intolerant (P=0.008, Table 1). Maternal L-carnitine treatment did not affect plasma insulin levels in the female offspring at any age. However, it normalised plasma TG levels at 20 days (P=0.001 vs SE) and 13 weeks (P=0.0007 vs SE), and AUC

value in the SE+LC offspring (P=0.02 vs SE+LC vs SE, Table 1).

2. Inflammatory markers in the liver

At 20 days, IL-6 mRNA was tripled by maternal SE (P=0.045 vs Sham, Figure 1b). No differences were observed in the liver mRNA expression levels of the other pro-inflammatory markers including IL-1β, TNF-α and toll-like receptor (TLR)4 and anti-inflammatory markers IL-10 and IL-22 (Figure 1). Maternal L-carnitine supplementation only showed a trend to increase the anti-inflammatory cytokine IL-10 (P=0.07, SE vs SE+LC Figure 1c).

At 13 weeks, hepatic mRNA expression of IL-1β (P=0.01, Figure 1g) and TNF-α (P=0.04, Figure 1k) were upregulated compared with Sham, whilst the anti-inflammatory marker IL-10 (P=0.01 vs Sham, Figure 1i) was downregulated in the SE offspring compared to the Sham offspring. IL-6 expression was doubled (P=0.08, Figure 1g); while IL-22 was nearly halved (P=0.43, Figure 1j) in the female SE offspring, it did not reach statistical significance due to large variance. Maternal L-carnitine supplementation normalized liver IL-1β (P=0.03 vs SE), IL-6 (P=0.03 vs SE), IL-22 and TNF-α (P=0.03 vs SE) mRNA expression (Figure 1), while IL-10 mRNA expression was increased by around 40% in the SE+LC offspring compared with the SE offspring (P=0.07 SE+LC vs SE by t test, Figure 1i).

3. Substrate metabolic markers in the liver

At 20 days, the lipid metabolic marker adipose triglyceride lipase (ATGL) was significantly downregulated in the liver of the SE offspring compared to the Sham offspring (P=0.049, Figure 2a). Liver mRNA expression of Carnitine palmitoyl transferase (CPT)-1α (Figure 2b), and peroxisome proliferator-activated receptor (PPAR)-γ (Figure 2c) had a trend to be reduced by 57% (P=0.08) and 54% (P=0.06) respectively. Maternal SE had no effect on the mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), forkhead box protein (FOXO)1, fatty acid synthase gene (FASN) and glucose transporter 4 (GLUT4; Figure 2). Maternal L-carnitine supplementation did not significantly affect the lipid metabolic markers measured at 20 days (Figure 2).

At 13 weeks, a trend towards increased liver PGC-1α and FASN mRNA expression was found (P=0.09 and 0.11 respectively, Figure 2k, 2l). However, ATG, CTP-1α, PPARγ, FOXO-1 and Glut4 mRNA levels were not changed. Maternal L-carnitine supplementation normalised PGC1α (P=0.04, SE+LC vs SE) and FASN mRNA expression (P=0.38, SE+LC vs SE Figure 2k, 2l) caused by

maternal SE, while Glut 4 mRNA expression in SE+LC offspring was less than half of the Sham and SE offspring albeit without statistical significance (P=0.18, Figure 2g).

4. Liver Fibrosis

Cigarette smoking increases the risk of developing fibrosis in multiple organ systems. Both collagen I and III protein expression were more than doubled in the livers from the SE offspring at weaning (P=0.0006 and P=0.005 SE vs SHAM respectively, Figure 3a,b). Both collagen I and III deposition in the SE+LC offspring was however normalized by maternal L-carnitine supplementation (collagen I, P=0.005 SE+LC vs SE, Figure 3a; collage III P=0.0003, SE+LC vs SE, Figure 3b).

At 13 weeks, although collagen I protein levels were not changed, collagen III protein expression was increased by nearly 3 fold in the SE offspring (P<0.0001, SE vs Sham, Figure 3b). Maternal L-carnitine supplementation significantly collagen I and III protein levels were significantly reduced (collagen I, P=0.0007 SE+LC vs SE; collagen III, P<0.0001 SE+LC vs SE, Figure 3c,d).

Discussion

Maternal SE prior to and during gestation and lactation significantly increased plasma lipid levels and caused glucose intolerance in the female offspring. This was associated with changes in liver lipid and glucose metabolic markers as well as increased fibrotic markers. The main finding in this study is that maternal L-carnitine supplementation during gestation and lactation can significantly ameliorate the adverse SE impacts on glucose intolerance and hyperlipidaemia in the female offspring, which is linked to improved metabolic markers in the liver in addition to ameliorating liver collagen deposition.

This study showed an increase in the liver weight expressed as a percentage of total body weight at weaning in the female SE offspring. A similar trend was observed in their male littermates at adulthood (6). This may reflect a catch up growth in less pivotal organs during development, which is commonly seen in the situation of intrauterine nutrition restriction such as maternal smoking (1). The addictive substance nicotine has been suggested to play a major role, whereas details on how the other ~5000 chemicals in the cigarette smoke contribute to such effects individually are largely unknown.

In humans, there is a dose-dependent association between prenatal and postnatal SE and the development of insulin resistance, a risk factor for type 2 diabetes, in children as young as 10 years

old (17). As nicotine is transmittable via breastmilk during breastfeeding, neonatal nicotine exposure during lactation may cause insulin resistance and metabolic disorders at adulthood (18). In this study, the female offspring from SE mothers developed glucose intolerance and hyperlipidaemia in adulthood. This is similar to the metabolic outcome in the male offspring at the same age, who had significant glucose intolerance albeit similar body weight as the controls (15). Here at weaning, random blood glucose levels were also increased in the female SE offspring, followed by significantly increased glucose intolerance at adulthood, suggesting a continuous impairment in systemic glucose metabolism. Similar disorders in glucose metabolism have also been found in the male offspring from weaning to adulthood (6, 15). It needs to be noted that in the laboratory setting, the rodent's diet is tightly controlled by using nutritionally balanced food, whereas in humans postnatal obesogenic/diabetogenic diet is an additional environment insult to the metabolic homeostatic regulation. Indeed, children of smokers tend to choose a high caloric diet which further accelerates the development of diabetes and related complications (1).

In this study, normal plasma insulin levels with hyperglycaemia in the female SE offspring at 20 days are suggestive of insulin resistance. Even at 13 weeks, plasma insulin levels were only marginally increased in the female offspring of SE dams (20%) compared to Sham offspring, with significant glucose intolerance; whereas in the male littermates, plasma insulin levels were similar to the control offspring (15). Maternal nicotine exposure during gestation and lactation has been shown to induce permanent β-cell loss and subsequent impairment in glucose tolerance in animal models (19). This may explain why there was no significant increase in blood insulin levels in both male and female SE offspring at adulthood. Furthermore, glucose intolerance at adulthood also indicates the insensitivity of insulin receptors. However, in a previous study we failed to observe a difference in the liver response to acute insulin stimulation (2). Since exogenous insulin injection in that study was not as physiological as that released by the pancreas in the present study in response to postprandial glucose increase, this finding can't exclude the existence of liver insulin resistance. Previously, maternal supplementation of a mixture of antioxidant (vitamin E, coenzyme Q10 and α-lipoic acid) during pregnancy and lactation has been shown to increase beta cell proliferation and tissue insulin in offspring of nicotine treated rat mothers; however there were no measurements of either blood insulin level or glucose level in that study (20).

Inflammation may play a key role in maternal smoking induced glucose intolerance in the offspring. Maternal smoking has been shown to change liver inflammatory markers in male but not female foetuses in humans and rats (10). Inflammation was also found to be increased in newborn livers from

the mice of SE mothers, however livers from both sexes were combined in this study (21). Here we showed significant increase in the liver inflammatory marker expression in female offspring at both weaning and adulthood. The association between insulin resistance, hyperglycaemia and increased liver inflammatory cytokine production (such as IL-1β and IL-6) has been well documented (22). The mechanism is suggested to be via the inhibition of two essential markers involved in cellular glucose uptake, PPAR-γ and GLUT4 (23). PPAR-γ regulates glucose homeostasis and insulin sensitivity in a tissue and age dependent manner which also involves GLUT4, the insulin dependent glucose transporter (24). TNF-α causes insulin resistance and glucose metabolic disorders through multiple mechanisms, including the downregulation of GLUT4, the inhibition on insulin signalling cascade, the stimulation of lipolysis to elevate free fatty acid level which induces insulin resistance, as well as the suppression of PPAR-γ expression and function (23). Here, an upregulation of IL-6 with reduced PPAR-γ mRNA expression at weaning may initiate the development of insulin resistance leading to glucose intolerance at adulthood; whereas upregulated PPAR-γ at adulthood could be an adaptive response to glucose intolerance. The anti-inflammatory cytokine IL-10 acts on macrophages to lower the pro-inflammatory cytokines such as TNF-α, and serum IL-10 is reduced in patients with insulin resistance (25). Reduced IL-10 mRNA expression in the SE offspring at 13 weeks may suggest an attenuation of the anti-inflammatory capacity, in line with the upregulation of pro-inflammatory TNF-α and IL-1β. Another cytokine IL-22 has been reported to alleviate metabolic disorders, such as improving insulin sensitivity and lipid metabolism, decreasing chronic inflammation in the liver, and restoring postprandial glucose clearance in obese mice (26). The trend reduction in IL-22 mRNA expression in the SE offspring at both ages also suggests a reduced capacity to counteract metabolic disorders (26).

Smoking is an independent risk factor for liver fibrosis due to cell injury induced by pro-inflammatory cytokines, such as IL-1, IL-6 and TNFα (12). Here in adult female offspring, the increase in these pro-inflammatory cytokines were indeed accompanied by more collagen deposition. In this study, we found that liver collagen I and III proteins were increased in the SE offspring at 20 days, whereas only increased collagen III deposition occurred at adulthood.

FOXO1 interacts with PCG1α to suppress hepatic gluconeogenesis in response to insulin, whereas the mutation of FOXO1 can demolish such regulation (27). FOXO1 mRNA expression was significantly reduced in weaning female offspring by maternal SE with increased blood glucose levels. FOXO1 expression was somewhat restored at 13 weeks when there was still significant glucose intolerance, suggesting that reduced tissue glucose uptake may play a major role. Interestingly liver

GLUT4 was somehow higher in the SE offspring, which was normalised by maternal L-carnitine treatment albeit improved glucose tolerance. This reflects the limitations of mRNA expression which does not reflect protein transcription. However, such upregulation may be an adaptive response to reduced protein levels, which explained increased AUC level during IPGTT. Therefore, the confirmation of protein levels and translocation in response to high glucose challenge is needed in future studies.

Smoking is a contributor to hypertriglyceridemia in humans (28). Our results showed that maternal SE during pregnancy and lactation led to increased plasma TG in female offspring. The exact mechanism for such effect is still unclear. The downregulation of regulators involved in lipid metabolism may play a key role. CPT-1α catalases the transportation of long-chain fatty acid into the mitochondria for oxidation to synthesise ATP (29); while ATGL regulates liver TG turnover and free fatty acid β-oxidation (30). PGC-1α is an essential metabolic switch for substrate metabolic functions (31), which also promotes fatty acid oxidation (32). Here both liver CPT-1α and ATGL mRNA expression is lower in the SE offspring at 20 days, which could potentially contribute to increased blood TG level. At 13 weeks, FASN was high in the female SE offspring, which may lead to increased *de novo* TG synthesis. This is consistent with a previous study using prenatal nicotine injection where FASN pathway was upregulated due to epigenetic modification (33). The non-significant increase in PGC-1α can be an adaptive response to dysregulated glucose and lipid substrate metabolism.

We have previously demonstrated that maternal L-carnitine supplementation during gestation and lactation improves renal and neurological alterations in the male SE offspring of SE dams (15, 16). In relation to the focus of this prior study, maternal L-carnitine supplementation also normalised low birth weight and kidney underdevelopment, as well as glucose intolerance in the SE male offspring in adulthood (15), suggesting a beneficial effect on foetal development and metabolic outcome. In the present study, we demonstrated that maternal L-carnitine supplementation also ameliorated the effect of maternal SE on low birth weight and increased liver weight in the female offspring with normalised glucose homeostasis and lipid profile at adulthood. As such, these effects are similar between the male and female offspring from the SE mothers. The unchanged blood insulin level in both male and female offspring suggests that L-carnitine may improve peripheral insulin action, but not insulin production. In humans, dietary L-carnitine supplementation has been shown to improve hepatic insulin resistance by decreasing TNF-α levels and improving lipid profiles (34); The direct anti-inflammatory effect of L-carnitine supplement has also been reported in a trial in patients with coronary artery disease (35). Here in the female offspring, maternal L-carnitine supplementation had

extended anti-inflammatory effects on their liver, evidenced by reduced levels of IL-1β, IL-6, and TNF-α expression in adulthood, all key players in insulin resistance. Interestingly, suppression of the metabolic regulator IL-22 was also reversed in these female offspring by maternal L-carnitine supplementation, which may contribute to normalised pro-inflammatory cytokines and glucose metabolism. Previously, L-carnitine has been shown to prevent the development of fibrosis in multiple organs, including the liver (36), as well as ameliorate alcohol-induced liver damage (37). In this study, maternal L-carnitine supplementation suppressed or normalised liver collagen deposition in the weaning and adult female offspring. It needs to be noted that the liver contains a low percentage of collagen - in the order of less than 4%. A second insult that promotes fibrosis such as viral hepatitis or non-alcoholic fatty liver disease is required to further determine whether offspring of SE dams are linked to high risk of liver cirrhosis, and whether maternal L-carnitine supplementation can prevent such changes.

Maternal L-carnitine supplementation normalised blood lipid profiles in the female SE offspring at both 20 days and 13 weeks. In rats fed a high fat diet, L-carnitine was shown to reduce liver TG synthesis and/or release (38), as well as improve lipid β-oxidation (39). In this study, maternal L-carnitine nearly normalised the TG lipase ATGL expression at weaning, PCG-1α and FASN expression at adulthood, suggesting different markers are involved in lipid dysfunction at different ages.

In summary, maternal SE led to glucose intolerance and hyperlipidaemia in the female offspring which is associated with abnormal hepatic inflammatory and lipid metabolic markers. This further increased their risk of developing fibrosis in adulthood. L-carnitine supplementation during gestation and lactation in the mothers can normalise maternal smoking induced metabolic disorders by reducing liver inflammation and normalising lipid metabolic and fibrotic markers. This suggests that L-carnitine supplementation may be beneficial if used by smokers during pregnancy.

Methods

1. Animal model

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). Female Balb/c mice (6 weeks old, n=36, Animal Resources Centre, Perth, Australia) were weighed and divided into two groups, the sham exposure group (Sham, n=12) and the SE group (SE, n=24). After 6 weeks of SE, the female mice were mated as previously described (8). SE was not performed on the day of delivery. Neither the male breeder

nor the offspring were exposed to cigarette smoke at any time during the study. A subgroup of the SE dams was treated with L-carnitine (SE+LC, n=12) via drinking water (1.5mM) starting at the time of mating and continued throughout gestation and lactation as previously described (15).

Female offspring were sacrificed at 20 days and at 13 weeks. Briefly, the non-fasted pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia, NSW, Australia). Blood was collected using heparinised syringes through cardiac puncture. Blood glucose was measured. The plasma was separated by centrifugation at 12,000g for 8 minutes and stored at -20^{o}C for later analysis. Mice were killed by decapitation. The livers were harvested, weighed, snap frozen in liquid nitrogen and then stored at -80^{o}C. One offspring from each litter was used for tissue analysis.

2. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed at 12 weeks of age. After 5 hours fasting, a baseline glucose level was taken from the tail blood (Accu-Chek, Roche Diagnostics, IN, USA). Then the glucose was administered (2 g/kg , n=9-10, ip) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection as previously described (2). The Area Under the Curve (AUC) of the glucose levels was calculated for each mouse.

3. Bio-assays

Plasma TG, NEFA and insulin concentrations were measured using an in-house TG assay (TG reagent, Roche Diagnostics, Mannheim, Germany, intra-assay variation 0.5%, inter-assay variation 3.4%) (40), NEFA-HR kit (WAKO, Osaka, Japan, intra-assay variation 0.75%, inter-assay variation 4.91%), and Insulin ELISA Kit (Abnova, Taipei, Taiwan, intra and inter assay variation 2.2% and 5.0%, respectively).

4. Real-time PCR

Total RNA was extracted from liver tissues using RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. The first strand of cDNA was synthesised from RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The genes of interest were measured using manufacturer pre-optimized and validated TaqMan® primers and probes (Supplementary Table 1, Thermo Fisher Scientific, MA, USA) and Platinum® qPCR SuperMix-UDG kit (Thermo Fisher Scientific) using 18s rRNA as the housekeeping gene. The probes of the target genes were labelled with FAM and 18s rRNA was labelled with VIC. The average mRNA

expression of the Sham group was used as the calibrator, and the values are expressed as fold change from control.

5. Immunohistochemistry

For direct visualisation of collagen fibres, formalin fixed livers were embedded in paraffin and sectioned to 5μm. Three non-consecutives sections were collected from each sample (n=6/group). Immunohistochemistry was performed with the Dako EnVision+ System-HRP labelled Polymer Anti-Rabbit kit (Aglient, CA, USA). In brief, the sections were deparaffinised and treated with xylene and decreasing graded ethanol to distilled water for hydration. The sections were then heated in pressure cooker (Homemaker, Mulgrave, Australia) for 10 mins with citrate buffer (pH 6.0) followed by cooling in water bath for 15 mins for heat-induced epitope retrieval. The sections were blocked with peroxidase block solution followed by 5% goat serum in phosphate buffered saline with Tween-20. The sections were then incubated for 30 minutes with either Collagen I (1:2000, Abcam, Cambridge, UK) or III (1:5000, Abcam, Cambridge, UK) diluted in antibody diluent solution (Aglient, CA, USA). This was followed by incubated with peroxidase labelled polymer (anti-rabbit) solution for 30 minutes. The sections were then incubated with substrate-chromogen for 10 minutes. Finally, they are counterstained with hematoxylin and coverslipped. The sections were then scanned with NanoZoomer SQ slide scanner (Hamamatsu, Hamamatsu city, Japan). Three fields of images were taken at 40X from each section. The images were assessed with (ImageJ National Institute of Health, Bethesda, Maryland, USA) to determine the percentage of collagen stain.

6. Statistical analysis

The results are expressed as mean ± SEM. The differences between the groups were analysed using one-way ANOVA followed by Turkey's *post hoc* tests (Prism 6, Graphpad CA, USA). Student's unpaired t-test was used for selected two groups with marked difference. $P<0.05$ is considered significant.

Acknowledgement:

This project was funded by a Postgraduate Research Support by Faculty of Science, University of Technology Sydney. Dr Ibrahim Al-Odat was supported by an Australian Postgraduate Award.

References

1. Chen H, Morris MJ. Maternal smoking——A contributor to the obesity epidemic? *Obesity Research & Clinical Practice* 2007; **1**:155-63.
2. Chen H, Iglesias MA, Caruso V, Morris MJ. Maternal cigarette smoke exposure contributes to glucose intolerance and decreased brain insulin action in mice offspring independent of maternal diet. *PLoS One* 2011; **6**:e27260.
3. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; **444**:860-7.
4. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. *Nature medicine* 2005; **11**:183-90.
5. Conceição EP, Peixoto-Silva N, Pinheiro CR, Oliveira E, Moura EG, Lisboa PC. Maternal nicotine exposure leads to higher liver oxidative stress and steatosis in adult rat offspring. *Food and Chemical Toxicology* 2015; **78**:52-9.
6. Al-Odat I, Chen H, Chan YL, et al. The impact of maternal cigarette smoke exposure in a rodent model on renal development in the offspring. *PLoS One* 2014; **9**:e103443.
7. Chan YL, Saad S, Al-Odat I, et al. Impact of maternal cigarette smoke exposure on brain and kidney health outcomes in female offspring. *Clin Exp Pharmacol Physiol* 2016; **43**:1168-76.
8. Chan YL, Saad S, Pollock C, et al. Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring. *Sci Rep* 2016; **6**:25881.
9. Mattsson K, Källén K, Longnecker MP, Rignell-Hydbom A, Rylander L. Maternal smoking during pregnancy and daughters' risk of gestational diabetes and obesity. *Diabetologia* 2013.
10. Filis P, Nagrath N, Fraser M, et al. Maternal smoking dysregulates protein expression in second trimester human fetal livers in a sex-specific manner. *The Journal of Clinical Endocrinology & Metabolism* 2015; **100**:E861-E70.
11. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; **11**:183-90.
12. Corpechot C, Gaouar F, Chrétien Y, Johanet C, Chazouillères O, Poupon R. Smoking as an independent risk factor of liver fibrosis in primary biliary cirrhosis. *Journal of Hepatology*; **56**:218-24.
13. Gulcin I. Antioxidant and antiradical activities of L-carnitine. *Life sciences* 2006; **78**:803-11.
14. Su C-C, Chang C-S, Chou C-H, et al. L-carnitine ameliorates dyslipidemic and hepatic disorders induced by a high-fat diet via regulating lipid metabolism, self-antioxidant capacity, and inflammatory response. *Journal of Functional Foods* 2015; **15**:497-508.
15. Nguyen LT, Stangenberg S, Chen H, et al. l-Carnitine reverses maternal cigarette smoke exposure-induced renal oxidative stress and mitochondrial dysfunction in mouse offspring. *Am J Physiol Renal Physiol* 2015; **308**:F689-96.
16. Chan YL, Saad S, Al-Odat I, et al. Maternal L-Carnitine supplementation improves brain health in offspring from cigarette smoke exposed mothers. *Frontiers in Molecular Neuroscience* 2017; **10**.
17. Thiering E, Brüske I, Kratzsch J, et al. Prenatal and postnatal tobacco smoke exposure and development of insulin resistance in 10 year old children. *International Journal of Hygiene and Environmental Health* 2011; **214**:361-8.
18. de Oliveira E, Moura EG, Santos-Silva AP, et al. Neonatal nicotine exposure causes insulin and leptin resistance and inhibits hypothalamic leptin signaling in adult rat offspring. *Journal of Endocrinology* 2010; **206**:55-63.
19. Bruin JE, Gerstein HC, Holloway AC. Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol Sci* 2010; **116**:364-74.
20. Bruin JE, Woynillowicz AK, Hettinga BP, et al. Maternal antioxidants prevent beta-cell apoptosis and promote formation of dual hormone-expressing endocrine cells in male offspring following fetal and neonatal nicotine exposure. *J Diabetes* 2012; **4**:297-306.

21. Diniz MF, Dourado VA, Silva ME, Pedrosa ML, Bezerra FS, Lima WG. Cigarette Smoke Causes Changes in Liver and Spleen of Mice Newborn Exposed During Pregnancy. *Journal of Cytology & Histology* 2013; **4**:168.
22. Fujimoto S, Mochizuki K, Shimada M, et al. Insulin resistance induced by a high-fat diet is associated with the induction of genes related to leukocyte activation in rat peripheral leukocytes. *Life sciences* 2010; **87**:679-85.
23. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-α, overexpressed in human fat cells from insulin-resistant subjects. *Journal of Biological Chemistry* 2003; **278**:45777-84.
24. Ahmadian M, Suh JM, Hah N, et al. PPAR [gamma] signaling and metabolism: the good, the bad and the future. *Nature medicine* 2013; **99**:557-66.
25. de Luca C, Olefsky JM. Inflammation and insulin resistance. *FEBS Letters* 2008; **582**:97-105.
26. Wang X, Ota N, Manzanillo P, et al. Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. *Nature* 2014; **514**:237-41.
27. Matsumoto M, Pocai A, Rossetti L, DePinho RA, Accili D. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. *Cell Metabolism* 2007; **6**:208-16.
28. Craig WY, Palomaki GE, Haddow JE. Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. *BMJ* 1989; **298**:784-8.
29. Kralisch S, Klein J, Lossner U, et al. Isoproterenol, TNFα, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Molecular and Cellular Endocrinology* 2005; **240**:43-9.
30. Ong KT, Mashek MT, Bu SY, Greenberg AS, Mashek DG. ATGL is a major hepatic lipase that regulates TAG turnover and fatty acid signaling and partitioning. *Hepatology (Baltimore, Md.)* 2011; **53**:116-26.
31. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature* 2005; **434**:113-8.
32. Sugden MC, Caton PW, Holness MJ. PPAR control: it's SIRTainly as easy as PGC. *Journal of Endocrinology* 2010; **204**:93-104.
33. Ma N, Nicholson CJ, Wong M, Holloway AC, Hardy DB. Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicology and Applied Pharmacology* 2014; **275**:1-11.
34. Malaguarnera M, Gargante MP, Russo C, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis—a randomized and controlled clinical trial. *The American journal of gastroenterology* 2010; **105**:1338-45.
35. Lee B-J, Lin J-S, Lin Y-C, Lin P-T. Antiinflammatory effects of l-carnitine supplementation (1000 mg/d) in coronary artery disease patients. *Nutrition* 2015; **31**:475-9.
36. Demiroren K, Dogan Y, Kocamaz H, et al. Protective effects of L-carnitine, N-acetylcysteine and genistein in an experimental model of liver fibrosis. *Clinics and Research in Hepatology and Gastroenterology* 2014; **38**:63-72.
37. Bykov I, Järveläinen H, Lindros K. L-carnitine alleviates alcohol-induced liver damage in rats: Role of tumour necrosis factor-alpha. *Alcohol & Alcoholism* 2003; **38**:400-6.
38. Amin KA, Nagy MA. Effect of Carnitine and herbal mixture extract on obesity induced by high fat diet in rats. *Diabetology & Metabolic Syndrome* 2009; **1**.
39. Turpin S, Hoy A, Brown R, et al. Adipose triacylglycerol lipase is a major regulator of hepatic lipid metabolism but not insulin sensitivity in mice. *Diabetologia* 2011; **54**:146-56.
40. Chen H, Simar D, Pegg K, Saad S, Palmer C, Morris M. Exendin-4 is effective against metabolic disorders induced by intrauterine and postnatal overnutrition in rodents. *Diabetologia* 2014; **57**:614-22.

Table 1: Body weight, liver weight, and blood metabolic markers in the female offspring at 20 days and 13 weeks

P 20	**Sham**	**SE**	**SE + LC**
Body weight (g)	10.4 ± 0.54	9.59 ±0.18	9.45 ±0.28
Liver weight (g)	0.45 ± 0.03	0.45 ±0.01	0.43 ±0.02
Liver/body (%)	4.27 ± 0.08	4.56 ±0.08 *	4.44 ±0.08
Glucose (mM)	10.8 ± 0.4	12.6 ±0.4 *	11.8 ±0.5
NEFA (mM)	2.11 ± 0.3	1.95 ± 0.2	1.50 ± 0.1
TG (mM)	0.86 ± 0.2	1.90 ± 0.2 *	0.66 ± 0.1$^{\#}$
Insulin (ng/ml)	0.51 ± 0.01	0.52 ± 0.02	0.50 ± 0.02
Week 13	**Sham**	**SE**	**SE + LC**
Body weight (g)	21.7 ± 0.3	20.9 ± 0.3	21.0 ± 0.2
Liver weight (g)	1.00 ± 0.03	0.96 ± 0.03	0.90 ± 0.02
Liver/body (%)	4.54 ± 0.06	4.47 ± 0.5	4.29 ± 0.07 $^{\#}$
Glucose (mM)	10.5 ± 0.4	10.5 ± 0.3	11.2 ± 0.2
NEFA (mM)	8.87 ± 0.5	8.14 ±0.7	7.18 ± 0.4
TG (mM)	1.43 ± 0.1	2.34 ± 0.1*	1.43 ± 0.2 $^{\#}$
Insulin (ng/ml)	0.54 ± 0.10	0.64 ± 0.03	0.64 ± 0.03
AUC during IPGTT (mM•min)	854 ± 30	1000 ± 25 *	884 ± 28 $^{\#}$

Results are expressed as Mean ± SEM. n=6-10. The data was analysed by One-way ANOVA followed by Turkey's post hoc tests. * $P < 0.05$ vs Sham, $^{\#}$ $P < 0.05$ vs SE

AUC: area under the curve; IPGTT: intraperitoneal glucose tolerance test; NEFA: Non-esterified fatty acids; TG: triglycerides;

Figures

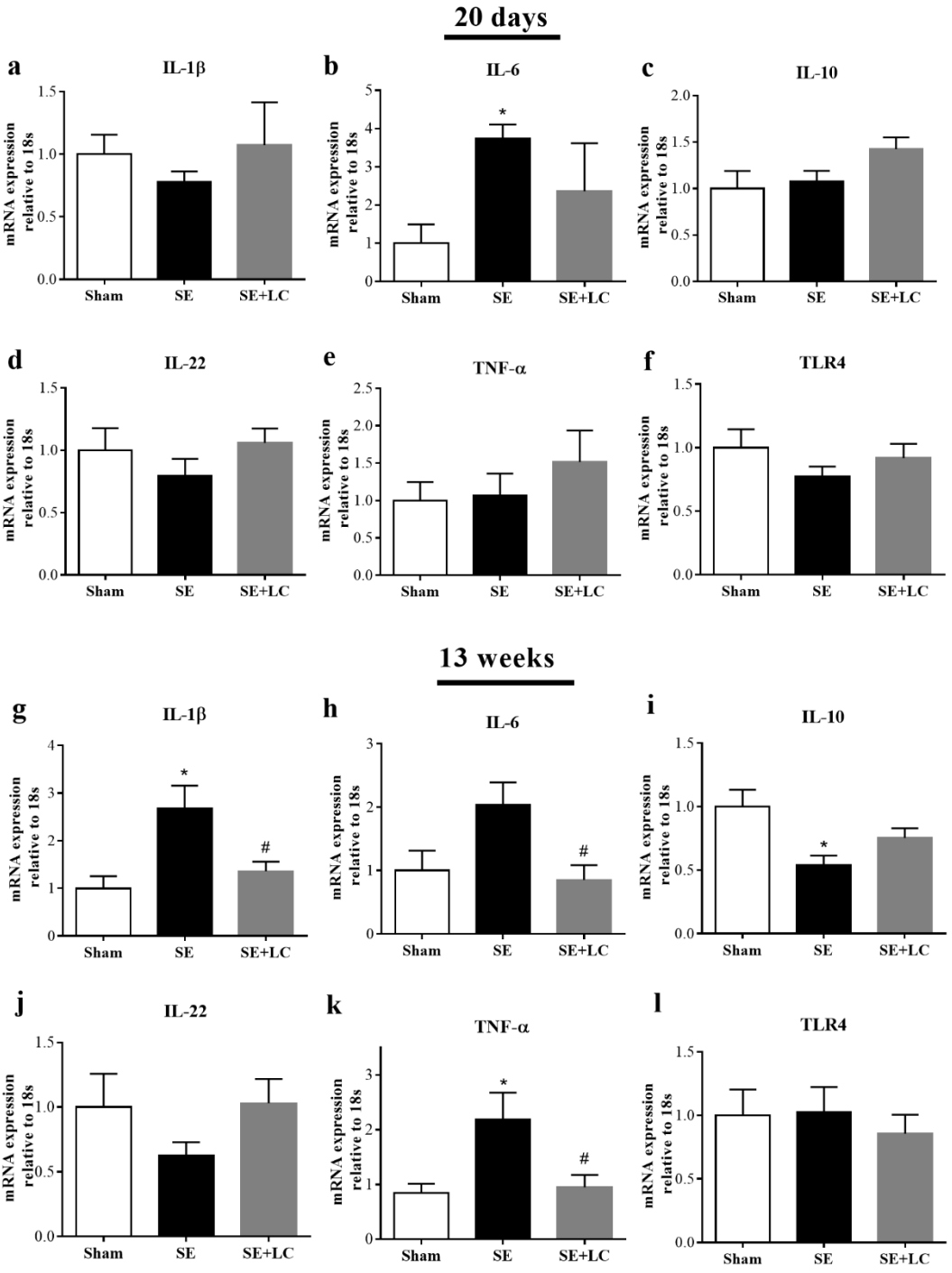

Figure 1: Hepatic mRNA expression of inflammatory markers, IL-1β (a, g), IL-6 (b, h), IL-10 (c, i), IL-22 (d, j), TNF-α (e, k), and TLR4 (f, l) in the female offspring at 20 days and 13 weeks of age. Results are expressed as mean ± SEM, n=6. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE. IL: interleukin; SE: mother exposed to cigarette smoke; SE+LC: mother exposed to cigarette smoke and treated with L-carnitine during gestation and lactation; TLR: toll-like receptor.

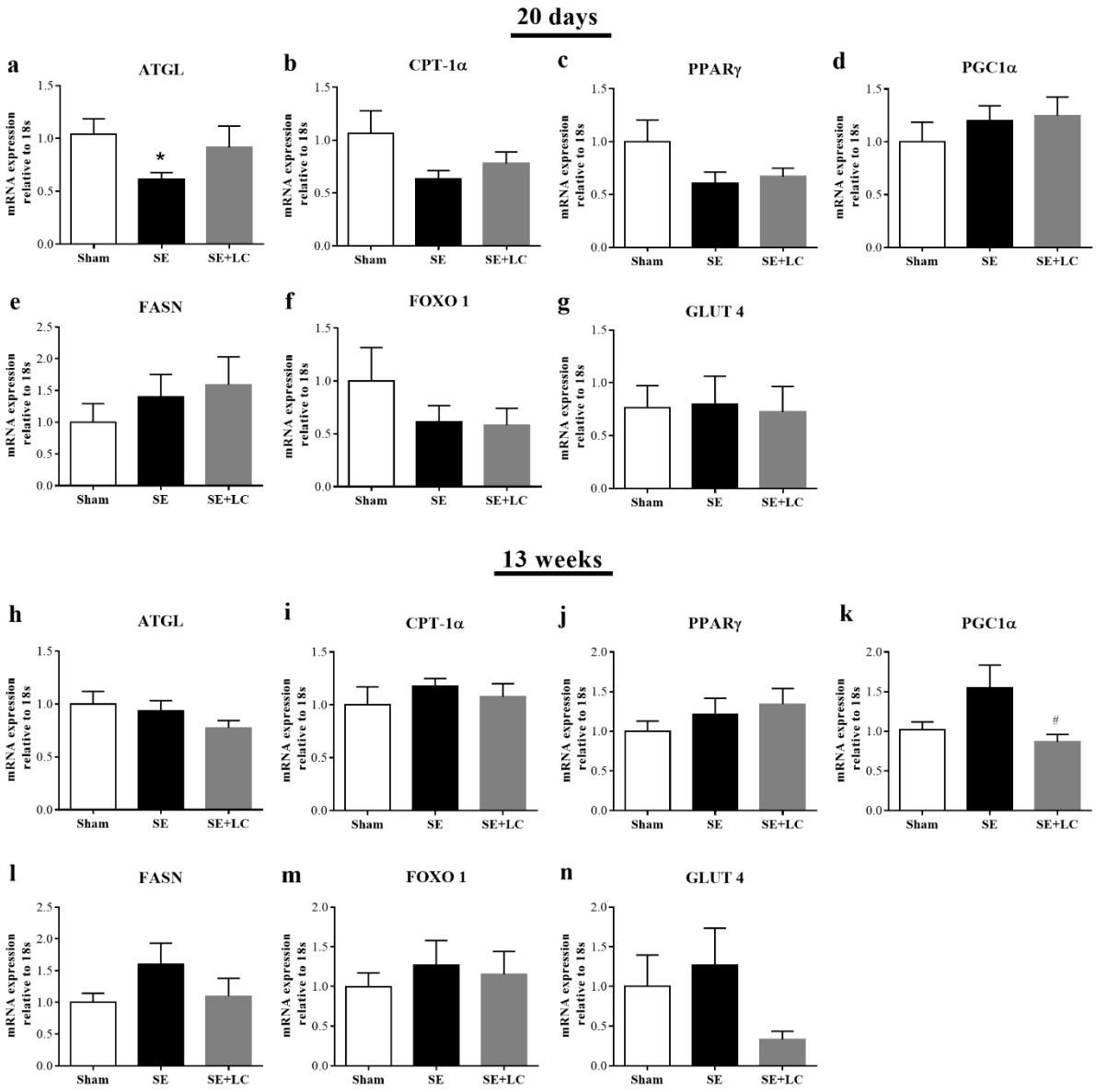

Figure 2: Hepatic mRNA expression of lipid metabolic markers, ATGL (a, h), CPT-1α (b, i), PPAR-γ (c, j), PGC1α (d, k), FASN (e, l), FOXO 1 (f, m), and glucose transporter GLUT 4 (g, n) in the female offspring at 20 days and 13 weeks of age. Results are mean ± SEM, n = 6. * $P < 0.05$ SE vs Sham. ATGL: adipose triglyceride lipase; CPT: carnitine palmitoyl transferase; FASN: fatty acid synthase gene; FOXO: forkhead box protein; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR: peroxisome proliferator-activated receptor; SE: mother exposed to cigarette smoke; SE+LC: mother exposed to cigarette smoke and treated with L-carnitine during gestation and lactation.

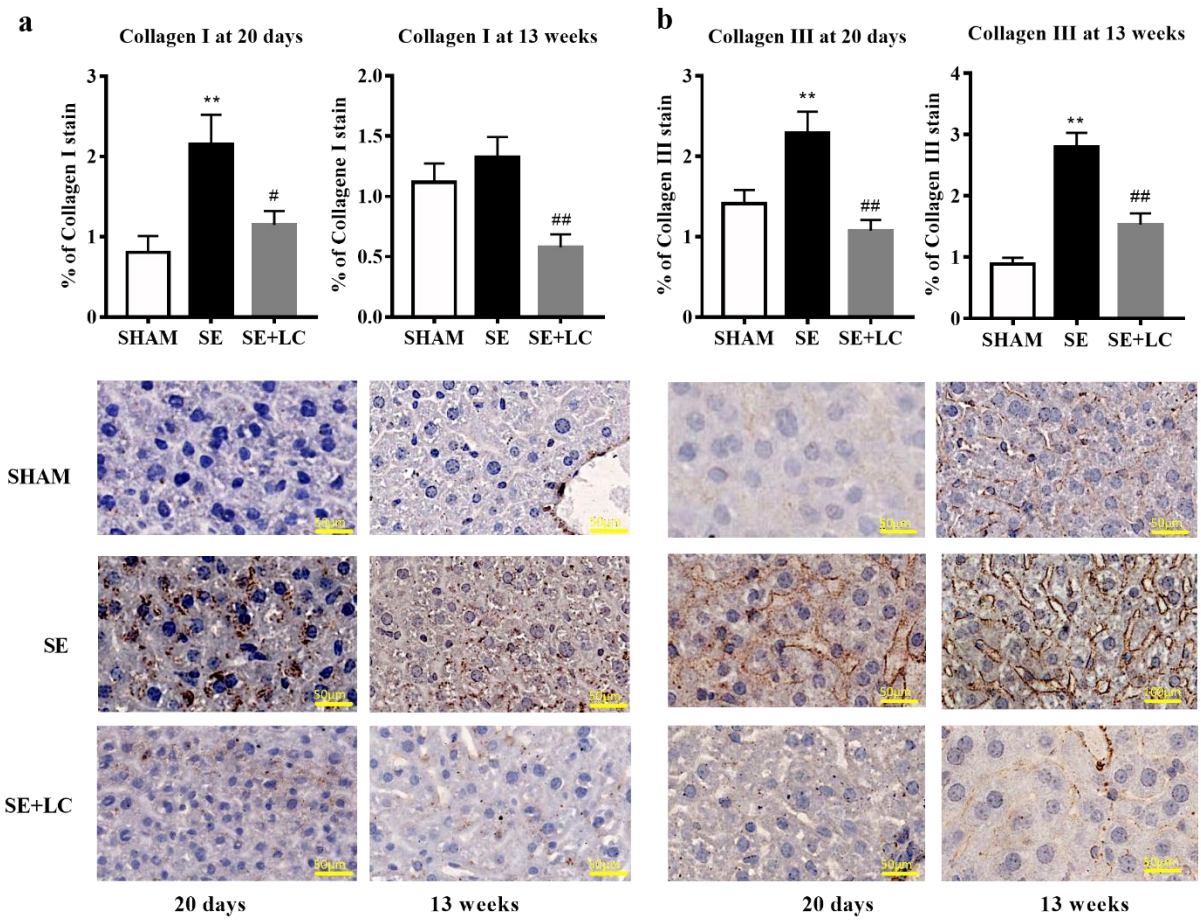

Figure 3: Hepatic fibrotic markers collagen I (a) and collagen III (b) staining quantification and representative photomicrographs of immunohistochemistry staining (mag 40x, scale bar = 50 µm) in the female offspring at 20 days and 13 weeks of age. Results are expressed as mean ± SEM, n = 6. * P < 0.05, **P<0.01, SE vs Sham; # P < 0.05, ## P < 0.01, SE+LC vs SE. SE: mother exposed to cigarette smoke; SE+LC: mother exposed to cigarette smoke and treated with L-carnitine during gestation and lactation.

Supplementary Table 1: Body weight changes and litter information of the dams.

	Sham	**SE**	**SE+LC**
Body weigh at mating (g)	19.1 ± 0.4	17.8 ± 0.5	18.0 ± 0.5
Body weight at weaning (g)	24.5 ± 0.7	22.5 ± 0.7	23.9 ± 0.8
Weight gain (g)	0.29 ± 0.03	0.27 ± 0.03	0.33 ± 0.03
Litter size (pup / litter)	6.2 ± 0.8	5.3 ± 1.0	6.0 ± 1.0
Male pup / litter	3.5 ± 0.5	2.9 ± 0.7	3.0 ± 0.6
Female pup / litter	2.6 ± 0.6	2.4 ± 0.6	3.0 ± 0.5

Results are expressed as Mean ± SEM. n=9-12. The data was analysed by One-way ANOVA followed by Turkey's post hoc tests.

Supplementary Table 2: Probes sequences provided by the manufacture used in rt-PCR (Life Technologies, CA, USA).

Gene	**NCBI references**	**Probe Sequence**	**Assay ID**
18S	X03205.1	ACCGCAGCTAGGAATAATGGA	4319413E
ATGL	NM_025802.2	GGCCTGCCTGGGTGAAGCAGGTGCC	Mm00503040_m1
CPT-1α	NM_013495.2	TACCGTGAGCAGGTACCTGGAGTCT	Mm00550438_m1
FASN	NM_007988.3	CAATTGTGGATGGAGGTATCAACCC	Mm00662319_m1
FOXO 1	NM_001191846	AAGAGTTAGTGAGCAGGCTACATTT	Mm00490672_m1
GLUT 4	NM_009204.2	CTCTGCTGCTGCTGGAACGGGTTCC	Mm00436615_m1
IL-1β	NM_008361.3	TCCTTGTGCAAGTGTCTGAAGCAGC	Mm01336189_m1
IL-6	NM_031168.1	ATGAGAAAAGAGTTGTGCAATGGCA	Mm00446190_m1
IL-10	NM_010548.2	GAAGACTTTCTTTCAAACAAAGGAC	Mm00439614_m1
IL-22	NM_016971.2	TGGCCAAGGAGGCCAGCCTTGCAGA	Mm00444241_m1
PGC-1α	NM_008904.1	CGCAACATGCTCAAGCCAAACCAAC	Mm00447183_m1
PPAR-γ	NM_0011273330.1	ATGCTGTTATGGGTGAAACTCTGG	Mm01184322_m1
TLR4	NM_021297.2	CCCTGCATAGAGGTAGTTCCTAATA	Mm00445273_m1
TNFα	NM_013693.2	CCCTCACACTCAGATCATCTTCTCA	Mm00443259_g1

ATGL: adipose triglyceride lipase, CPT: carnitine palmitoyl transferase, FASN: Fatty acid synthase, FOXO: Forkhead box protein, GLUT: Glucose transporter, IL: interleukin, PGC: Peroxisome proliferator-activated receptor gamma coactivator, PPAR: Peroxisome proliferator-activated receptor, TLR: Toll-like receptor, TNF: tumour necrosis factor.