

Suboptimal maternal nutrition, during early fetal liver development, promotes lipid accumulation in the liver of obese offspring

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Abstract

Maternal nutrition during the period of early organ development can modulate the offspring's ability to metabolise excess fat as young adults when exposed to an obesogenic environment. This study examined the hypothesis that exposing offspring to nutrient restriction coincident with early hepatogenesis would result in endocrine and metabolic adaptations that subsequently lead to increased ectopic lipid accumulation within the liver. Pregnant sheep were fed either 50 or 100% of total metabolisable energy requirements from 30 to 80 days gestation and 100% thereafter. At weaning, offspring were made obese, and at ~1 year of age livers were sampled. Lipid infiltration and molecular indices of gluconeogenesis, lipid metabolism and mitochondrial function were measured. Although hepatic triglyceride accumulation was not affected by obesity *per se*, it was nearly doubled in obese offspring born to nutrient-restricted mothers. This adaptation was accompanied by elevated gene expression for peroxisome proliferator-activated receptor γ (PPARG) and its co-activator PGC1 α , which may be indicative of changes in the rate of hepatic fatty acid oxidation. In contrast, maternal diet had no influence on the stimulatory effect of obesity on gene expression for a range of proteins involved in glucose metabolism and energy balance including glucokinase, glucocorticoid receptors and uncoupling protein 2. Similarly, although gene expressions for the insulin and IGF1 receptors were suppressed by obesity they were not influenced by the prenatal nutritional environment. In conclusion, excess hepatic lipid accumulation with juvenile obesity is promoted by suboptimal nutrition coincident with early development of the fetal liver.

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Introduction

Chronic excess energy intake combined with sedentary behaviour results in obesity, hyperlipidaemia and insulin resistance (Bellisari 2008). These adaptations are associated with the development of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD; Dixon *et al.* 2001), conditions now occurring in substantial numbers of children and adults (Moscatiello *et al.* 2007, Lidofsky 2008). We have recently established an ovine model for the metabolic syndrome, which demonstrates differential adaptations following exposure to a post-weaning obesogenic environment that is dependent on the animal's prenatal nutritional environment (Williams *et al.* 2007, Sebert *et al.* 2009). This is important because in fast developing countries, such as

India and China, the adoption of a more western lifestyle has resulted in generations of children and young adults, many previously undernourished *in utero*, being exposed to an environment of plenty, potentially predisposing them to the early development of obesity and its complications.

Irrespective of the *in utero* environment, young adult obese sheep exhibit raised fasting plasma non-esterified fatty acid (NEFA), leptin and insulin (Williams *et al.* 2007, Sebert *et al.* 2009), together with molecular markers of inflammation in the kidney and adipose tissue (Sharkey *et al.* 2009a, 2009c). However, obese offspring born to nutrient-restricted mothers demonstrate greater insulin resistance as indicated by raised insulin (Sebert *et al.* 2009), enhanced ectopic lipid accumulation in

the heart (Chan *et al.* 2009) and impaired function of the endoplasmic reticulum in adipose tissue (Sharkey *et al.* 2009b). These findings raise the question as to whether *in utero* nutritional programming followed by exposure to an obesogenic environment fits with a 'two hit' hypothesis for the pathogenesis of NAFLD (Day 2002). The first challenge would cause insulin resistance and chronic inflammation (Xu *et al.* 2003), while the second, following exposure to an obesogenic environment, would result in hepatic steatosis (Brunt 2007), fibrosis and abnormal mitochondrial β oxidation (Tessari *et al.* 2009). We therefore hypothesised that ectopic lipid accumulation would be amplified with obesity in offspring born to nutrient-restricted mothers as hepatic endocrine sensitivity to both glucocorticoids and insulin-like growth factors (IGF) is reset during development in these animals (Hyatt *et al.* 2007).

To date, numerous small animal models dependent on the presence of a genetic defect and/or the consumption of more extreme diets either in adulthood (Weltman *et al.* 1996, Enriquez *et al.* 1999, Lieber *et al.* 2004, Ayala *et al.* 2009, Fu *et al.* 2009, Li *et al.* 2009) or in early life (Bruce *et al.* 2009) have been adopted to study NAFLD. They have shown that peroxisome proliferator-activated receptor α (PPARA), a regulator of a number of mitochondrial fatty acid metabolising enzymes in the liver, underpins the development of NAFLD. We, therefore, examined some of these primary regulators of hepatic energy metabolism in order to determine whether these are differentially affected by obesity and *in utero* exposure to maternal nutrient restriction. Ultimately, we aimed to establish a developmental model of NAFLD based on a large mammal and mediated by changes in dietary intake, rather than composition, through pregnancy. Studies in NAFLD are particularly confusing due to the multiple potential sources of excess triglyceride (TAG), i.e. *de novo* synthesis, uptake of NEFA released from adipose tissue lipolysis, uptake of dietary TAG, reduced hepatic fatty acid oxidation and reduced release of TAG in the form of very low-density lipoproteins. In a recent study (Fabbrini *et al.* 2008, 2009), NAFLD was associated with changes in all of these indices. Sheep therefore represent a particularly interesting model for examining lipid trafficking between tissues because *de novo* synthesis of NEFA is restricted to adipose tissue, which is the only source of long-chain TAG (Ingle *et al.* 1972).

Results

Body composition, liver weight and metabolic profile

Complete body composition and metabolic profiles at 1 year of age for all lean (L), obese (O) and obese offspring born to nutrient-restricted mothers (NRO) have been described previously (Williams *et al.* 2007, Sebert *et al.* 2009) but data essential for the full interpretation of the new

data obtained from the liver are included in this study. Obesity resulted in an increased total fat mass irrespective of the prenatal nutritional environment (i.e. L: 2.5 ± 0.3 ; O: 8.0 ± 0.3 ; NRO: 7.7 ± 0.6 g/kg ($P < 0.001$)) and had no effect on relative liver weight (i.e. L: 10.3 ± 0.3 ; O: 9.9 ± 0.6 ; NRO: 10.1 ± 0.6 g/kg). Obesity was accompanied by raised fasting plasma NEFA, leptin and insulin concentrations, with the insulin response being enhanced in the NRO animals (insulin – L: 0.6 ± 0.1 ; O: 1.0 ± 0.3 ; NRO: 1.6 ± 0.3 ng/ml ($P < 0.05$)) but all animals remained normoglycaemic (glucose – L: 4.6 ± 0.3 ; O: 5.9 ± 0.7 ; NRO: 5.3 ± 0.7 mmol/l).

Hepatic TAG concentration and histological analysis

Although juvenile-onset obesity alone had no effect on hepatic TAG content, this was significantly raised in obese offspring born to nutrient-restricted mothers (Fig. 1a). Hepatic lipid content, determined by Oil red O staining as an index of macrovesicular lipid infiltration, was only raised in obese animals born to control fed mothers (Fig. 1b). In contrast, microvesicular steatosis was observed in the NRO group (Fig. 2). This was confirmed by the raised Kleiner scores for both steatosis (L: 0; O: 1; NRO: 3) and ballooning (L: 0; O: 0; NRO: 1) (but not inflammation) that is in accord with the TAG analysis (Brunt 2007).

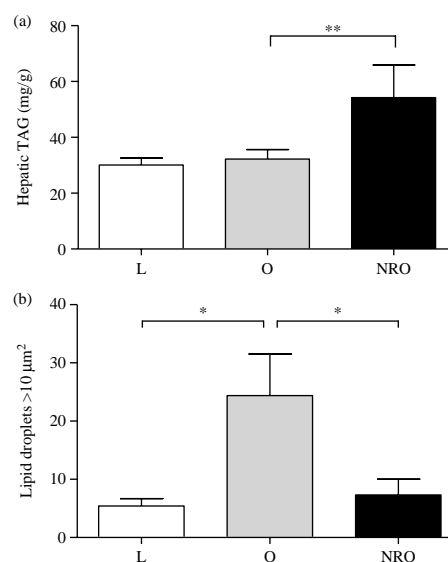


Figure 1 Effects of juvenile-onset obesity and early-to-mid gestational maternal nutrient restriction on hepatic (a) triglyceride content and (b) macrovesicular lipid infiltration as assessed by Oil red O staining. Values are expressed as means \pm s.e.m. for lean (L, $n=8$), obese (O, $n=8$) and offspring born to nutrient-restricted mothers during early-mid gestation (NRO, $n=9$) at 1 year of age. Significant effects of juvenile-onset obesity (i.e. L versus O) and prenatal nutrient restriction (O versus NRO) were assessed using Student's unpaired *t*-test: * $P < 0.01$, ** $P < 0.005$.

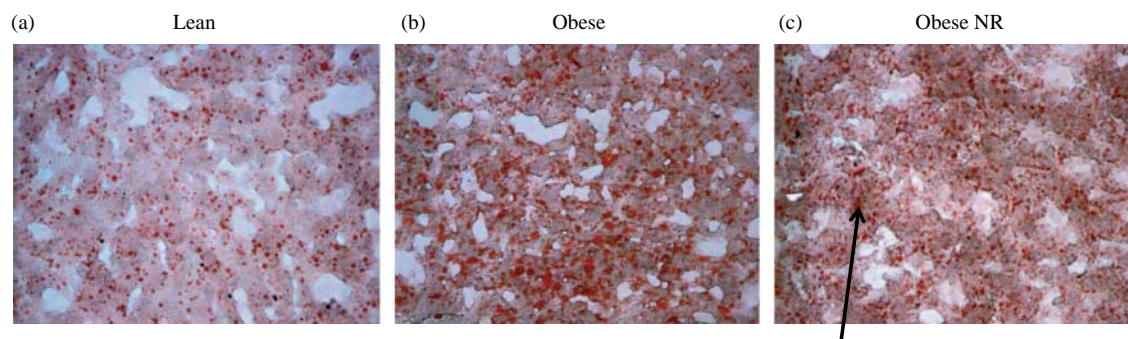


Figure 2 Effects of juvenile-onset obesity and early-to-mid gestational maternal nutrient restriction on hepatic macrovesicular lipid infiltration as assessed by Oil red O staining with typical examples of liver histology from each of the following groups (a) lean; (b) obese and (c) obese offspring born to nutrient-restricted (NR) mothers. Magnification is $\times 20$; note the macrovesicular lipid infiltration in the obese animals (b) compared with the microvesicular steatosis (as indicated by the arrow) as observed in the obese offspring born to nutrient-restricted mothers (c).

Hepatic gene expression

Following obesity, expression of genes that determine hepatic sensitivity to cortisol (i.e. the glucocorticoid receptor and 11β hydroxysteroid dehydrogenase (HSD) type 2) and glucose (i.e. phosphoenolpyruvate carboxykinase (PCK2 (PEPCK)) and glucokinase IV) were all significantly increased while insulin-responsive genes including insulin receptor (IR), IGF1 receptor (IGF1R), IGF-binding protein 3 (IGFBP3), GH receptor (GHR) and PPARA were all decreased irrespective of prenatal diet (Table 1).

Mitochondrial uncoupling protein 2 (UCP2) was also significantly increased in obese animals, a response that was unaffected by maternal diet during pregnancy. Maternal nutrient restriction followed by juvenile-onset obesity resulted in an upregulation of PPARG, its co-activator (PGC1 α) and IGFBP1 in adult livers of 1-year-old obese sheep. There was no effect of either

prenatal diet or postnatal obesity on hepatic mRNA abundance for energy-sensing AMP-related kinase (AMPK), insulin-sensitive adiponectin or inflammatory markers TNF and interleukin 6 (IL6; Table 1).

Discussion

We have shown that while exposure to a sedentary post-weaning obesogenic environment alone has no effect on hepatic TAG content, when this is preceded by prenatal nutrient restriction coincident with early organogenesis of the fetal liver, this is significantly raised by early adulthood. These findings are in accord with adverse adaptations previously established within adipose tissue (Sharkey *et al.* 2009b) and the heart (Chan *et al.* 2009). In addition, hepatic TAG accumulation was accompanied with microvesicular steatosis that is indicative of advanced steatosis and, therefore, NAFLD (Reddy & Sambasiva Rao 2006). Taken together, these findings

Table 1 Hepatic gene expression for lean (L, $n=8$), obese (O, $n=8$) animals and NR obese (NRO, $n=9$) at 1 year of age.

Metabolic pathways	mRNA	L	O	NRO	Juvenile-onset obesity	Prenatal restriction
Glucose metabolism	PCK2	1.0 \pm 0.1	2.7 \pm 0.9	4.4 \pm 1.1	*	NS
	Glucokinase IV	1.0 \pm 0.4	11 \pm 3.4	30 \pm 8.4	*	NS
Insulin sensitivity and growth	Insulin receptor	1.0 \pm 0.4	0.3 \pm 0.06	0.2 \pm 0.1	*	NS
	IGF1R	1.0 \pm 0.2	0.3 \pm 0.05	0.6 \pm 0.1	*	NS
	IGFBP1	1.0 \pm 0.3	1.1 \pm 0.2	2.7 \pm 0.4	NS	*
	IGFBP3	1.0 \pm 0.2	0.4 \pm 0.1	0.4 \pm 0.1	*	NS
	GHR	1.0 \pm 0.2	0.4 \pm 0.1	0.4 \pm 0.1	*	NS
Glucocorticoids and inflammation	Glucocorticoid receptor	1.0 \pm 0.2	3.8 \pm 1.1	2.9 \pm 0.5	†	NS
	HSD11B1	1.0 \pm 0.1	1.7 \pm 0.4	4.7 \pm 1.1	NS	*
	HSD11B2	1.0 \pm 0.1	2.0 \pm 0.2	4.6 \pm 1.0	*	*
	TNF (α)	1.0 \pm 0.3	1.0 \pm 0.4	1.2 \pm 0.5	NS	NS
	IL6	1.0 \pm 0.2	0.5 \pm 0.1	0.3 \pm 0.1	NS	NS
	PRKAA2	1.0 \pm 0.2	2.2 \pm 0.8	2.6 \pm 1.0	NS	NS
Lipid metabolism and mitochondrial bioactivity	PPARA	1.0 \pm 0.3	0.2 \pm 0.05	0.2 \pm 0.1	*	NS
	PPARG	1.0 \pm 0.7	2.2 \pm 0.6	6.3 \pm 1.2	NS	†
	PGC1 α	1.0 \pm 0.1	0.4 \pm 0.03	6.4 \pm 0.1	†	†
	UCP2	1.0 \pm 0.1	3.7 \pm 0.2	3.9 \pm 0.3	‡	NS

Significant effects of juvenile-onset obesity (i.e. L versus O) and prenatal nutrient restriction (O versus NRO) were assessed using Student's unpaired *t*-test: * $P<0.05$, † $P<0.01$, ‡ $P<0.005$.

support the 'two hit' hypothesis and offer the possibility of using the sheep as a model for investigating the pathogenesis of NAFLD. The lack of NAFLD in those obese animals born to normally fed mothers is likely to reflect the much longer duration of exposure to an obesogenic challenge required to produce these symptoms in offspring that have not been nutritionally programmed (Bruce *et al.* 2009). Importantly, the hepatic TAG content of 3% in lean sheep is in accord with lean adult humans (Szczepaniak *et al.* 2005), as is the doubling in TAG accumulation with NAFLD (Kleiner *et al.* 2005). This is despite the fact that, in sheep, *de novo* synthesis of NEFA is restricted to adipose tissue, which is, therefore, the only source of long-chain TAG (Ingle *et al.* 1972).

Obesity and hepatic mitochondrial function

Our study confirms that, as in humans, *PPARG* gene expression is normally minimal in the liver and was only raised in those offspring born to nutrient-restricted mothers in which lipid accumulation was occurring, indicative of the onset of NAFLD (Medina-Gomez *et al.* 2007). Previously, the significance of this adaptation has been overlooked in small animal models of liver disease as it is not consistently observed, apparently being confined to those models showing leptin resistance, i.e. *ob/ob* mice (Friedman *et al.* 1991). Upregulation of hepatic *PPARG* in obese offspring born to nutrient-restricted mothers could, therefore, be a consequence of raised TAG and/or an increase in *IGFBP1* mRNA abundance, which would, if translated into protein, reduce hepatic bioavailability of both IGF1 and 2 (Jones & Clemmons 1995). One consequence of this would be a decrease in cell proliferation and/or apoptosis in steatotic hepatocytes (Kmiec 2001).

Obesity, alone, induced a reduction in *PGC1 α* , an expected response that is normally associated with hyperglycaemia (Medina-Gomez *et al.* 2007). *PPARG* and its co-activator *PGC1 α* induce the expression of genes involved in adipogenesis and have a prominent role in mitochondrial β oxidation of NEFA (Medina-Gomez *et al.* 2007). Upregulation of both of these genes in the livers from offspring born to nutrient-restricted mothers may represent a compensatory mechanism to the increased fat within the liver. Conversely, decreased mRNA abundance of both *PGC1 α* and *PPARA* in obese sheep suggests a decrease in hepatic NEFA oxidation but this was not accompanied by any lipotoxic effects. Mice deficient in *PPARA* show a minimal steatotic phenotype under fed conditions but manifest an exaggerated steatotic response to fasting (Lee & Gonzalez 1996), indicating that defects in *PPARA*-inducible NEFA oxidation determine the severity of fatty liver phenotype in conditions reflecting energy-related stress. The severity of NAFLD would, therefore, be expected to progress faster with obesity in offspring born to

nutrient-restricted mothers. Despite an increased abundance of *UCP2* mRNA with obesity, indicative of increased reactive oxygen species production (Arsenijevic *et al.* 2000), *PRKAA2* (*AMPK*) mRNA remained unchanged and, therefore, would not provide a protective effect against oxidative stress, which is consistent with some, but not all, animal (Chavin *et al.* 1999) and clinical (Ribeiro *et al.* 2004) studies.

Obesity and hepatic glucose metabolism

In this study, although obesity was accompanied with hyperinsulinaemia all animals were able to remain normoglycaemic. Plasma insulin was, however, raised further in offspring born to nutrient-restricted mothers, which reflects a resetting of insulin-glucose homeostasis (Sebert *et al.* 2009). This response may be mediated in part through compromised pancreatic development (Jones & Ozanne 2009) in conjunction with a marked insulin resistance within the adipose tissue (Sharkey *et al.* 2009b). All obese sheep exhibited raised expression of glucose-sensitive genes including *GR* (*NR3C1*), *PCK2* and glucokinase IV. However, it is unclear whether this is due to increased cortisol sensitivity as *HSD11B* types 1 and 2 were both raised by the same amount and are likely to be mirrored by changes in activity (Whorwood *et al.* 2001). Therefore, it appears unlikely that hepatic sensitivity to cortisol was reset.

Adult obese sheep were able to maintain normoglycaemia despite increased transcription of gluconeogenic genes. The extent to which this adaptation is mediated by an increase in glycolysis, glycogen storage or increased uptake in glucose-sensitive tissues remains to be determined. However, increased glycogen storage seems unlikely due to the presence of raised plasma NEFA concentrations (Williams *et al.* 2007, Sebert *et al.* 2009), which would be predicted to decrease glucose conversion into glycogen for storage (Melania *et al.* 2004). Regardless of the prevailing plasma glucose concentration, lipid influx and *de novo* synthesis in NRO sheep exceeds hepatic lipid export and utilisation, thereby resulting in the storage of excess glucose as intrahepatic TAG. Increased glucokinase gene expression and TAG deposition in NRO animals were accompanied by the formation of microvesicular lipid droplets within the hepatocytes. This is an indication that macrovesicles have burst and is, therefore, a marker of the severity of NAFLD (Reddy & Sambasiva Rao 2006) in these animals. In order to clarify this, further studies should investigate the effects on signalling molecules (Jump *et al.* 2005) that act by the transcription factors hepatocyte nuclear factor 4 (HNF4) and sterol regulatory element-binding protein-1c (SREBF1 (SREBP-1c)) to regulate hepatocyte differentiation and overall lipid metabolism (Jump *et al.* 2005, Hayhurst *et al.* 2008). Modification of both HNF4 and SREBF1 has been found associated with obesity-related diseases and may explain

the link between insulin resistance and lipid-related alterations (Ueki *et al.* 2004). Their influence on NAFLD as yet to be uncovered but their unique ability to use NEFA as co-factors raises the question as to whether increase fat deposition in the liver promotes alteration upon the activity of the factors thus altering lipid metabolism.

A further finding of this study is that insulin-sensitive genes within the liver including *IR*, *GHR* and *IGF1R*, *IGFBP3*, *PPARA* and *PGC1 α* were downregulated following juvenile-onset obesity despite the absence of TAG accumulation. Insulin resistance is an important mediator of hepatic TAG accumulation (Day 2002), promoting lipolysis in adipose tissue and influx of NEFA into the liver. The hyperinsulinaemia that accompanied obesity is, therefore, likely to have promoted *de novo* lipogenesis as well as indirectly inhibiting NEFA oxidation (Azzout-Marniche *et al.* 2000). This was potentially mediated by the decrease in both *PPARA* and *PGC1 α* gene expression. The exacerbated insulin response, in conjunction with reduced expression of the *IR*, seen in obese offspring born to NR mothers is predicted to promote insulin resistance (Sanderson & Smyrk 2005) and is likely to be an important contributory factor to the onset of NAFLD. Taken together, our findings suggest that, with time, these animals would become metabolically unstable, ultimately leading to hyperglycaemia, oxidative stress and the development of hepatic fibrosis.

Obesity and hepatic inflammation

Cytokines such as IL6 and TNF, produced by the liver, are established to play a role in the pathogenesis of NAFLD (Kmiec 2001, Jarrar *et al.* 2008), a process that has been suggested to be ameliorated by adiponectin (Kmiec 2001). In our sheep model, however, we did not observe an associated local hepatic inflammatory response as seen in more advanced clinical cases (Larter & Farrell 2006, Tessari *et al.* 2009). The absence of inflammation is not unexpected, as adult clinical studies of NAFLD have also demonstrated a limited degree of hepatic inflammation (Brunt *et al.* 2009). We can now use this model to follow the more progressive disorders associated with NAFLD, which may be specific to fibrosis and the onset of non-alcoholic steatohepatitis (Larter & Farrell 2006, Larter & Yeh 2008). These studies could be combined with an assessment of the changes in cytokine receptor expression, which are likely to have an important role in the pathogenesis of NAFLD.

In conclusion, a combination of *in utero* nutrient restriction targeted during the period of early liver development is necessary to promote ectopic lipid accumulation within the liver following obesity. This developmental model of an early life nutritional intervention is confined to changes in the amount of feed consumed, rather than composition, now offers

the potential to further understand developmental influences on fatty acid composition and liver enzymes with respect to fatty acid oxidation, lipid partitioning, fibrosis and on both the onset and severity of NAFLD.

Materials and Methods

Experimental animals and tissue collection

The experimental design of this study has been published previously (Williams *et al.* 2007, Chan *et al.* 2009, Seibert *et al.* 2009). Briefly, 25 twin-bearing sheep of a similar weight and body condition were randomly assigned to receive either a control (~7–8 MJ/day of metabolisable energy, $n=16$) or nutrient-restricted diet (50% of control, $n=9$) from 30 to 80 days of gestation (term 147 d) and were fed to requirements at all other times (~12–13 MJ/day). Offspring were delivered spontaneously and reared by their mothers as singletons (one twin was killed on day 7) until weaning (10 weeks) with no mothers producing twins of discordant body weight. There was no difference in growth rates between the groups either over the first week of life or subsequently up to the time of weaning. Then after weaning, control offspring were randomly assigned to either a lean (L; field ($n=8$)) or an obesogenic (O; group housed in a barn 50 m², i.e. restricted activity ($n=8$)) environment with the ratio of male to females being 1:2. NR offspring were also exposed to an obesogenic environment (i.e. NRO). Obese and NRO offspring had access to hay and concentrate pellets (crude protein 140 g/kg, oil 3%, 12.7 MJ/kg dry matter; Manor Farm Feeds, Oakham, UK) *ad libitum* in a restricted physical activity environment to promote increased fat deposition and juvenile-onset obesity. Obese offspring had ~30% increase in food intake and ~65% reduction in physical activity, as described previously (Williams *et al.* 2007). In contrast, lean animals remained at pasture and had unrestricted physical activity. Adult offspring were humanely killed by electrocortical stunning and exsanguination at 1 year of age to enable liver tissue sampling. After weighing, a representative liver sample from the right hepatic lobe was flash frozen in liquid nitrogen and stored at –80 °C until analysis was undertaken, in duplicate, for each animal. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and approved by the local ethics committee of the University of Nottingham.

Molecular and biochemical analyses

Lipid extraction and TAG assay

Lipids were extracted from frozen liver tissue (~400 mg) with a mixture of chloroform and methanol (1:1) and dissolved in tert-butyl alcohol/Triton X-100 mixture (1/1 by volume) before enzymatic measurement (Infinity Triglycerides Liquid Stable Reagent; Thermo Electron, Worthing, UK; Folch *et al.* 1957, Danno *et al.* 1992).

Histological analyses

Approximately, 1 cm³ blocks of frozen liver tissue were cut from the centre of the right hepatic lobe, embedded in

optimum cutting temperature compound (OCT; Tissue-Tek; Miles, Naperville, IL, USA) and stored at -80°C until being processed. Frozen OCT liver tissue was cut into 10 μm serial sections using a cryostat (Bright OTF model cryostat, specimen temperature set at -21°C). Three random liver sections per animal were fixed in 3.7% formaldehyde, stained with haematoxylin–eosin and imaged with a Leica DMRB microscope connected to Hamamatsu-Open Lab imaging system under $\times 20$ and $\times 40$ magnification lenses to assess liver architecture and density of voids resulting from lipid infiltration.

Oil red O staining for assessment of intracellular TAG content and assessment of severity of NAFLD

Oil red O staining was used for quantitative determination of accumulated TAG in hepatocytes. Briefly, hepatic cryosections were fixed for 1 h in 3.7% formaldehyde, incubated in a 37% Oil red O solution for 30 min and washed with distilled water. Sections were counterstained with Harris's haematoxylin for 1 min, washed with hydrochloric acid (0.1%) for 30 s, air dried and mounted with 10% glycerol in Tris-buffered saline solution. The staining procedure was repeated for three separate sections per animal to obtain representative results. Once stained, sections were imaged as above and quantified by histomorphometry using Volocity software (Improvision, PerkinElmer, Coventry, UK). The slides were analysed three times at $\times 40$ magnification under a light microscope (Leica Microsystems, Milton Keynes, UK) for Oil red O staining. The average surface area of stained lipid droplets was calculated. Stained cryosections were further analysed for macro- and microvesicular steatosis. The extent of macrovesicular steatosis, ballooning and lobular inflammation was graded based on the Kliener scoring system (Kleiner *et al.* 2005). The intra- and inter-assay coefficients of variances were <5 and 10% respectively. All histological analyses were undertaken while blinded to each intervention group.

Total RNA isolation and RT

Total RNA was extracted from ~ 500 mg of frozen liver tissue using Tri-Reagent (Sigma). Total RNA samples were treated for potential genomic DNA contamination with DNase 1 (Promega Ltd) and their A260/A280 ratio was assessed to confirm purity and concentration. cDNA was synthesised from 3 μg RNA using 200 U Superscript II (Invitrogen Ltd) by RT in accordance with the manufacturer's protocol. Standard curves comprising a 1 in 10 dilution of primer-specific gel-purified amplicon: $1-10^{-8}$ ng/ μl was used to ensure PCR amplification efficiency (1.95–2.0) as described previously (Chan *et al.* 2009); *18S* rRNA was used as a housekeeping gene and was not found to be different between any of the study groups. All results were then calculated using the $2^{-\Delta\text{Ct}}$ method (Livak & Schmittgen 2001).

Quantitative real-time PCR analyses

The relative abundance of mRNA transcripts for each gene together with *18S* was determined by quantitative PCR amplification, using a real-time thermocycler (Quantica,

Techne Incorporated, Barloword Scientific Ltd, Stone, UK), Quantitect SYBR green PCR kit (Qiagen Ltd), diluted RT reactions and 10–15 pmol of the forward and reverse primers as previously described (Chan *et al.* 2009). Primer sequences have been previously published (Bispham *et al.* 2005, Muhlhausler *et al.* 2007, Williams *et al.* 2007, Sebert *et al.* 2009) with the exception of *GHR* (F: AAG CCT GGA AAC CAT ACG, R: TGC CAC TGC CAA GGT CAA C), IGF1 receptor (*IGF1R*) (F: TCT AAC TTT GTC TTT GCA AGA A, R: TCA CTG GCC CAG GAA ATG TC), glucokinase IV (F: GAC ATT GAC AAG GGC ATC CT, R: GTG GCC ACA GTG TCA TTC AC), IGF-binding protein 1 (*IGFBP1*) (F: GAT TAG CCA GGG AGC AGC AGA AG, R: CCG TCC AGC GAA GTC TCA CAC) and *IGFBP3* (F: TCC AAG CAT GAG ACA GAA TAC GG, R: TTA TCC ACA CAC CAG CAG AAA CC). Real-time PCR conditions were 95 $^{\circ}\text{C}$ (15 min); 35–40 cycles of 94 $^{\circ}\text{C}$ (45 s), 60 $^{\circ}\text{C}$ (30 s), 72 $^{\circ}\text{C}$ (45 s); 72 $^{\circ}\text{C}$ (15 min) for all primer sets. Gene expression data were normalised to the lean group (assigned as 1.0) and are, therefore, presented as a fold change.

Statistical analysis

Normality of data was assessed using the Kolmogorov–Smirnov test, using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) and log transformed where necessary. Hepatic lipid content and gene expression data were first analysed by a univariate general linear model (GLM) procedure with the treatment group as a fixed factor. Owing to the unbalanced design of the study, offspring gender was added as a covariate. Selected *a priori* comparisons to test for the effects of prenatal nutrition (O versus NRO) and postnatal obesity (L versus O) were performed using Student's unpaired *t*-tests. Data are expressed as mean values with their standard errors. For all comparisons, following Bonferroni correction, statistical significance was accepted when a probability of 5% was observed ($P < 0.05$).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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