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Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Sex-specific alterations in hepatic cholesterol metabolism in low birth weight adult guinea pigs

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BACKGROUND: Intrauterine growth restriction and low birth weight (LBW) have been widely reported as an independent risk factor for adult hypercholesterolaemia and increased hepatic cholesterol in a sex-specific manner. However, the specific impact of uteroplacental insufficiency (UPI), a leading cause of LBW in developed world, on hepatic cholesterol metabolism in later life, is ill defined and is clinically relevant in understanding later life liver metabolic health trajectories.
METHODS: Hepatic cholesterol, transcriptome, cholesterol homoeostasis regulatory proteins, and antioxidant markers were studied in UPI-induced LBW and normal birth weight (NBW) male and female guinea pigs at 150 days.
RESULTS: Hepatic free and total cholesterol were increased in LBW versus NBW males. Transcriptome analysis of LBW versus NBW livers revealed that "cholesterol metabolism" was an enriched pathway in LBW males but not in females. Microsomal triglyceride transfer protein and cytochrome P450 7A1 protein, involved in hepatic cholesterol efflux and catabolism, respectively, and catalase activity were decreased in LBW male livers. Superoxide dismutase activity was reduced in LBW males but increased in LBW females.
CONCLUSIONS: UPI environment is associated with a later life programed hepatic cholesterol accumulation via impaired cholesterol elimination in a sex-specific manner. These programmed alterations could underlie later life cholesterol-induced hepatic lipotoxicity in LBW male offspring.

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IMPACT:

- Low birth weight (LBW) is a risk factor for increased hepatic cholesterol.
- Uteroplacental insufficiency (UPI) resulting in LBW increased hepatic cholesterol content, altered hepatic expression of cholesterol metabolism-related genes in young adult guinea pigs.
- UPI-induced LBW was also associated with markers of a compromised hepatic cholesterol elimination process and failing antioxidant system in young adult guinea pigs.
- These changes, at the current age studied, were sex-specific, only being observed in LBW males and not in LBW females.
- These programmed alterations could lead to further hepatic damage and greater predisposition to liver diseases in UPI-induced LBW male offspring as they age.

INTRODUCTION

Cholesterol is a critical biological molecule, acting as a precursor for the synthesis of steroid hormones, bile acids, and vitamin D, and also being critical as a central modulator of cell membrane proteins, receptor trafficking, signal transduction, and cell membrane fluidity.[1] In mammals, the liver is the central organ regulating cholesterol homoeostasis through it actions in cholesterol uptake, export, conversion into bile acids, biosynthesis, and storage.[2–7] When cholesterol homoeostasis is disrupted, large concentrations of cholesterol accumulate in the liver resulting in a lipotoxic state associated with oxidative stress and can culminate in nonalcoholic steatohepatitis (NASH).[8–14]

An increase in the consumption of dietary cholesterol and/or genetic susceptibility to hypercholesterolaemia can underlie impaired cholesterol homoeostasis, cholesterol accumulation, and a lipotoxic state in the liver.[10,15,16] However, it is now becoming apparent that insults early in the life cycle, such as in prenatal life can also contribute, in a sex-specific manner, to aberrant cholesterol metabolism and hepatic cholesterol overload in adulthood. Animal studies have indicated that a wide variety of experimental in utero insult/stress situations (maternal protein undernutrition, dietary restriction, hypoxia, or prenatal nicotine) resulting in intrauterine growth restriction (IUGR)/Low Birth Weight (LBW) lead to increased serum and/or hepatic cholesterol in weaned and adult male rat offspring.[17–20] Transcriptome analysis in the liver of prenatal maternal food restricted-adult IUGR rat offspring revealed that activated cholesterol biosynthesis and down-regulated bile acid biosynthesis pathways are main targets for intrauterine reprogramming.[21] Studies have highlighted that IUGR models such as maternal protein undernutrition,

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bilateral uterine vessel ligation, or intrauterine stress induced via 'sham' surgery, yield different phenotypes and specific transcriptional patterns in organs such as placenta and kidney.[22,23] Therefore, understanding in utero insult-specific mechanisms is key to identifying both conserved pathways and model-specific alterations which increase our understanding and applicability of how these modifications in organ systems and phenotype manifest. Collectively, this will allow for development of better-informed target interventions.

Observational human studies have reported that impaired in utero growth and the resulting LBW are associated with differentially increased serum concentrations of total and low-density lipoprotein (LDL) cholesterol in men and women in later life with sex difference by age groups in adults.[24–29] Although, Uteroplacental Insufficiency (UPI) is the leading cause of IUGR/LBW in the developed world,[30,31] investigations attempting to understand the specific impact of UPI-induced IUGR/LBW on later life hepatic cholesterol metabolism and cholesterolemia are currently limited to rat models.[32,33] New reports have begun to examine the molecular pathways that may underly the association between abnormal foetal growth in utero and later life cholesterol metabolism dysregulation in humans, but to date they are limited to the analysis of cord blood plasma of IUGR at birth,[34] with limited follow-up on hepatic cholesterol metabolism and associated pathways.

In the present study, we sought to unravel the hepatic metabolic pathways underlying the specific impact of UPI on hepatic cholesterol metabolism in young adult female and male LBW offspring. We have chosen to use the well-established preclinical animal model of UPI-induced IUGR/LBW in guinea pigs,[35] especially given the close similarities between humans and guinea pigs with respect to uteroplacental development and function[36] and later life hepatic and whole-body trafficking and processing of cholesterol.[37] With the use of transcriptome analysis, we postulated that LBW offspring, in a sex-specific manner, would display increased hepatic cholesterol by young adulthood in association with alterations in key regulators of cholesterol metabolism.

METHODS

Animals

All animal procedures were conducted in accordance with guidelines and standards of the Canadian Council on Animal Care. Animal Use Protocol (AUP-#2009-229) was approved and post approval monitoring conducted by the Western University Animal Care Committee. All investigators understood and followed the ethical principles outlined by Grundy,[38] and study design was informed by ARRIVE guidelines.[39]

Time-mated pregnant Dunkin-Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were housed in 12 h light and dark cycles in a temperature (20 ± 2 °C) and humidity (30–40%)-controlled environment, with access to guinea pig chow (LabDiet diet 5025) and tap water ad libitum. All pregnant guinea pigs underwent uterine artery ablation[35,40] at mid-gestation (~32 days, term ~69 days) and sows delivered spontaneously. At the end of the experimental pupping period, male and female pups were defined as normal birth weight (NBW) or low birth (LBW) as per previously reported criteria.[40,41] At weaning (postnatal day 15), pups were housed individually in clear perplex containers and fed ad libitum a normal diet (TD.110239; Harlan Laboratories, Madison, WI) until young adulthood (postnatal day 150). Only one LBW and NBW pup of each sex, from a single litter, was used in order to minimize compounding effects of litter. At postnatal day (PND) 150, NBW ($n = 8$) and LBW ($n = 8$) offspring of each sex were fasted overnight, euthanized via CO_2 inhalation[42] in the morning (~10 a.m.) of the following day. The whole liver was removed and weighed immediately and the right lobe liver was frozen in liquid nitrogen and stored at −80 °C for later biochemical and molecular analyses.

Hepatic biochemical analysis

Hepatic triglycerides were measured using 50 mg of frozen liver and following the Triglyceride Colorimetric Assay Kit (Item No. 10010303; Cayman Chemical, Ann Arbor, MI). Liver content of total cholesterol, free cholesterol, and cholesteryl esters was measured as previously described.[43,44] Enzymatic reagents for total cholesterol (WAKO Diagnostics: Cholesterol E (CHOD-DAOS method) #439-17501) and free cholesterol (WAKO Diagnostics: Free cholesterol (COD-DAOS) method #435-35801) were used as per the manufacturer's instructions. Cholesteryl ester was determined as the difference between total cholesterol and free cholesterol.

RNA isolation

Total RNA for microarray analysis was isolated from a subcohort of five NBW and five LBW livers of each sex at the Genome Québec Innovation Centre (Montreal, QC, Canada) while independent validation of microarray data was performed using RNA extracted from all NBW and LBW animals of the experiment, including the microarray cohort. For the microarray analysis, 50 mg of frozen liver was homogenized in 1 mL of Trizol (Invitrogen, Burlington, ON, Canada). The homogenate was treated with 100 µL of gDNA eliminator solution and then shaken vigorously and maintained at room temperature for 2–3 min. The homogenate was centrifugated at 12,000*g* for 15 min at 4 °C. RNA in the aqueous phase was then extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Toronto, ON, Canada). Total RNA for validation of microarray data was isolated from frozen ground liver (100 mg) using Trizol reagent and the Purelink RNA Mini Kit according to the manufacturer's instructions (Invitrogen). The quantity and quality of extracted RNA was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. RNA samples with RNA Integrity Number (RIN) above 7 were considered of high integrity and considered for microarray and quantitative reverse transcription PCR (RT-qPCR) analyses.[45]

RNA labelling, microarray hybridization, scanning, and processing

A Whole-Transcript Expression Analysis Gene Titan was conducted (Genome Québec Innovation Centre). Sense-strand cDNA was synthesized from 100 ng of total RNA, and fragmentation and labelling were performed to produce ss-cDNA with the GeneChip® WT Terminal Labeling Kit according to the manufacturer's instructions (Thermo Fisher Scientific). After fragmentation and labelling, 2.1 µg cDNA was hybridized onto Guinea Pig Gene 1.1 ST Array Plate (Thermo Fisher Scientific) and processed on the GeneTitan® Instrument (Thermo Fisher Scientific) for Hyb-Wash-Scan automated workflow. The microarray scanned images were imported into the Transcriptome Analysis Console (TAC) (version 4.0) (Life Technologies, Carlsbad, CA) and the raw microarray data (.CEL files) were normalized using the Robust Multiple-Array Averaging (RMA) method. Differentially expressed genes were identified using the TAC Expression (Gene) analysis method. Microarray data have been deposited in the National Center for Biotechnology information Gene Expression Omnibus (GEO; accession number GSE161124).

Quantitative reverse transcription PCR

Independent validation of microarray data was performed by examining levels of selected differentially expressed transcripts using RT-qPCR as previously described.[46] Detailed information for primer sequences is provided in Supplementary Table 1. Gene expression analysis was performed with the CFX Maestro software (Bio-rad, Mississauga, ON, Canada) using the $2^{-\Delta\Delta Ct}$ method and GAPDH and beta actin as reference genes. GAPDH and beta actin were classified as acceptable reference genes by GeNorm algorithm

Table 1. Phenotypic traits of the offspring.

	Males		Females		*P* value		
Characteristics at birth	NBW	LBW	NBW	LBW	BW	Sex (S)	BW × S
Birth weight (BW; g)	110.43 ± 2.56	79.04 ± 1.30****	105.75 ± 4.28	69.50 ± 2.81****	**<0.0001**	0.318	0.710
BPD (mm)	25.18 ± 1.74	21.53 ± 0.44*	21.13 ± 0.65	19.61 ± 0.57	**0.008**	**0.003**	0.240
AC (mm)	108.80 ± 5.78	106.71 ± 4.09	122.83 ± 1.92	110.00 ± 7.37	0.170	0.130	0.310
CRL (mm)	148.40 ± 11.66	137.86 ± 4.21	145.33 ± 7.42	131.03 ± 7.20	0.090	0.509	0.935
Length (mm)	181.96 ± 17.09	178.24 ± 4.52	193.35 ± 6.81	166.43 ± 12.68	0.223	0.656	0.882
BW: length	0.641 ± 0.08	0.441 ± 0.01***	0.555 ± 0.02	0.336 ± 0.01***	**<0.0001**	**0.007**	0.778
Lee index	0.212 ± 0.03	0.145 ± 0.00**	0.183 ± 0.01	0.142 ± 0.01**	**<0.0001**	0.180	0.967
Characteristics at postnatal day (PND) 150							
Body weight (g)	802.20 ± 46.63	737.75 ± 32.57	652.75 ± 27.25	5.99.86 ± 33.04	0.111	**<0.001**	0.872
Liver weight (g)	30.85 ± 2.27	28.85 ± 2.17	20.90 ± 1.30	19.78 ± 0.94	0.393	**<0.001**	0.807
Liver:body weight	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.712	**0.011**	0.512
Free cholesterol (mg/g wet liver)	2.48 ± 0.13	3.05 ± 0.18*	1.89 ± 0.17	2.30 ± 0.19	**0.005**	**<0.001**	0.156
Cholesteryl ester (mg/g wet liver)	0.43 ± 0.06	0.74 ± 0.17	0.58 ± 0.17	0.65 ± 0.13	0.175	0.827	0.402
Total cholesterol (mg/g wet liver)	2.90 ± 0.13	3.79 ± 0.29*	2.44 ± 0.32	2.96 ± 0.27	**0.012**	**0.020**	0.485
Triglycerides (mg/g wet liver)	0.03 ± 0.00	0.039 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.143	0.721	0.896

Birth weight (BW), biparietal diameter (BPD), abdominal circumference (AC), crown–rump length (CRL), naso-anal length, BW:naso-anal length and Lee index (BW*0.33:naso-anal length) at birth, body and liver weights, hepatic content of different cholesterol types and triglycerides at postnatal day 150 in male and female offspring are displayed. $n = 8$ NBW and 8 LBW for each sex group with the exception of BPD, AC, CRL, naso-anal length, BW:naso-anal length ratio, and Lee index where $n = 5$–7 for each birth weight/sex group. All data were checked for normality using a Shapiro–Wilk test. Data that were not normally distributed, including birth weight, AC, naso-anal length, CRL, Lee index, liver:body ratio, and free cholesterol, were Box–Cox-transformed prior to ANOVA. Displayed data are mean ± SEM of untransformed data. The main effect of birth weight (BW), sex and BW and sex interaction, using a two-way ANOVA, are displayed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ when comparing NBW/Males versus LBW/Males or NBW/females versus LBW/females by Bonferroni post hoc test.
Bolded *p* values indicate statical significance.

in the CFX Maestro software with a calculated expression stability value (*M*) of 0.57 for both genes. Threshold (Ct) values were also consistent between the NBW and LBW groups.

Immunoblot analysis

Proteins were extracted from frozen ground liver (50 mg) in radioimmunoprecipitation assay buffer as previously described.[46] Equal amounts of total proteins (20 µg) were separated on a 7.5 or 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were then blocked with 5% non-fat milk in 0.1% TBS-Tween-20 and probed with primary and secondary antibodies (Supplementary Table 2). The chemiluminescence signal was captured with the ChemiDoc MP Imaging System (Bio-Rad), and protein band densitometry was determined using the Image Lab software (Bio-Rad). Ponceau staining was used as the loading control, as previously published.[47,48]

SOD, CAT, GSH, and GSSG assays

Superoxide dismutase (SOD; kit no. 706002, Cayman Chemical) and catalase (CAT; kit no. 707002, Cayman Chemical) enzyme activities, reduced (GSH), and oxidized (GSSG) glutathione (kit no. 703002, Cayman Chemical) in livers were measured following the kit instructions.

Statistical analysis

Non-microarray data. A Shapiro–Wilks test was used to determine if data were normally distributed. The few of the data sets that did not pass the normality test and comparing four experimental groups (birth weight, abdominal circumference, CRL, naso-anal length and Lee index at birth, liver:body weight ratio and free cholesterol at PND 150) were thus Box−Cox-transformed before ANOVA. A two-way ANOVA was used to determine the main effect of birth weight, sex, and possible interactions, followed by Bonferroni post hoc test using GraphPad 8 (GraphPad Software, San Diego, CA). When comparing NBW and LBW only, a Student's *t*-test was used for normally distributed and a Mann–Whitney was performed on non-normally distributed data. Data are presented as mean ± SEM and a probability of 5% ($p < 0.05$) was considered significant for all analyses.

Microarray data. Only genes that met the criteria of |fold change|≥2 and *p* value <0.05 were defined as differentially expressed. To explore biological processes and pathways associated with the differentially expressed genes, we performed gene ontology (GO) biological process term enrichment (adjusted $p < 0.05$) using *human* as target organism and the g:GOSt functional profiling in g:Profiler web server (https://biit.cs.ut.ee/gprofiler/gost).

RESULTS

Phenotypic traits

By using diathermy partial ablation of branches of the uterine artery, UPI was induced to generate LBW, as described previously.[35] Those pups that at birth were <85 g, were classified as LBW and as a group weighed less than sex-matched NBW pups ($p < 0.0001$; Table 1). At birth, crown–rump length (CRL) tended to decrease ($p < 0.090$) and biparietal diameter (BPD) as well as body weight over naso-anal length (BW:length) decreased ($p < 0.01$) in LBW offspring (Table 1). In addition, at birth, Lee index (body weight × 0.33:naso-anal length), a measure of fatness,[49] decreased in both male and female LBW offspring ($p < 0.01$; Table 1). At the time of tissue collection (PND 150; Table 1), LBW and NBW offspring had similar mean body and absolute and relative liver weights within each sex group. However, at this age, females displayed overall lower body, absolute and relative liver weights than males, independent of the birth weight ($p < 0.05$; Table 1). Male offspring overall exhibited higher hepatic free cholesterol and total cholesterol than females ($p < 0.01$; Table 1). Interestingly, hepatic-free cholesterol and total cholesterol contents were

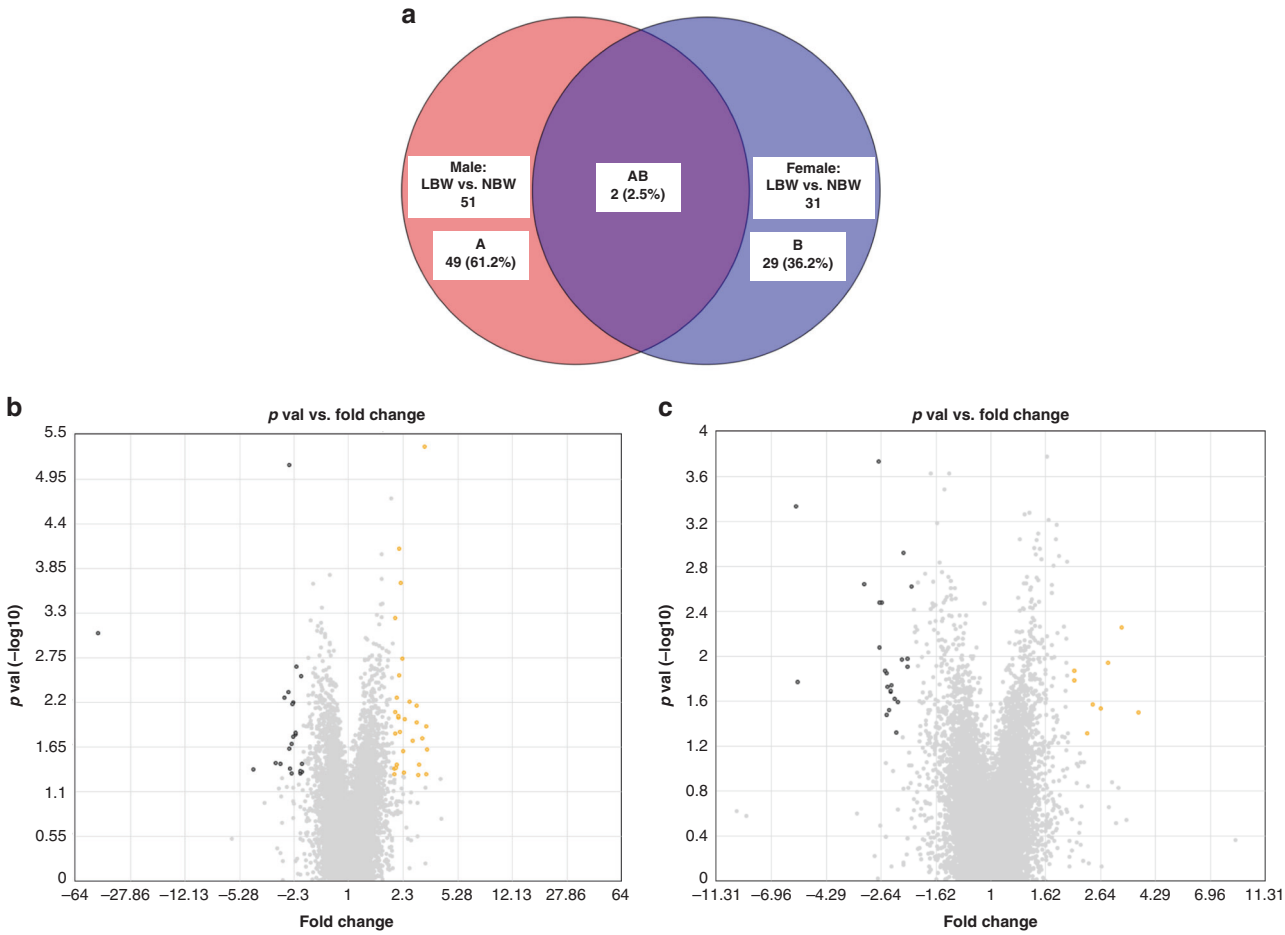

Fig. 1 Differential gene analysis, volcano plots, and hierarchical clustering chart/heat maps. a Venn diagram demonstrating differentially up- and down-regulated genes in five NBW versus five LBW offspring. Volcano plots were generated to visualize the differentially expressed genes (DEGs) between LBW and NBW in males (**b**) and females (**c**). The *x*-axis indicates the fold change and the *y*-axis represents the log10 *p* values. The orange and red points indicate the up-regulated and down-regulated mRNAs with statistical significance, respectively.

increased in LBW males compared to NBW males ($p < 0.01$; Table 1). No significant difference was observed for hepatic-free cholesterol and total cholesterol content in NBW or LBW females (Table 1). Birth weight or sex at PND 150 had no impact upon hepatic cholesteryl ester and triglyceride contents (Table 1).

Hepatic transcriptome

Analysis of whole hepatic transcriptome results identified that between LBW and NBW offspring livers, 51 (29 up-regulated and 22 down-regulated), and 31 (8 up-regulated and 23 down-regulated) genes were differentially expressed (|fold change| ≥ 2 and *p* value <0.05) in males and females respectively (Fig. 1 and Supplementary Tables 3 and 4). The Venn diagram (Fig. 1a), the volcano plots (Fig. 1b, c), and 2D hierarchical clustering chart/heat maps (Fig. 2a, b) indicate the degree of separation among the LBW and NBW groups in males and females. Only two genes encoding thioredoxin pseudogene and small nucleolar RNA SNORA2/SNORA34 family transcripts were differentially expressed between LBW and NBW in both male and female offspring (Fig. 1a).

Functional analysis of the hepatic transcriptomic profile and validation of microarray data

In the biological process analysis of the differentially expressed genes in LBW versus NBW males, 18 enriched GO biological processes were observed, with positive regulation of hepatic fatty acid metabolism process, lipid transport, lipid localization, regulation of fatty acid biosynthetic process and positive regulation of lipid metabolic process being the five top ranked processes (Fig. 3a and Table 2). No significant changes to GO biological processes were observed in LBW females (Fig. 3b). The KEGG functional enrichment analysis identified that cholesterol metabolism was a significantly enriched pathway in LBW males (adjusted $p < 0.05$, Fig. 3a). Specifically, in LBW males, the reverse cholesterol transport-related genes (*Apoa1* and *Angplt4)* were up-regulated while *Ldlr* gene associated with the internalizing of circulating LDL cholesterol was down-regulated (Table 2 and Supplementary Table 3).

The PPAR signalling pathway was also an up-regulated pathway in LBW versus NBW offspring (adjusted $p < 0.05$, Fig. 3a). This latter pathway included *Adipoq* in addition to *Apoa1* and *Angplt4* (Table 2 and Supplementary Table 3). FoxO signalling pathway was the only significantly enriched pathway for differentially expressed genes in LBW females (adjusted $p < 0.05$, Fig. 3B). This pathway included down-regulated *Bcl6* and *Gadd45g* (Table 2 and Supplementary Table 4).

Genes involved in cholesterol metabolism, PPAR and FoxO signalling pathways and other differentially expressed genes not related to these pathways were selected to be validated by RT-qPCR independent RNA samples (Fig. 4). *Apoa1* gene was increased 4.76-fold ($p = 0.037$) in LBW relative to NBW males, similar to the fold change in the microarray data of 2.92-fold ($p = 0.037$). *Angplt4* was also confirmed to be increased by 3.7 ($p = 0.023$) similar to microarray fold change of 2.84 and 1.2 ($p = 0.011$). In LBW versus

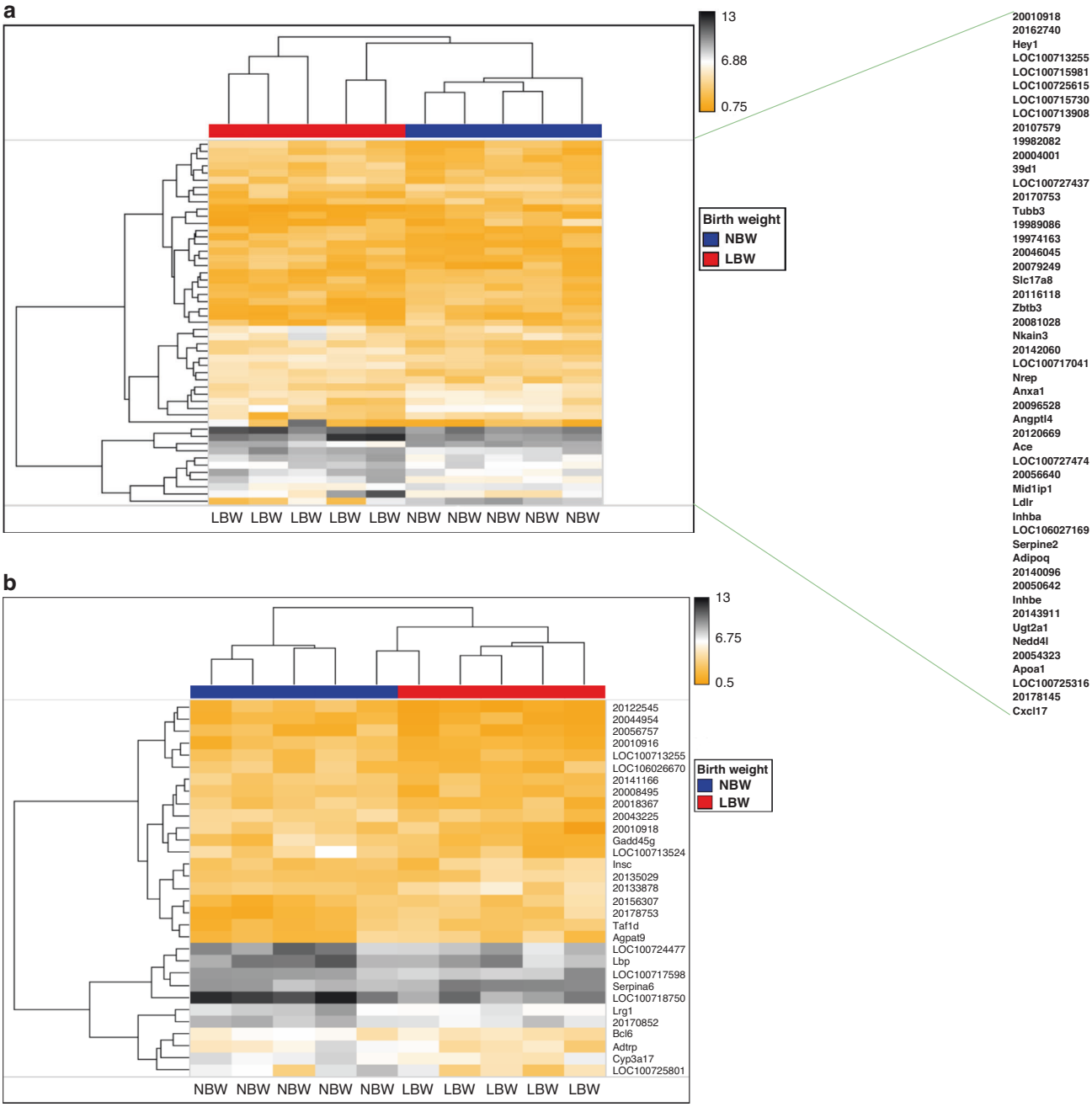

Fig. 2 Heat maps looking at differential changes of genes. Hierarchical clustered heat maps of DEGs between LBW and NBW in males (**a**) and females (**b**). Each column represents one sample and value to the right of colour scale indicates the (log2) gene expression. The intensity of the colour indicates the expression level, with black representing a high expression level and orange representing a low expression level. Gene symbols are indicated on the right of the heat maps.

NBW males, *Ldlr* and *Gstt2* genes were also confirmed to be decreased by −2.16 and −3.05-fold ($p = 0.008$ and 0.020) and similar to microarray fold changes of −2.39 and −2.44, respectively ($p = 0.021$ and 0.041). *Inhba*, which was −2.82-fold lower in the microarray data ($p = 0.036$), was relatively lower in the LBW male livers by 2.89-fold ($p = 0.061$). Although not significant, *Anxa1*, which was 2.18-fold higher in the microarray data ($p = 0.015$), was relatively higher in the LBW male livers by 1.72-fold ($p = 0.721$). *Lbp* was significantly lower by −2.28-fold in the LBW female livers in the validation cohort ($p = 0.038$), similar to the −2.96-fold decreased in the LBW microarray female cohort ($p = 0.026$). Lastly, while down-regulated in LBW females in the microarray data, *Lcn2* tended to decrease in the validation cohort ($p = 0.105$).

Hepatic content of proteins related to cholesterol metabolism

Selected proteins were studied on a priori based on their known biological role in hepatic cholesterol metabolism. The levels of hepatic proteins involved in cholesterol uptake (Ldlr, Apoe), biosynthesis (Srebp2), catabolism (Cyp7a1), efflux into bile acids (Abcg8), and export to blood (Fas, Acc, Abca1, and Mtp) were determined in LBW and NBW livers (Fig. 5). Female livers displayed significantly higher levels of Ldlr and Srebp2 protein than males ($p < 0.01$). Neither birth weight nor sex impacted Apoe protein. Females displayed lower Mtp and Acc protein levels than males ($p < 0.01$) and Fas protein levels were not affected by birth weight nor sex. Cyp7a1 and Mtp protein levels were both reduced in male LBW compared to male NBW livers.

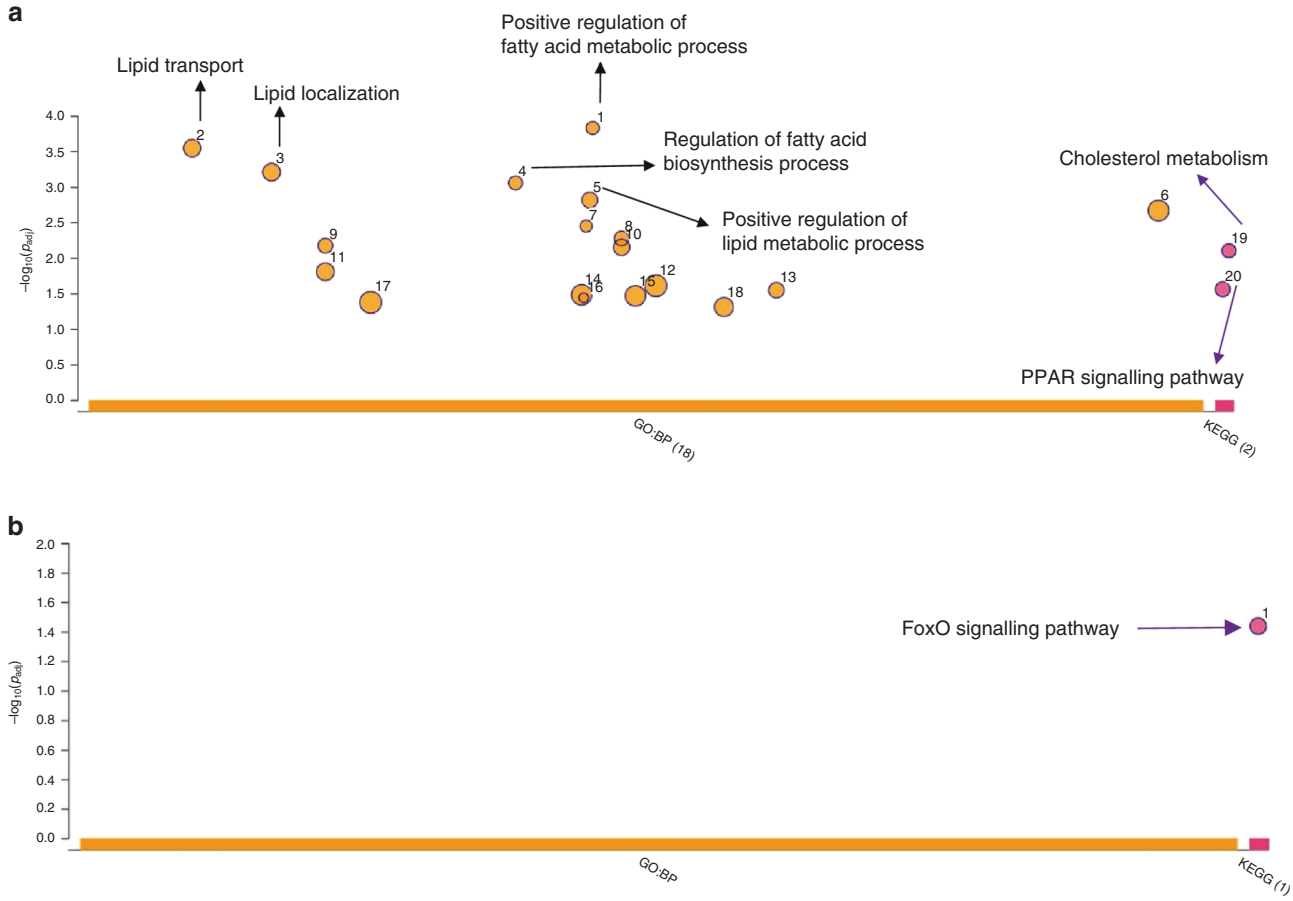

Fig. 3 Gene function analysis. Manhattan plots illustrate the enrichment analysis results on DEGs between LBW and NBW in males (**a**) and females (**b**). The *x*-axis represents functional terms that are grouped and colour-coded by data sources (e.g. biological processes (BP) from gene ontology (GO) are orange circles and enriched KEGG pathways are purple circles). The circle sizes are in accordance with the corresponding term size, i.e. larger terms have larger circles. The *y*-axis shows the adjusted enrichment *p* values in negative log10 scale.

Hepatic antioxidant systems

As elevated hepatic cholesterol is associated with oxidative stress,[9,10] we quantified markers of this latter. The protein levels and activities of antioxidant enzymes SOD and CAT, as well as GSH and GSSG concentrations were determined in LBW and NBW livers. Sex had a significant effect on SOD1 protein level, which were lower in females than in males ($p < 0.0001$; Fig. 6a). While hepatic CAT protein was not impacted by birth weight nor sex, hepatic CAT activity was lower in male LBW ($p < 0.05$) (Fig. 6a, b). SOD activity was decreased in male LBW but increased in female LBW (Fig. 6c). GSH activity was not significantly affected by birth weight or sex (Fig. 6d). While the concentration of GSSG was significantly reduced in male LBW compared to male NBW ($p < 0.05$; Fig. 6e), the ratio of GSH:GSSG was not significantly impacted by birth weight or sex (Fig. 6f).

DISCUSSION

An adverse environment during in utero life has been associated with gene reprogramming and modification of organ functions that can persist throughout the entire lifespan and are associated with an increased risk of developing the metabolic syndrome.[50] Using a pre-clinical guinea pig animal model of UPI, we demonstrated that LBW is associated with increased hepatic cholesterol content and aberrant expression of cholesterol metabolism-related signalling pathway genes in young adulthood (PND150) liver. These changes occur in conjunction with markers of a compromised hepatic cholesterol elimination process and indications of antioxidant stress in young adults. Interestingly, these changes, at the current age studied, were sex-specific, only being observed in LBW males.

The impact of an adverse in utero environment on cholesterol metabolism has been mostly studied using mouse and rat models of IUGR arising from maternal protein undernutrition, in utero dietary restriction, prenatal hypoxia, or nicotine exposure.[17–20] These different species and intrauterine insults collectively lead to elevated serum or hepatic cholesterol levels at weaning or in the adulthood in male offspring, similar to what was observed in this current study. The specific aetiology of the IUGR insult is of critical importance in determining the adult metabolic outcomes and phenotype[22,23] and hence in the current study, UPI was investigated as it is the most common in utero insult associated with IUGR and LBW in developed world.[51,52] Moreover, the guinea pig was utilized given its greater similarities with human pregnancy and outcomes, including a relatively long gestation, haemomonochorial placenta, luteo-placental shift in hormone production, foetal development of metabolic tissues, and pre-cocial offspring.[36] The reported outcomes here are similar to the observed increased in blood total cholesterol at 15 weeks of age observed in UPI-induced IUGR male rat offspring.[32] Additionally, the observed increase in hepatic cholesterol without increased hepatic triglycerides in male LBW offspring is also in alignment with other species studies that report IUGR-induced alterations in the cholesterol metabolizing pathway, without changes in hepatic fatty acid metabolism.[17,33] The current work highlights not only that the UPI environment is associated with programming of later life hepatic cholesterol metabolic dysfunction, but also validates

Table 2. Gene ontology (GO) terms and KEGG pathways overrepresented among differentially expressed genes in livers of LBW versus NBW offspring.

Sex	Source	Term name	Adjusted *p* value	Genes
Male	GO:BP	Positive regulation of fatty acid metabolic process	0.0001	*Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Lipid transport	0.0003	*Inhba, Ldlr, Midlip1, Anxa1, Ace, Adipoq, ApoA1*
	GO:BP	Lipid localization	0.0006	*Inhba, Ldlr, Midlip1, Anxa1, Ace, Adipoq, ApoA1*
	GO:BP	Regulation of fatty acid biosynthetic process	0.0009	*Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Positive regulation of lipid metabolic process	0.0015	*Ldlr, Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Regulation of multicellular organismal development	0.0022	*Inhba, Serpine2, Ldlr, Nell1, Nedd4l, Anxa1, Ace, Angptl4, Adipoq, Apoa1, Hey1, Nrep*
	GO:BP	Positive regulation of fatty acid biosynthetic process	0.0036	*Midlip1, Anxa1, ApoA1*
	GO:BP	Positive regulation of lipid biosynthetic process	0.0053	*Ldlr, Midlip1, Anxa1, ApoA1*
	GO:BP	Regulation of fatty acid metabolic process	0.0067	*Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Regulation of lipid biosynthetic process	0.0071	*Ldlr, Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Regulation of lipid metabolic process	0.0157	*Ldlr, Midlip1, Anxa1,Angptl4, Adipoq, ApoA1*
	GO:BP	Regulation of developmental process	0.0250	*Inhba, Serpine2, Ldlr, Nell1, Nedd4l, Anxa1, Ace, Angptl4, Adipoq, Apoa1, Hey1, Nrep*
	GO:BP	Positive regulation of small molecule metabolic process	0.0285	*Midlip1, Anxa1, Adipoq, ApoA1*
	GO:BP	Regulation of cell differentiation	0.0332	*Inhba, Serpine2, Ldlr, Nell1, Nedd4l, Anxa1, Adipoq, Apoa1, Hey1, Nrep*
	GO:BP	Cell development	0.0343	*Inhba, Serpine2, Inhbe, Ldlr, Nedd4l, Tubb3, Adipoq, Apoa1, Hey1, Nrep*
	GO:BP	Negative regulation of macrophage differentiation	0.0365	*Inhba, Adipoq*
	GO:BP	Cell differentiation	0.0423	*Inhba, Serpine2, Inhbe, Ldlr, Krtap12-1, Nell1, Nedd4l,Tubb3, Anxa1, Ace, Adipoq, Apoa1, Hey1, Nrep*
	GO:BP	Regulation of cell development	0.0492	*Serpine2, Ldlr, Nedd4l, Adipoq, Apoa1, Hey1, Nrep.*
	KEGG pathway	Cholesterol metabolism	0.0080	*Ldlr, Angptl4, Apoa1*
	KEGG pathway	PPAR signalling pathway	0.0277	*Angptl4, Adipoq, Apoa1*
Female	KEGG pathway	FoxO signalling pathway	0.0366	*Bcl6, Gadd45g*

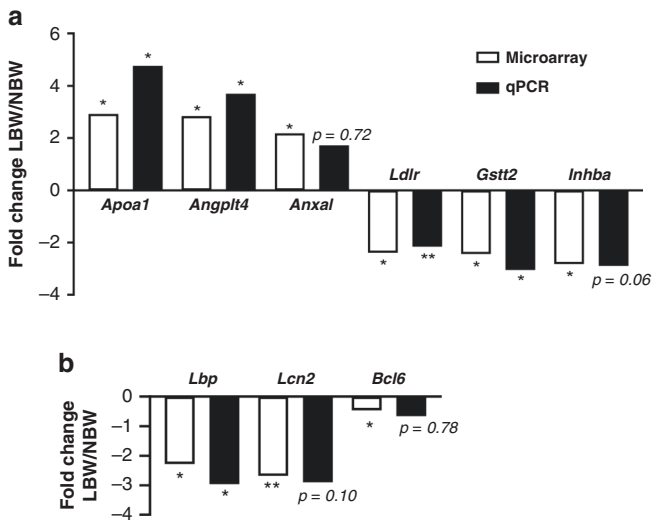

Fig. 4 RT-qPCR validation for selected differentially expressed genes. Transcriptomic differences between NBW and LBW in males (**a**) and females (**b**) were evaluated by RT-qPCR. RT-qPCR fold change values are the ratios of LBW to NBW; $n = 7$–8 samples per group. Data are mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ using a Student's *t*-test or a Mann–Whitney test for *Anxa1*, *Lcn2,* and *Bcl6*.

the experimental in utero insults/stress situations used in other species. Collectively, this provides external validation for the central nature of this pathway and later life cholesterol metabolic dysregulation across species when subjected to in utero environments associated with LBW outcomes.

The present study also highlights that cholesterol metabolism and PPAR signalling pathways are impacted and associated genes are differentially expressed in LBW versus NBW male offspring, but not in females. Hepatic *Apoa1*, *Angplt4*, and *Adipoq* genes, components of both cholesterol metabolism and PPAR signalling pathways, were up-regulated exclusively in LBW males. Apoa1 is the main apolipoprotein of high-density lipoprotein (HDL), playing a key role in regulating lipid transport and in the process of reverse cholesterol transport acting through promotion of the efflux of excess cholesterol from peripheral tissues and returning it to the liver for biliary excretion.[53] Hepatic *ApoA-1* gene expression is up-regulated in liver disease states,[54,55] and ApoA-1 protein among other liver-specific proteins in extracellular vesicles, has been suggested to potentially serve as a specific biomarker for hepatotoxicity in drug- and alcohol-mediated hepatic injury.[56] Additionally, Angptl4 and Adipoq, like ApoA-1, collectively serve a number of important roles in cholesterol metabolism. Angptl4 is present in HDLs physically protecting HDLs from endothelial lipase hydrolysis,[57] and upregulates cholesterol synthesis in liver secondary to inhibition of lipoprotein lipase- and hepatic lipase-dependent hepatic cholesterol uptake.[58] In addition, Adipoq accelerates reverse cholesterol

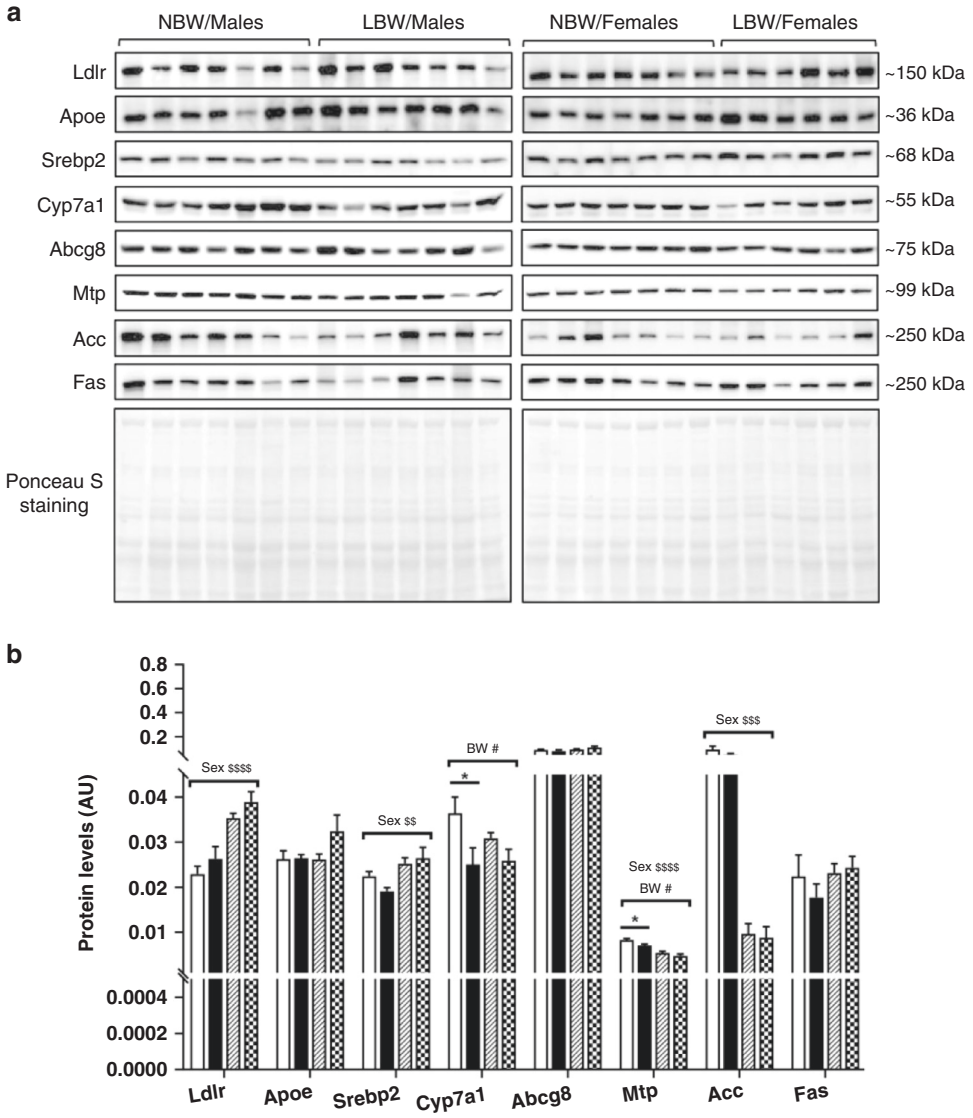

Fig. 5 Immunoblotting for proteins related to cholesterol metabolism. Panel **a** shows representative blots of the targeted proteins and ponceau S as a loading control. Panel **b** indicates normalized densitometry values (targeted protein: ponceau) of targeted proteins. Data are mean ± SEM of 6–7 animals per birth weight/sex group. $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ and $^{\$\$\$\$}p < 0.0001$ for the main effect of sex, $^{\#}p < 0.05$ for the main effect of birth weight, using a two-way ANOVA. $^{*}p < 0.05$ when comparing NBW/Males versus LBW/Males by Bonferroni post hoc test. The empty bars ▭ represent NBW/Males, the filled black bars ■ show LBW/Males and the hatched ▨ and checkerboarded ▩ patterns indicate NBW/Females and LBW/Females, respectively.

transport by increasing HDL assembly through enhanced ApoA-1 synthesis in the liver.[59] Conversely, the decreased *Ldlr* gene expression and unaltered Ldlr protein in LBW males may reflect an unaltered LDL cholesterol uptake into these livers. In agreement with this assumption, it is well established that overaccumulation of cholesterol in the liver supresses *Ldlr* gene transcription and accelerates its mRNA decay.[60] Finally, the intersection of cholesterol and PPAR pathways is of note given the relationship between PPARs in the regulation of bile acid and cholesterol homoeostasis.[61] Therefore, the current changes in gene expression and the previously observed unchanged serum cholesterol levels in this model[62] collectively emphasize a potential increased HDL assembly and/or reverse cholesterol transport. This likely occurs in conjunction with an unaltered LDL uptake in LBW male liver, which may underly the higher hepatic cholesterol content observed, though this remain to be more thoroughly investigated.

Although unaltered at transcriptional level, hepatic Cyp7a1 and Mtp were reduced at the protein level in LBW males. The Cyp7a1 enzyme catalyses the initial step in cholesterol catabolism and bile acid synthesis and decreased Cyp7a1 gene expression and protein levels have been reported in conjunction with increased hepatic cholesterol in adult IUGR male rats from protein-restricted mothers.[17] Further, Cyp7a1-deficient mice are observed to have elevated hepatic and serum cholesterol and decreased total bile acids,[63] supporting the concept that Cyp7A1 acts in a similar manner in guinea pigs and rats and that alterations in its level/activity impact hepatic cholesterol content. It is of further interest to note that the human *CYP7A1* mutation results in substantial cholesterol accumulation in the liver as well as decreased classic bile acid synthesis.[64] Our current findings are in line with the concept that Cypa7a1 is critical in the control of hepatic cholesterol homoeostasis and provide evidence that Cyp7a1-induced cholesterol catabolism appears sensitive to changes in the intrauterine environment, specifically those associated with UPI such as hypoxia and altered nutrient supply.[17,19] The parallel decrease in Mtp protein is of great interest considering that Mtp

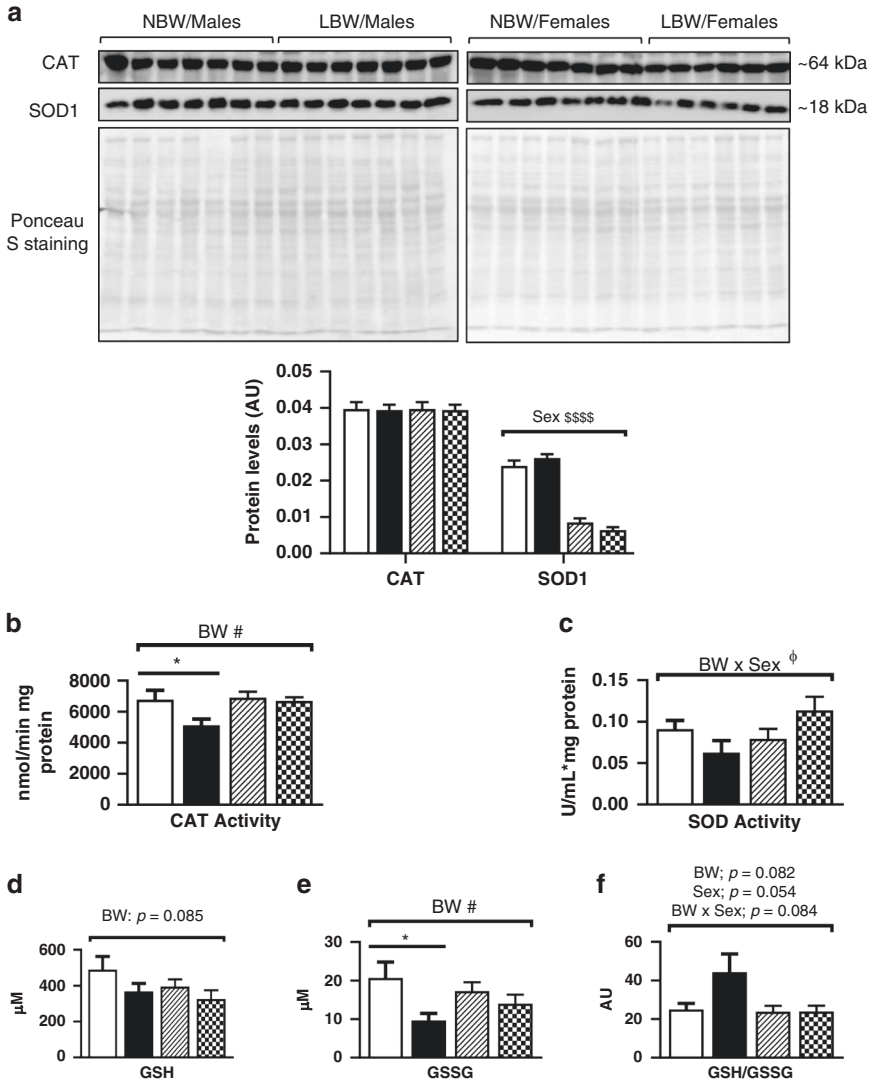

Fig. 6 Immunoblotting and colorimetric assays of antioxidant system. Panel **a** shows representative blots of CAT and SOD1 proteins and ponceau as well as normalized densitometry values of proteins. Panel **b** shows hepatic activity of CAT and panel **c** indicates hepatic activity of SOD. Panels **d**–**f** show reduced glutathione (GSH), oxidized GSH (GSSG), and GSH/GSSG ratio, respectively. Data are means ± SEM of 7–8 animals per birth weight/sex group. $^{\$\$\$\$}p < 0.0001$ for the main effect of sex; $^{\#}p < 0.05$ or the indicated p value for the main effect of birth weight (BW) and $^{\phi}p < 0.05$ for the effect BW and sex interaction, using a two-way ANOVA. $^{*}p < 0.05$ when comparing NBW/Males versus LBW/Males by Bonferroni post hoc test. The empty bars ☐ represent NBW/Males, the filled black bars ■ show LBW/Males and the hatched ▨ and checkerboarded ▩ patterns indicate NBW/Females and LBW/Females, respectively.

catalyses the assembly of cholesterol, triglycerides, and apolipoprotein B to VLDL for their export outside of the liver.[65] Therefore, this impairment in hepatic cholesterol catabolism/efflux-related proteins could reflect a lower elimination rate of hepatic cholesterol, programmed by an adverse in utero environment, which culminates in increased cholesterol content in LBW males born from pregnancies complicated by UPI.

Antioxidant enzyme defense and non-enzymatic antioxidant defense systems are critical in reducing oxidative stress and maintaining redox homoeostasis within liver.[66] SOD reduces the radical superoxide to form hydrogen peroxide and oxygen[67] and CAT catalytically decomposes hydrogen peroxide into water and oxygen.[68] At the same time, glutathione peroxidase (GPx) also reduces hydrogen peroxide to water while converting GSH to GSSG.[69] During oxidative stress there is decrease in levels of GSH and increase in levels of GSSG and thus GSH/GSSG ratio decreases.[70] Liver oxidative injury and abnormal activities of hepatic antioxidant enzymes are reported in male neonates from IUGR pregnancies.[71,72] In the current report, UPI-induced LBW male offspring displayed a significantly depressed CAT activity, and a reduced total SOD activity, a result not observed in the LBW female livers. The ratio of GSH to GSSG was however not reduced in the livers of LBW males. Collectively, these data suggest that whereas female offspring appear to have functional postnatal hepatic oxidative stress recovery mechanisms, the in utero defects in male offspring appear to persist, and this could manifest with the altered hepatic cholesterol metabolic pathways observed. Indeed, in adult animal cholesterol feeding studies, elevated hepatic cholesterol is associated with oxidative stress.[9,10] It is then possible that alterations in the functional postnatal hepatic oxidative stress of male LBW start at the level of SOD and CAT enzymes and may later extend to the glutathione redox couple GSH/GSSG system, as previously highlighted.[70] This notion maybe supported by the observed reduction in the expression of *inhba* and *Gstt2* genes, involved in hepatocyte regeneration and antioxidant system, respectively.[73,74] Therefore, our current observations point to a lower antioxidant capacity in young adult LBW male offspring born from UPI pregnancies, which may

promote the development of liver diseases in later life, especially when challenged with an elevated cholesterol environment.

In the current study, we observed lower body and liver weights at young adulthood in females than males, independent of the birth weight. Previous studies have also reported a sexual dimorphism in guinea pig with males consistently larger than females in skeletal measurements and body weight.[75,76] It has been proposed that rapid and early growth in males leads to male-biased sexual dimorphism in these cases. In conjunction with these growth differences, we report sexual dimorphism in hepatic Ldlr, Srebp2, Mtp, Acc, and SOD1 proteins as well as liver cholesterol content, irrespective of birth weight, but also sex-specific programming of *Apoa1, Angplt4, and ldlr* genes and Cyp7a1 and Mtp proteins in male LBW offspring. Epidemiologic studies have demonstrated that LBW predisposes to adult onset hypercholesterolaemia in men,[26–28] and women[29] with sex difference by age groups in adults.[24] In the current study, male offspring displayed higher hepatic free cholesterol and total cholesterol than females, despite higher protein abundance of Ldlr and Srebp2 and reduced Mtp and Acc proteins in female livers. We speculate that oestrogens levels may be protective for cholesterol overaccumulation within livers of female guinea pigs, given that physiological levels of oestrogen increased CYP7A1 activity along with small transient increases in bile acid production in hepatocytes.[77] These results also indicate that protective compensatory mechanisms of elevated Ldlr and Srebp2 protein in males and reduced Mtp and Acc protein in females occurs.

A limitation of our study is that the concentrations of sex steroids in the serum as well as the steroid receptors in the liver were not measured. Cholesterol is the precursor for endogenous sex steroid biosynthesis.[1] Furthermore, testosterone replacement therapy enhances liver cholesterol uptake, suppressing cholesterol removal, and promoting cholesterol storage.[78] Oestrogen together with oestrogen receptor-α also plays a role in preventing liver malfunctioning and reducing liver damage.[79] Thus, a role of serum sex steroids and downstream signalling on the observed hepatic outcomes cannot be ruled out. Future work should also examine serum concentrations of triglycerides and cholesterol types in order to better characterize uptake/export of these lipids from livers of LBW animals.

In conclusion, the present study demonstrates that LBW occurring as a result of UPI results in increased hepatic cholesterol content, likely through compromised hepatic cholesterol elimination in young male, but not female, guinea pigs. The mechanisms underlying this potential programing effect and continued postnatal presentation of the defect are yet to be fully delineated. Certainly UPI is associated with hypoxia[80] and foetal hypoxia is associated with promoted oxygenated blood flow to the heart and reduced umbilical blood supply to foetal liver, an adaption which is understood to reprogramme liver carbohydrate and lipid metabolism in utero[81–84] likely through altered epigenetic regulation of hepatic metabolism.[17] Furthermore, the observed disrupted hepatic cholesterol metabolism may contribute to permanent alterations in hepatic oxidative stress defense system, ultimately leading to further hepatic damage and greater predisposition to liver diseases in UPI-induced LBW male offspring as they age.

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AUTHOR CONTRIBUTIONS

O.S. and T.R.H.R. designed the experiments, analysed data and drafted the manuscript. O.S., K.E.M., C.V., K.W., A.D., L.Z., and T.R.H.R. performed the experiments. All authors reviewed and edited the manuscript.

ADDITIONAL INFORMATION

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