Research Article

Lipid metabolism is altered in maternal, placental, and fetal tissues of ewes with small for gestational age fetuses[†]

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Abstract

Nutrient restriction (NR) has the potential to negatively impact birthweight, an indicator of neonatal survival and lifelong health. Those fetuses are termed as small for gestational age (SGA). Interestingly, there is a spectral phenotype of fetal growth rates in response to NR associated with changes in placental development, nutrient and waste transport, and lipid metabolism. A sheep model with a maternal diet, starting at Day 35, of 100% National Research Council (NRC) nutrient requirements ($n = 8$) or 50% NRC ($n = 28$) was used to assess alterations in fetuses designated NR SGA ($n = 7$) or NR NonSGA ($n = 7$) based on fetal weight at Day 135 of pregnancy. Allantoic fluid concentrations of triglycerides were greater in NR SGA fetuses than 100% NRC and NR NonSGA fetuses at Day 70 ($P < 0.05$). There was a negative correlation between allantoic fluid concentrations of triglycerides ($R^2 = 0.207$) and bile acids ($R^2 = 0.179$) on Day 70 and fetal weight at Day 135 for NR ewes ($P < 0.05$). Bile acids were more abundant in maternal and fetal blood for NR SGA compared to 100% NRC and NR NonSGA ewes ($P < 0.05$). Maternal blood concentrations of NEFAs increased in late pregnancy in NR NonSGA compared to NR SGA ewes ($P < 0.05$). Protein expression of fatty acid transporter SLC27A6 localized to placental maternal and fetal epithelia and decreased in Day 70 NR SGA compared to 100% NRC and NR NonSGA placentomes ($P < 0.05$). These results identify novel factors associated with an ability of placentae and fetuses in NR NonSGA ewes to adapt to, and overcome, nutritional hardship during pregnancy.

Summary sentence

Allantoic fluid concentrations of triglycerides and bile acids from nutrient restricted ewes at mid-gestation are negatively correlated with fetal weights near term, while placental fatty acid transporter SLC27A6 protein is reduced in small for gestational age fetuses.

Key words: lipid, nutrient restriction, placenta, sheep, small for gestational age, triglyceride.

Introduction

Birth weight is an influential indicator of neonatal survival and life-long health in humans and livestock. Low birth weight is associated with increased perinatal morbidity and mortality, and an increased incidence of metabolic and cardiovascular diseases in adulthood [1–5]. The 2018 Global Nutrition Report found that 462 million adults are currently classified as underweight, a factor that contributes to over 20 million babies born annually with low birth weight, and highlights the often-overlooked issue of food security that still plagues much of the world [6]. Insufficient placental development and function, which impairs the transfer of nutrients, gases, and wastes between the mother and fetus, often results in fetuses that fail to reach their growth potential in utero. Clinically, fetuses that fall below the 10th percentile for weight at a specific gestational age are classified as small for gestational age (SGA) [7, 8].

Alterations in maternal lipid metabolism during normal pregnancy occur in response to increasing concentrations of estrogen and progesterone in serum, as well as changes in insulin sensitivity [9, 10]. Those changes include enhanced lipogenesis early in pregnancy, followed by increased lipolysis as pregnancy progresses, resulting in greater concentrations of non-esterified fatty acids (NEFAs), cholesterol, triglycerides, and bile acids in serum [11–13]. Those changes are due to mobilization of maternal stores of those molecules for transport and use by the fetus as pregnancy progresses, in addition to removal of waste products from the fetus. Pregnancy is, therefore, a period of dynamic changes in lipid metabolism in the mother, even under the most optimal of conditions.

When the ideal environment is not obtainable during pregnancy, one of the results can be SGA fetuses. Meta analyses of multiple human SGA studies indicated that concentrations of certain fatty acids, phospholipids, and cholesterol in amniotic fluid, maternal serum, and maternal hair can be predictive of SGA fetuses [14, 15]. Additionally, total caloric nutrient restriction (NR), a potential cause of SGA fetuses, induces greater mobilization of lipids, resulting in lipid metabolism disorders, such as hypercholanemia, hypercholesterolemia, and cholestasis [16–18]. While maternal NR during pregnancy in sheep results in SGA fetuses with perturbations in glucose and amino acid metabolism, few studies have explored effects of NR on lipid metabolism in the mother, placenta, and fetus [19–22].

During pregnancy, the placenta assumes the role of multiple organs, including the liver, and plays a pivotal role in lipid homeostasis. In addition to regulated and un-regulated lipid transport from the mother to the fetus, the placenta also synthesizes triglycerides, and transports bile acids. Enzymes for synthesis of triglycerides and fatty acid transporters (SLC27A family) have been identified in placentae and are altered in SGA fetuses, as well as fetuses from obese mothers [23–26], but have not been studied in normal weight fetuses born to nutrient restricted mothers.

The majority of published studies investigated maternal consequences of NR only considering fetuses from NR dams as a single experimental group, regardless of fetal growth rate. Our previous research identified a greater spectrum of fetal growth in NR fetuses, likely due to the ewe's ability to adapt to nutritional deficiencies while supporting fetal growth [27, 28]. Therefore, the long-term objective of our research is to elucidate mechanisms by which the ewe, placenta, and fetus adapt to nutritional deficiencies, and determine whether fetuses from NR ewes that are not SGA also have perturbations in lipid metabolism. The aim of the present study was to determine the effect of NR on lipid and bile acid profiles in maternal and fetal fluids during mid- and late-gestation,

and on placentomal gene expression of fatty acid transporters and enzymes for triglyceride synthesis enzymes, by utilization of a novel surgical approach to remove placentomes from pregnant ewes during mid-gestation.

Materials and methods

Ethics statement

All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of Texas A&M University and were conducted in accordance with National Institutes of Health guidelines.

Animal study and tissue collection

Mature Hampshire ewes of similar parity, frame size, and initial body condition were fed to meet 100% of their National Research Council (NRC) [29] nutritional requirements and served as embryo transfer recipients. Embryo transfer was utilized to produce singleton pregnancies as sheep will naturally produce both singles and twins, which would confound the model. Ewes were synchronized into estrus, and a single embryo from a superovulated Hampshire donor ewe of normal body condition was transferred into the uterus of a recipient ewe on Day 6 post-estrus. Pregnancy was diagnosed by ultrasound on Day 28 of gestation. All ewes were individually housed in pens with concrete flooring from Days 28 to 135 of gestation and fed once daily. Beginning on Day 28 of gestation, body weight was measured weekly, and feed intake was adjusted based on changes in body weight. On Day 35 of pregnancy, ewes were assigned randomly to either a control-fed group (100% NRC; $n = 8$) or a NR group (50% NRC; $n = 28$). Composition of the diet was as published previously [20]. Briefly, ewes were fed a wheat, cottonseed, rice mill, and alfalfa-based diet including mineral and vitamin mixtures. Nutrient-restricted ewes were provided 50% of the total weight of feed that the control-fed group was provided to induce a total caloric restriction that was equal across macromolecule groups. Blood samples were collected from ewes on Days 35, 70, 105, and 135 with plasma harvested and stored for further analyses.

On Day 70 of pregnancy, a single placentome was surgically removed as previously described [30]. Briefly, care was taken to remove a placentome from near the antimesometrial greater curvature of the gravid uterus and proximal to the anterior end of the amniotic membrane. A cross section of the placentome was subjected to paraformaldehyde fixation for further histological processing, while the remainder was snap-frozen in liquid nitrogen for RNA and protein analyses. Maternal blood samples and allantoic fluid were also collected on Day 70, centrifuged to remove cellular debris and stored at -20°C . Necropsies were performed on Day 135 of gestation. At this time, blood samples from maternal vein, fetal umbilical vein, and fetal heart were collected and, after centrifugation, plasma was stored at -20°C . The volume of allantoic fluid was recorded, aliquoted, and stored at -20°C . Placentomes were dissected, weighed, and then processed as on Day 70.

Fetuses from ewes fed 100% NRC were the control group ($N = 8$; $N = 3$ female). Fetuses within the NR group ($N = 28$; $N = 15$ female) were segregated into quartiles based on fetal weights. The highest (NR NonSGA; $N = 7$; $N = 4$ female) and lowest (NR SGA; $N = 7$; $N = 2$ female) quartiles were selected for further investigation, except when regression analyses were performed, during which all fetuses from NR ewes were analyzed [27].

Cholesterol, triglycerides, NEFAs, and bile acids

Concentrations of cholesterol, triglycerides, NEFAs, and bile acids were quantified in maternal plasma, fetal plasma, and allantoic fluid using commercially available kits. All assays were validated for linearity in plasma from sheep and all samples were run in duplicate. Total cholesterol was measured using a Cholesterol Fluorometric Assay Kit (Cat#10007640; Caymen Chemical Company, Ann Arbor, MI, USA) according to manufacturer's instructions (intra-assay variation = 3.0%; inter-assay variation = 2.7%). Triglycerides were measured using a Triglyceride Colorimetric Assay Kit (Cat# 10010303; Caymen Chemical Company, Ann Arbor, MI, USA) according to manufacturer's instructions (intra-assay variation = 4.5%; inter-assay variation = 6.7%). NEFAs were measured using a NEFA-HR Assay (Wako Diagnostics, Mountain View, CA, USA) according to manufacturer's instructions (intra-assay variation = 6.2%; inter-assay variation = 7.4%). Bile acids were measured with a colorimetric Total Bile Acid Assay Kit (Cat# STA-631; Cell Biolabs, Inc., San Diego, CA, USA) according to manufacturer's instructions (intra-assay variation = 3.5%; inter-assay variation = 5.2%).

RNA extraction and cDNA synthesis

Total RNA was extracted from snap-frozen placentomes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. After DNase I treatment (Qiagen, Hilden, Germany), the RNA was quantified and quality was assessed using a Nanodrop and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. An RNA integrity number (RIN) of ≥ 8 and a 260/230 value of >1.8 were considered acceptable. Extracted RNA was stored at -80°C . First-strand cDNA was synthesized from 1 μg of RNA using the Superscript First Strand Synthesis System (Invitrogen) according to manufacturer's instructions. Negative controls with reverse transcriptase omitted were included to confirm the absence of genomic contamination. The cDNA was stored at -20°C and diluted 5-fold before use in the quantitative PCR (qPCR) reactions.

qPCR analyses

Primers for qPCR were designed using National Center for Biotechnology Information Genbank sequences and Primer-BLAST (<http://www.ncbi.nlm.nih.gov/>). Primer sequences were submitted to BLAST against the known ovine genome to confirm specificity. Primer information is summarized in [Supplementary Table 1](#).

The qPCR assays were performed using SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) in 10 μL reactions, with 0.5 mM of each specific primer (Integrated DNA Technologies, Coralville, IA, USA), on a 7900HT Real-Time PCR System (Applied Biosystems) with approximately 10 ng of cDNA per reaction. The PCR program began with 5 min at 95°C followed by 40 cycles of 95°C denaturation for 10 s and 60°C annealing/extension for 30 s. A dissociation curve was produced with every run to verify a single gene-specific peak. Standard curves with 2-fold serial dilutions (eight points) of pooled cDNA were run to determine primer efficiencies. All primer correlation coefficients were greater than 0.97 and efficiencies were 95–105%. The geometric mean of the reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase complex flavoprotein A (*SDHA*), and tyrosine 3,5-monooxygenase activation protein zeta (*YWHAZ*) was used for normalization and all samples were run in triplicate. The $2^{-\Delta\Delta\text{Ct}}$

method was utilized and fold changes were subjected to statistical analyses.

Immunohistochemistry

Immunohistochemical localization of solute carrier family 27 member 6 (SLC27A6) in paraffin-embedded sheep placentomes (5 μm thick) was performed as previously described [31]. Briefly, rabbit anti-human SLC27A6 (GTX31878; GeneTex, Irvine, CA, USA) was used at 10 mg/ml and subjected to boiling citrate antigen retrieval. The negative control was nonimmune rabbit IgG diluted to the same concentration as the primary antibody. Immunoreactive protein was visualized using the Vectastain Elite ABC Kit (PK-6101; Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions with 3,3'-diaminobenzidine tetrahydrochloride (D5637; Sigma-Aldrich) as the chromagen. Sections were counterstained with Harris-modified hematoxylin (Fisher Scientific), dehydrated, and coverslips affixed using Permount mounting medium (Fisher Scientific).

Protein extraction and western blots

Total protein was extracted from placentomes by homogenization of tissue in lysis buffer (60 mM Tris-HCl, 1 mM Na_3VO_4 , 10% Glycerol, 1% SDS w/v, EDTA-free protease inhibitors). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions. Proteins (20 μg) were separated by gel electrophoresis in TGX Stain-free Gels (4–20% gradient; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and gels were activated with 1 min of UV light [32]. Proteins were transferred and membranes were blocked with 5% milk protein in Tris-buffered saline-0.1% Tween20 (TBST) for 1 h at room temperature. Rabbit anti-human SLC27A6 (GTX31878; GeneTex) was incubated with the membrane overnight at 4°C after being diluted 1:1500 in 2.5% milk protein in TBST. Membranes were probed with goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody (7074S; Cell Signaling Technology, Danvers, MA, USA) diluted 1:20 000 in 2.5% milk protein in TBST and incubated for 1 h at room temperature. After washing in TBST, membranes were exposed to UV light and imaged to determine total protein to be used as a loading control [33]. Antibody-bound proteins were visualized using SuperSignal West Dura Substrate (ThermoFisher) according to the manufacturer's instructions. Imaging and quantification were performed using a ChemiDocXRS (Bio-Rad Laboratories) interfaced with Quantity One software (v 4.6.1; Bio-Rad Laboratories). Background was subtracted from bands and total protein independently, and then band density was divided by total protein density for each sample. The ratio was subjected to statistical analysis.

Imaging

Representative brightfield illumination images of histology and immunohistochemistry were captured using a Nikon Eclipse Ni-E microscope (Nikon Corp, Tokyo, Japan) and a 10x or 40x objective, interfaced with a DS-Ri1 camera (Nikon), and NIS-Elements AR software (V 4.30.02; Nikon). Photographic plates were assembled using GNU Image Manipulation Program (GIMP; v2.10.14; www.gimp.org).

Statistical analyses

Metabolite, gene expression, and protein expression data were subjected to analysis using a mixed model ANOVA with ewe considered a random effect (100% NRC, $N = 8$; NR NonSGA, $N = 7$; NR SGA,

Table 1. Concentrations of bile acids, cholesterol, NEFAs, and triglycerides in maternal plasma.

Column 1	Day	100% NRC	NR NonSGA	NR SGA	Mean $\pm$ SEM
NEFAs (mM)	35	0.56 ^{ab}	0.64 ^{abc}	0.66 ^{abc}	0.62 $\pm$ 0.03
	70	0.43 ^a	0.56 ^{ab}	0.69 ^{bc}	0.56 $\pm$ 0.08
	105	0.49 ^{ab}	0.96 ^d	0.69 ^{bc}	0.71 $\pm$ 0.14
	135	0.54 ^{ab}	0.86 ^{cd}	0.41 ^a	0.60 $\pm$ 0.13
	Mean $\pm$ SEM	0.50 $\pm$ 0.03	0.76 $\pm$ 0.09	0.62 $\pm$ 0.07	
Triglycerides (mg/dl)	35	9.70	11.44	10.81	10.65 $\pm$ 0.51
	70	12.96	10.09	10.05	11.03 $\pm$ 0.96
	105	12.13	14.07	12.13	12.78 $\pm$ 0.65
	135	12.27	13.47	11.24	12.32 $\pm$ 0.64
	Mean $\pm$ SEM	11.77 $\pm$ 0.71	12.26 $\pm$ 0.92	11.06 $\pm$ 0.43	
Bile Acids (μ M)	35	14.88	12.23	16.70	14.60 $\pm$ 1.30 ^a
	70	15.10	12.65	19.62	15.79 $\pm$ 2.04 ^a
	105	21.81	22.89	26.77	23.82 $\pm$ 1.51 ^b
	135	6.58	14.90	29.55	17.01 $\pm$ 6.71 ^a
	Mean $\pm$ SEM	14.59 $\pm$ 3.12 ^a	15.67 $\pm$ 2.48 ^a	23.16 $\pm$ 3.00 ^b	
Cholesterol (mM)	35	1.81	1.78	1.91	1.84 $\pm$ 0.04 ^a
	70	2.30	1.99	2.89	2.39 $\pm$ 0.27 ^b
	105	2.19	2.29	2.75	2.41 $\pm$ 0.17 ^b
	135	2.00	2.25	2.23	2.16 $\pm$ 0.08 ^b
	Mean $\pm$ SEM	2.07 $\pm$ 0.11 ^a	2.08 $\pm$ 0.12 ^a	2.44 $\pm$ 0.23 ^b	

Means with different superscript letters are different ($P < 0.05$) for the treatment by day interaction (NEFAs) or main effects (bile acids, cholesterol). Means without superscripts are not different ($P > 0.05$).

$N = 7$). Main effects (treatment, day) and interactions were included. Least-squares means are presented with Tukey's honestly significant difference test used for multiple comparisons among means. Fetal sex was not included in the model due to insufficient statistical power due to small group size. Data were log transformed when necessary after observation of residual plots. Linear regression and multiple linear regression analyses of data on triglycerides and bile acids from Day 70 of gestation included all NR fetuses ($N = 28$). Significance was set at $P < 0.05$ and all data are presented as means $\pm$ SEM.

Results

Maternal characteristics

All ewes were assigned to the study at Day 35 with similar body weights (NR NonSGA, 78.28 kg; NR SGA 70.05 kg; 100% NRC, 73.94 kg). By Day 135 of gestation, the body weights of all NR ewes were significantly decreased (NR NonSGA, 63.96 kg; NR SGA, 56.31 kg; 100% NRC, 80.40 kg) and had greater changes in body weight (NR NonSGA, -14.32 kg; NR SGA, -13.74 kg; 100% NRC, 6.46 kg) than 100% NRC ewes (Supplementary Table 2; $P < 0.05$). Additionally, weights of maternal gastrocnemius muscle (NR NonSGA, 146.1 g; NR SGA, 124.7 g; 100% NRC, 183.9 g) and liver (NR NonSGA, 679.4 g; NR SGA, 623.4 g; 100% NRC, 880.6 g) decreased in NR ewes compared to 100% NRC ewes (Supplementary Table 2; $P < 0.05$). Total placentome weight was lower in the NR SGA ewes (NR SGA, 306.8 g; NR NonSGA, 523.7 g; 100% NRC 546.2 g), while placentome numbers (NR NonSGA, 86; NR SGA, 66; 100% NRC, 81) were significantly greater for NR NonSGA compared to NR SGA ewes, while 100% NRC ewes had an intermediate number of placentomes (Supplementary Table 2; $P < 0.05$).

Maternal blood samples were collected throughout the duration of the study to determine changes in lipid profiles (Table 1). Concentrations of NEFAs in plasma of ewes were affected by a day

by treatment interaction with the highest concentrations on Days 105 and 135 for NR NonSGA ewes ($P < 0.05$). In contrast, plasma concentrations of NEFAs from 100% NRC ewes were similar across all days studied, and concentrations of NEFAs in plasma of NR SGA ewes decreased to Day 135 of pregnancy. Plasma concentrations of triglycerides in ewes were not affected by day or treatment ($P > 0.05$). Plasma concentrations of bile acids were greater on Day 105 than Days 35, 70, and 135 of pregnancy. Additionally, plasma concentrations of bile acids were greater in NR SGA ewes compared to 100% NRC and NR NonSGA ewes ($P < 0.05$). Plasma concentrations of cholesterol were lowest on Day 35 and increased to Day 70 of pregnancy, and values were greater for NR SGA ewes compared to 100% NRC and NR NonSGA ewes ($P < 0.05$).

Lipids in fetal blood

Blood samples were collected from fetuses on Day 135 of pregnancy to determine differences in concentrations of NEFA, triglycerides, bile acids, and cholesterol (Table 2). Plasma concentrations of NEFA were greater in NR NonSGA fetuses compared to 100% NRC fetuses ($P < 0.05$). Plasma concentrations of triglycerides were lower in NR SGA versus NR NonSGA fetuses ($P < 0.05$). Plasma concentrations of bile acids were greater for NR SGA fetuses compared to 100% NRC and NR NonSGA fetuses ($P < 0.05$). There were no treatment effects on concentrations of cholesterol in fetal plasma ($P > 0.05$).

Lipids in allantoic fluid

Allantoic fluid samples were collected from each conceptus during the placentectomy procedure at Day 70 and again at Day 135 of gestation to determine concentrations of NEFA, triglycerides, bile acids, and cholesterol (Table 3). Concentrations of NEFA in allantoic fluid were below the detectable limit of the assay. There was a day $\times$ treatment interaction for concentrations of triglycerides as concentrations for Day 70 NR SGA fetuses were greater than

Table 2. Concentrations of bile acids, cholesterol, NEFAs, and triglycerides in fetal plasma at Day 135 of pregnancy.

Group	100% NRC	NR NonSGA	NR SGA	SEM
NEFAs (mM)	0.03 ^a	0.06 ^b	0.04 ^{ab}	0.01
Triglycerides (mg/dl)	8.80 ^{ab}	10.67 ^b	6.79 ^a	0.83
Bile acids (μM)	28.38 ^a	29.82 ^a	55.55 ^b	11.16
Cholesterol (mM)	0.59	0.60	0.59	0.08

Means with differing superscript letters are different within rows ($P < 0.05$).

Table 3. Allantoic fluid concentrations of bile acids, cholesterol, and triglycerides on Days 70 and 135 of pregnancy.

Column 1	Day	100% NRC	NR NonSGA	NR SGA	Mean ± SEM
Triglycerides (mg/dl)	70	15.08 ^a	19.56 ^a	26.89 ^b	20.51 ± 3.44
	135	123.30 ^c	125.10 ^c	124.40 ^c	124.30 ± 0.51
	Mean ± SEM	69.19 ± 14.05	72.31 ± 14.66	75.65 ± 13.61	
Bile Acids (μM)	70	2.07	2.20	3.03	2.44 ± 0.30 ^a
	135	12.10	15.59	13.41	13.69 ± 1.02 ^b
	Mean ± SEM	7.08 ± 1.63	8.89 ± 2.82	8.22 ± 3.22	
Cholesterol (μM)	70	10.92	4.80	6.98	7.57 ± 1.80
	135	6.71	5.81	5.72	6.08 ± 0.30
	Mean ± SEM	8.81 ± 2.11 ^b	5.31 ± 0.50 ^a	6.35 ± 0.63 ^{ab}	

Means with differing superscript letters are different ($P < 0.05$) for the treatment by day interaction (triglycerides) or main effects (bile acids, cholesterol).

those on Day 70 for 100% NRC and NR NonSGA, but there was no effect of treatment at Day 135 of gestation ($P < 0.05$). Based on those results, a linear regression was used to assess changes in concentrations of triglycerides in allantoic fluid from all Day 70 NR pregnancies (Figure 1A). Indeed, there was a significant correlation between concentrations of triglycerides in allantoic fluid at mid-gestation and fetal weight at Day 135 of pregnancy as smaller fetuses had higher triglyceride levels at mid-gestation ($R^2 = 0.2065$; $P < 0.05$). Concentrations of bile acids increased from Days 70 to 135 of gestation ($P < 0.05$), but there was no treatment effect ($P > 0.10$). Since bile acids are necessary for degradation of triglycerides, a linear regression was performed (Figure 1B) to reveal a significant correlation between concentrations at mid-gestation and fetal weight at Day 135 of pregnancy with higher concentrations being detected in smaller fetuses ($R^2 = 0.1785$; $P < 0.05$). Since concentrations of triglycerides and bile acids in allantoic fluid on Day 70 of gestation had negative correlations with Day 135 fetal weight, a multiple linear regression was performed. The combined effects of concentrations of triglycerides and bile acids on Day 70 of gestation were negatively correlated with fetal weights on Day 135 of pregnancy ($R^2 = 0.350$; $P < 0.01$). In comparison, total placenta weights and fetal weights were positively correlated on Day 135 of pregnancy ($R^2 = 0.514$; $P < 0.01$). Concentrations of cholesterol were greater for 100% NRC fetuses than NR NonSGA fetuses ($P < 0.05$).

Gene expression in placentomes for synthesis of triglycerides

Gene expression of enzymes involved in triglyceride synthesis was analyzed in placentomes (Supplementary Figure 1). Expression of *AGPAT3* mRNA was greater in placentomes from NR SGA compared to 100% NRC ewes, while expression in NR NonSGA placentomes was at intermediate levels ($P < 0.05$), but there were no other treatment effects. Expression of multiple mRNAs increased ($P < 0.05$) from Days 70 to 135, including those for *AGPAT2*, *AGPAT3*, *DGAT1*, *GPAT3*, *LPIN1*, and *LPIN2*, while expression of mRNAs for *AGPAT4*, *MGAT1*, and *MGAT2* decreased ($P < 0.05$).

Fatty acid transporter gene expression in placentomes

The expression of mRNAs for members of the fatty acid transporter family, *SLC27* was assessed in placentomes. In the placenta, *SLC27A6* mRNA expression was lower in NR SGA than 100% NRC and NR NonSGA pregnancies at Day 70, but there was no effect of treatment on Day 135 of gestation ($P < 0.05$; Figure 2). Expression of *SLC27A1*, *SLC27A2*, *SLC27A3*, and *SLC27A4* mRNAs increased from Days 70 to 135, but was not affected by treatment (Figure 2).

Protein expression and localization of SLC27A6 in the placenta

Due to treatment differences in expression of *SLC27A6* mRNA, *SLC27A6* protein was assessed for abundance and localization (Figure 3). At Day 70 of pregnancy, *SLC27A6* protein was localized to the maternal luminal epithelium (LE) near the capsule of the placenta. On the opposite side of the placenta, near the chorioallantois, *SLC27A6* protein localized to the fetal chorionic epithelium (FE). There was no protein detected in stromal cells in either the caruncular or the cotyledonary portions of the placenta. While the localization was similar at Day 135 of pregnancy, the abundance of *SLC27A6* protein was less near the capsule, as was the signal in placentomes of NR SGA ewes on Day 70 of pregnancy. This observation was confirmed by western blots indicating less total *SLC27A6* protein in placentomes from NR SGA ewes than 100% NRC and NR NonSGA placentomes at Day 70, and less *SLC27A6* protein between Days 70 and 135 in placentomes from 100% NRC and NR NonSGA ewes ($P < 0.05$).

Discussion

NR during pregnancy results in a large variation in birth weights of offspring. Although low birth weights are associated with poor health outcomes, little focus has been placed on characteristics of offspring with normal birth weights from nutrient restricted dams regarding metabolic state or compensatory mechanisms. Additionally, low fetal weight/birthweight is the single measure that defines SGA and can only be determined late in pregnancy, by ultrasound,

Figure 1. Regression analyses of allantoic fluid components at Day 70 of pregnancy versus fetal weight at Day 135 of pregnancy in NR animals. (A) High concentrations of triglycerides were correlated with low fetal weights ($R^2 = 0.207$; $P < 0.05$). (B) High concentrations of bile acids were correlated with low fetal weights ($R^2 = 0.179$; $P < 0.05$). (C) Total placentome weight and fetal weight were correlated positively at Day 135 of pregnancy ($R^2 = 0.514$; $P < 0.05$).

or at birth. There is no early detection marker available to predict if a fetus will be SGA. Results of the present study revealed that concentrations of triglycerides and bile acids in allantoic fluid on Day 70 of gestation are predictive of late-gestation SGA fetuses in nutrient restricted ewes. Also, the results indicate that NR NonSGA pregnancies adapt to and overcome NR as indicated by similarities in abundances of triglycerides and bile acids, and SLC27A6 protein between NR NonSGA and 100% NRC ewes.

The use of sheep as a model of human pregnancy has been well established and, repeatedly, the findings from sheep studies have proven applicable to humans for both normal and growth restricted pregnancies when appropriate technologies have become available [34–36]. While there are differences in placental type and blood flow between humans (hemochorial) and sheep (synepithelio-chorial), especially on the maternal side of the placenta, there are substantial similarities in the fetal vascular structure as well as in cellular function [34]. Additionally, many of the maternal metabolic adaptations to pregnancy, including lipid mobilization, are similar between the two species [36] as is relative timing of fetal organ development and growth [37]. The use of sheep as a model also allows for mid-gestational sampling of the placenta and fluids of both the mother and fetus without compromising the pregnancy, which provides the opportunity for retrospective analysis based on late gestation fetal weight.

Irrespective of the eventual growth rate of their fetuses, NR ewes in this study experienced total body weight loss, as well as catabolism of muscle and liver compared to their well-fed counterparts [38]. NR ewes also had elevated concentrations of NEFA, a normal response to NR in which lipids are mobilized through activation of lipolysis to increase the availability of degradation products of lipids as alternative energy sources. Indeed, NR ewes were reported to have elevated NEFAs during pregnancy, however, those studies did not consider variations in responses to NR for fetal weight, which may identify adaptive responses to maternal malnutrition influencing fetal growth [39, 40]. Interestingly, in the present study, concentrations of NEFA were only increased on Day 70 of gestation in NR ewes with SGA fetuses and at Days 105 and 135 for NR ewes with NonSGA fetuses. This indicates that NR ewes having SGA fetuses were not able to maintain a compensatory response even though they had equal depletion of body reserves compared to the NR ewes with NonSGA fetuses. The fetuses from all nutrient restricted ewes had similar concentrations of NEFA in their blood on Day 135 of pregnancy, which can be attributed to both availability from the ewe (for NR NonSGA fetuses) and increased NEFA production by the fetal liver as reported previously [19].

Allantoic fluid acts as a storage reservoir for nutrients and waste that can be easily moved into and out of fetal circulation by the substantial placental vascular network supporting the chorioallantoic membranes [41, 42]. This study confirmed that triglycerides are one of the nutrients stored in allantoic fluid, and that the concentrations are affected by NR. But concentrations are not only variable in NR ewes, they are predictive of SGA fetuses when sampled at Day 70 of pregnancy as evidenced by a negative correlation between concentrations of triglycerides and fetal weight at Day 135 of pregnancy. Day 70 of gestation is a dynamic period in pregnant ewes as the placenta is still growing and developing in preparation for exponential fetal growth during the last trimester of gestation. Therefore, the fetus must liberate triglycerides stored in allantoic fluid. Bile acids, along with lipases, are necessary for catabolism of triglycerides and high levels at Day 70 were correlated negatively with fetal weights on Day 135 of pregnancy. When analyzed conjointly, triglycerides and

Figure 2. Fatty acid transporters in placentomes. (A-D) Expression of *SLC27A1-4* mRNAs increased from Days 70 to 135 (** $P < 0.01$) of gestation, but there was no effect of treatment. (F) There was a day by treatment interaction for expression of *SLC27A6* mRNA as placentomes from NR SGA ewes had lower expression than placentomes from 100% NRC and NR NonSGA ewes at Day 70, but expression was lowest at Day 135 of gestation for all treatments (differing letters indicate differences at $P < 0.05$).

bile acids in allantoic fluid on Day 70 of pregnancy provided an even stronger predictor of fetal weight in late-gestation. Indeed, this combination of factors accounts for a significant proportion of variation in fetal weights similar to what is accounted for by variations in placental weights in this study and those of others, potentially making it a reliable biomarker for SGA pregnancies [28, 43–45].

Maternal concentrations of triglycerides and NEFA during early-to mid-pregnancy are positively associated with birthweight and childhood adiposity [46–48]. We reported that NonSGA fetuses from NR ewes had similar weights at Day 135 of pregnancy to those of fetuses from 100% NRC fed ewes, and both groups had greater fetal weights than SGA fetuses [27]. Fetal fluids were not sampled in the human studies, but the increase in concentrations of triglycerides in maternal plasma may indicate an increase in triglycerides available to support growth of the fetus and post-natal changes in adiposity. While there was no significant increase in triglycerides in plasma of ewes in the current study, the high concentrations of NEFA in maternal and fetal plasma in NR pregnancies with NonSGA fetuses may predispose the fetuses to adiposity in

post-natal life. Not only is this a concern in human health where long-term health consequences of childhood obesity include premature death, cardiovascular disease, and depression, but also in livestock where production efficiency and fertility would be severely impacted [49, 50].

Bile acids are synthesized from cholesterol in the liver to promote lipid absorption by aiding in the breakdown of triglycerides and acting as signaling molecules to regulate glucose metabolism [51]. Bile acid homeostasis is tightly regulated since high levels of bile acids, especially certain conjugates, are toxic [52]. Although concentrations of bile acids rise throughout pregnancy, abnormally high bile acid levels lead to cholestasis in human pregnancy, which is associated with abnormal vascularization of the placenta, vasoconstriction of placental blood vessels, and SGA fetuses [18]. Concentrations of bile acids rose throughout pregnancy in ewes fed 100% NRC, and then declined sharply near term, a pattern which was also seen in NR ewes with NonSGA fetuses. NR ewes with SGA fetuses had increases in bile acids that continued to increase to near term, as observed in individuals suffering from cholestasis.

Figure 3. SLC27A6 protein localization and quantification in placentomes. (A) Near the capsule, SLC27A6 protein immunolocalized to maternal LE, while near the chorioallantois, SLC27A6 localized to chorionic FE on Day 70 of gestation. At Day 135, localization was similar, but SLC27A6 protein was less abundant. (B) Total SLC27A6, as determined by western blot, was lower in placentomes from NR SGA ewes on Day 70 compared to those from 100% NRC and NR NonSGA ewes, but the abundance was similar for all groups at Day 135 of gestation. Width of fields is 820 μ m. Inset width of fields is 100 μ m. Legend: LE, luminal epithelium; FE, fetal epithelium; FS, fetal stroma; MS, maternal stroma; and FBV, fetal blood vessel.

In this study, concentrations of bile acids in blood of NR SGA fetuses were twice those for 100% NRC and NR NonSGA fetuses. This may result from abnormal production by fetal liver or, more likely, impaired transport of bile acids across the placenta. Multiple studies of bile acid transport across the sheep placenta indicated that the primary bile salts, chenodeoxycholate, and cholate, are predominately conjugated with taurine, and the resulting taurochenodeoxycholate and taurocholate are transported from the fetal to the maternal circulation, with very little maternal to fetal transfer of those bile salts or acids [53, 54]. This is in contrast to the other nutrients discussed in this study, which would be transported predominantly down a concentration gradient into the fetal-placental compartment. The bile acid gradient exists such that concentrations in fetal blood are higher than those in maternal blood. Thus, removal of those waste products protects the fetus from potentially toxic molecules that could come from the mother [55]. Regardless of the direction of movement, transport of bile acids is potentially impaired in SGA placentas due to decreased surface area and alterations in expression of bile acid transporters, which would allow bile acids

to accumulate in fetal blood [28]. This buildup of bile acids could potentially have detrimental effects on the fetal heart and lungs, or cause fetal distress resulting in stillbirth or perinatal mortality [18, 56, 57].

This is the first report of bile acids in allantoic fluid of pregnant sheep. Concentrations of bile acids increased in allantoic fluid as pregnancy progressed, but there was no effect of NR. There is evidence from research with humans and rats that the proportion of different types of bile acid is different in an adult as compared to a fetus [52]. Indeed, fetal lambs have a substantially greater proportion of chenodeoxycholate late in gestation compared to those for neonates and adults suggesting a major change in synthesis of bile acids at birth [58]. Further studies are warranted to determine the relative proportions of bile acids in allantoic fluid and in response to NR.

In early pregnancy, cholesterol is provided by the ewe, and then as the fetal liver becomes functional, it synthesizes most cholesterol in the fetal circulation [59]. In the present study, concentrations of cholesterol in maternal blood are greater in NR SGA ewes. While

concentrations of cholesterol in maternal blood increase in pregnancy, specifically in response to estrogens, there is little information regarding the consequences of the increases in cholesterol during pregnancy or its cause [12, 60]. Potentially, elevated cholesterol in maternal blood is due to a delayed functionality of the fetal liver and its ability to produce cholesterol, therefore, the mother is continuing to liberate her own cholesterol for us by the fetus. But by Day 135 of pregnancy, there were no differences among the three categories of fetuses regarding concentrations of cholesterol in their blood as all fetuses had adequate cholesterol available for cellular growth, and production of bile acids and steroids. Previous studies of cord blood in human infants found decreases in the high-density lipoprotein (HDL) fraction of cholesterol in SGA fetuses [61]. HDL was not determined in the present study, but there is the potential for similar results for fetal lambs.

Triglycerides are the storage form of fatty acids and cannot be transported in and out of cells. There is evidence that triglyceride synthesis occurs in the human placenta, but there is little information in the literature about triglycerides in the ruminant placenta [61]. Therefore, we assessed the gene expression of the enzymes involved in the pathway for synthesis of triglycerides in placentomes. The mRNAs for all enzymes required for synthesis of triglycerides were expressed in placentomes and expression of most of them increased between Days 70 and 135 of pregnancy. Interestingly, expression of *MGAT1* and *MGAT2* mRNAs decreased from Days 70 to 135 of pregnancy. Those two enzymes are from an alternative pathway for triglyceride synthesis in which monoacylglycerol is acylated to produce diacylglycerol [62]. The diacylglycerol then enters the traditional pathway for synthesis of triglycerides. There was little influence of NR, as only *AGPAT3*, an intermediate enzyme in the pathway, was expressed to a greater degree in placentomes of SGA fetuses from NR ewes. While there was little influence of NR on placental triglyceride synthesis enzymes, there was a decrease in plasma concentrations of triglycerides in SGA compared to Non-SGA fetuses. This could potentially be due to decreased triglyceride synthesis in the fetal circulation, as triglycerides themselves cannot be transported across membranes, and would allow the fetus to maintain plasma concentrations of NEFAs, a more readily usable form of energy.

Triglycerides, once they are broken down into NEFAs, must be transported across the placenta between the maternal and fetal circulations. These transporters, such as fatty acid transport protein family members SLC27A1-6 have been localized to placenta of humans and sheep [23, 26, 63], but this is the first study showing altered expression of SLC27A6 mRNA and protein placenta of NR ewes. SLC27A6 is differentially expressed in maternal or fetal tissue depending on the location within the placenta, and has lower total expression in placentomes of NR SGA ewes at mid-pregnancy. This transporter was of particular interest because it was the only family member studied that had higher expression in mid-pregnancy, when placental growth and development is occurring, compared to late pregnancy, when exponential fetal growth is occurring. Furthermore, in pregnant NR ewes, SLC27A6 was the only placental fatty acid transporter that was associated with fetal weight, which suggests that SLC27A6 has an influential role in providing fatty acids to the fetus.

The overall goal of placental transport, to move nutrients from mother to fetus and waste from fetus to mother, is the same between species, even though the placental types are variable. The hemochorial placental type found in humans results in maternal blood bathing fetal epithelial cells, which simplifies transport compared to the synepithelialchorial placental type of sheep where nutrients

must still be moved through an endothelium and, in some regions, an epithelium on the maternal side before ever reaching the fetal epithelium. But, there are similarities in nutrient transporters found in fetal placental cells between humans and sheep, including glucose and amino acid transporters [64, 65]. Indeed, SLC27A6 has been previously isolated to human fetal epithelium (syncytiotrophoblasts) [23], while also being localized to sheep fetal epithelium in this study.

Total weights and numbers of placentome were less for NR SGA ewes, indicating that there was less capacity for nutrient and waste transport. Indeed, placental weight is correlated with uterine and umbilical blood flow, so not only is there less surface area of placentomes in NR SGA ewes, but potentially decreased blood flow as well [28, 43, 66, 67]. These combined effects result in placental insufficiency, and impaired fetal growth. In this model, the NR NonSGA pregnancies do not reach placental insufficiency based on placental weights and numbers that are similar to those for 100% NRC pregnant ewes. The mechanism whereby this discrepancy in NR occurs remains to be elucidated.

Conclusion

Results of the present study revealed key differences in lipid metabolism in maternal, fetal, and placental tissues from NR ewes having either NonSGA or SGA fetuses. Negative correlations between concentrations of triglycerides and bile acids in allantoic fluid at mid-gestation with fetal weights near term, indicated that those molecules, when paired, may serve as biomarkers for compromised pregnancies. Finally, results highlight a potentially significant role for SLC27A6 fatty acid transport during the period of placental formation in delivering triglycerides to the fetus and preventing the development of SGA in NR ewes. Collectively, results of this study identified novel factors associated with placental adaptation to nutritional deficiencies, and those factors, particularly as they relate to fetal sex, can now be the focus of future research.

Supplementary material

Supplementary material is available at *BIOLRE* online.

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Conflict of interest

The authors have declared that no conflict of interest exists.

References

1. Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* 2006; 84:2316–2337.
2. Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Boileau P, Le Bouc Y, Deal CL, Lillycrop K, Scharfmann R, Sheppard A et al. Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* 2011; 32:159–224.
3. McMillen IC, MacLaughlin SM, Muhlhauser BS, Gentili S, Duffield JL, Morrison JL. Developmental origins of adult health and disease: the role of periconceptional and foetal nutrition. *Basic Clin Pharmacol Toxicol* 2008; 102:82–89.

4. Balasuriya CND, Stunes AK, Mosti MP, Schei B, Indredavik MS, Hals IK, Evensen KAI, Syversen U. Metabolic outcomes in adults born preterm with very low birthweight or small for gestational age at term: a cohort study. *J Clin Endocrinol Metab* 2018; **103**:4437–4446.
5. Delcurto H, Wu G, Satterfield MC. Nutrition and reproduction: links to epigenetics and metabolic syndrome in offspring. *Curr Opin Clin Nutr Metab Care* 2013; **16**:385–391.
6. Global Nutrition Report; 2018. globalnutritionreport.org. Accessed 23 July 2020.
7. Regnault TRH, Friedmann JE, Wilkening RB, Anthony RV, Hay WW. Fetoplacental transport and utilization of amino acids in IUGR - a review. *Placenta* 2005; **26**:S52–62.
8. Mayhew TM. A stereological perspective on placental morphology in normal and complicated pregnancies. *J Anat* 2009; **215**:77–90.
9. Newbern D, Freemark M. Placental hormones and the control of maternal metabolism and fetal growth. *Curr Opin Endocrinol Diabetes Obes* 2011; **18**:409–416.
10. Angueira AR, Ludvik AE, Reddy TE, Wicksteed B, Lowe WL, Layden BT. New insights into gestational glucose metabolism: lessons learned from 21st century approaches. *Diabetes* 2015; **64**:327–334.
11. Pascual MJ, Serrano MA, El-Mir MY, Macias RIR, Jiménez F, Marin JJG. Relationship between asymptomatic hypercholaemia of pregnancy and progesterone metabolism. *Clin Sci (Lond)* 2002; **102**:587–593.
12. Bartels A, Egan N, Broadhurst DI, Khashan AS, Joyce C, Stapleton M, O'Mullane J, O'Donoghue K. Maternal serum cholesterol levels are elevated from the 1st trimester of pregnancy: a cross-sectional study. *J Obstet Gynaecol (Lond)* 2012; **32**:747–752.
13. Butte NF. Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am J Clin Nutr* 2000; **71**:1256S–61S.
14. Leite DFB, Morillon AC, Júnior EFM, Souza RT, McCarthy FP, Khashan A, Baker P, Kenny LC, Cecatti JG. Examining the predictive accuracy of metabolomics for small-for-gestational-age babies: a systematic review. *BMJ Open* 2019; **9**:e031238.
15. Jin WY, Lin SL, Hou RL, Chen XY, Han T, Jin Y, Tang L, Zhu ZW, Zhao ZY. Associations between maternal lipid profile and pregnancy complications and perinatal outcomes: a population-based study from China. *BMC Pregnancy Childbirth* 2016; **16**:60.
16. Cal-Pereyra L, Benech A, González-Montaña JR, Acosta-Dibarrat J, Da Silva S, Martín A. Changes in the metabolic profile of pregnant ewes to an acute feed restriction in late gestation. *N Z Vet J* 2015; **63**:141–146.
17. Williamson C, Geenes V. Intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 2014; **124**:120–133.
18. McIlvride S, Dixon PH, Williamson C. Bile acids and gestation. *Mol Aspects Med* 2017; **56**:90–100.
19. Xue Y, Guo C, Hu F, Zhu W, Mao S. Maternal undernutrition induces fetal hepatic lipid metabolism disorder and affects the development of fetal liver in a sheep model. *FASEB J* 2019; **33**:9990–10004.
20. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE, Wu G. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. *J Nutr* 2010; **140**:1242–1248.
21. Wallace JM, Milne JS, Aitken RP, Horgan GW, Adam CL. Ovine prenatal growth restriction impacts glucose metabolism and body composition throughout life in both sexes. *Reproduction* 2018; **156**:103–119.
22. Dunlap KA, Brown JD, Keith AB, Satterfield MC. Factors controlling nutrient availability to the developing fetus in ruminants. *J Anim Sci Biotechnol* 2015; **6**:16.
23. Chassen SS, Ferchaud-Roucher V, Gupta MB, Jansson T, Powell TL. Alterations in placental long chain polyunsaturated fatty acid metabolism in human intrauterine growth restriction. *Clin Sci* 2018; **132**:595–607.
24. Tian L, Dong SS, Hu J, Yao JJ, Yan PS. The effect of maternal obesity on fatty acid transporter expression and lipid metabolism in the full-term placenta of lean breed swine. *J Anim Physiol Anim Nutr (Berl)* 2018; **102**:e242–e253.
25. Cetin I, Parisi F, Berti C, Mandó C, Desoye G. Placental fatty acid transport in maternal obesity. *J Dev Orig Health Dis* 2012; **3**:409–414.
26. Zhu MJ, Ma Y, Long NM, Du M, Ford SP. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. *Am J Physiol Regul Integr Comp Physiol* 2010; **299**:R1224–R1231.
27. Sandoval C, Lambo CAA, Beason K, Dunlap KAA, Satterfield MCC. Effect of maternal nutrient restriction on skeletal muscle mass and associated molecular pathways in SGA and non-SGA sheep fetuses. *Domest Anim Endocrinol* 2020; **72**:106443.
28. Edwards AK, McKnight SM, Askelson K, McKnight JR, Dunlap KA, Satterfield MC. Adaptive responses to maternal nutrient restriction alter placental transport in ewes. *Placenta* 2020; **96**:1–9.
29. NRC, 6th ed. Washington DC: National Academy of Sciences; 1985.
30. Lambo CA, Edwards AK, Bazer FW, Dunlap K, Satterfield MC. Development of a surgical procedure for removal of a placenta from a pregnant ewe during gestation. *J Anim Sci Biotechnol* 2020; **11**:48.
31. Steinhauser CB, Landers M, Myatt L, Burghardt RC, Vallet JL, Bazer FW, Johnson GA. Fructose synthesis and transport at the uterine-placental interface of pigs: cell-specific localization of SLC2A5, SLC2A8, and components of the Polyol pathway. *Biol Reprod* 2016; **95**:108.
32. Gilda JE, Gomes AV. Western blotting using in-gel protein labeling as a normalization control: stain-free technology. *Methods Mol Biol* 2014; **1295**:381–391.
33. Moritz CP. Tubulin or not tubulin: heading toward total protein staining as loading control in western blots. *Proteomics* 2017; **17**.
34. Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. *Theriogenology* 2008; **69**:55–67.
35. Pardi G, Cetin I. Human fetal growth and organ development: 50 years of discoveries. *Am J Obstet Gynecol* 2006; **194**:1088–1099.
36. Vuguin PM. Animal models for small for gestational age and fetal programming of adult disease. *Horm Res* 2007; **68**:113–123.
37. Morrison JL, Berry MJ, Botting KJ, Darby JRT, Frasch MG, Gattford KL, Giussani DA, Gray CL, Harding R, Herrera EA, Kemp MW, Lock MC et al. Improving pregnancy outcomes in humans through studies in sheep. *Am J Physiol Regul Integr Comp Physiol* 2018; **315**:R1123–R1153.
38. Satterfield MC, Dunlap KA, Keisler DH, Bazer FW, Wu G. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. *Amino Acids* 2013; **45**:489–499.
39. Jones AK, Hoffman ML, Pillai SM, McFadden KK, Govoni KE, Zinn SA, Reed SA. Gestational restricted- and over-feeding promote maternal and offspring inflammatory responses that are distinct and dependent on diet in sheep. *Biol Reprod* 2018; **98**:184–196.
40. Tygesen MP, Nielsen MO, Nørgaard P, Ranvig H, Harrison AP, Tauson AH. Late gestational nutrient restriction: effects on ewes' metabolic and homeostatic adaptation, consequences for lamb birth weight and lactation performance. *Arch Anim Nutr* 2008; **62**:44–59.
41. Reynolds LP, Redmer DA. Utero-placental vascular development and placental function. *J Anim Sci* 1995; **73**:1839–1851.
42. Bazer FW. Allantoic fluid: regulation of volume and composition. In: Brace R, Ross M, Robillard J (eds.), *Reproductive and Perinatal Medicine*, 11th ed. Ithaca, NY: Perinatology Press; 1989: 135–155.
43. Bell AW, Ehrhardt RA. Regulation of placental nutrient transport and implications for fetal growth. *Nutr Res Rev* 2002; **15**:211–230.
44. Long NM, Vonnahme KA, Hess BW, Nathanielsz PW, Ford SP. Effects of early gestational undernutrition on fetal growth, organ development, and placental composition in the bovine. *J Anim Sci* 2009; **87**: 1950–1959.
45. Morrison JL. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clin Exp Pharmacol Physiol* 2008; **35**:730–743.
46. Gademan MGJ, Vermeulen M, Oostvogels AJJM, Roseboom TJ, Visscher TLS, van Eijsden M, Twickler MTB, Vrijkotte TGM. Maternal prepregnancy BMI and lipid profile during early pregnancy are independently associated with offspring's body composition at age 5–6 years: The ABCD study. *PLoS One* 2014; **9**:e94594.
47. CDC Overweight & Obesity; 2017. www.cdc.gov. Accessed 7 February 2020.

48. Martin CL, Vladutiu CJ, Zikry TM, Grace MR, Siega-Riz AM. Maternal lipid levels during pregnancy and child weight status at 3 years of age. *Pediatr Obes* 2019; **14**:e12485.
49. Ryder JR, Xu P, Inge TH, Xie C, Jenkins TM, Hur C, Lee M, Choi J, Michalsky MP, Kelly AS, Urbina EM. Thirty-year risk of cardiovascular disease events in adolescents with severe obesity. *Obesity* 2020; **28**:616–623.
50. Lindberg L, Hagman E, Danielsson P, Marcus C, Persson M. Anxiety and depression in children and adolescents with obesity: a nationwide study in Sweden. *BMC Med* 2020; **18**:30.
51. Chiang JYL. Bile acid metabolism and signaling. *Compr Physiol* 2013; **3**:1191–1212.
52. Macias RI, Marin JJ, Serrano MA. Excretion of biliary compounds during intrauterine life. *World J Gastroenterol* 2009; **15**: 817.
53. Sewell RB, Hardy KJ, Smallwood RA, Hoffman NE. Fetal bile salt metabolism: placental transfer of taurocholate in sheep. *Am J Physiol Gastrointest Liver Physiol* 1980; **2**:354–357.
54. Sewell RB, Hardy KJ, Smallwood RA, Hoffman NE. Fetal bile salt metabolism: placental transfer of dihydroxy bile salts in sheep. *Am J Physiol Gastrointest Liver Physiol* 1982; **6**:G172–5.
55. Macias RIR, Pascual MJ, Bravo A, Alcalde MP, Larena MG, St-Pierre MV, Serrano MA, Marin JJG. Effect of maternal cholestasis on bile acid transfer across the rat placenta-maternal liver tandem. *Hepatology* 2000; **31**:975–983.
56. Zecca E, De Luca D, Baroni S, Vento G, Tiberi E, Romagnoli C. Bile acid-induced lung injury in newborn infants: a bronchoalveolar lavage fluid study. *Pediatrics* 2008; **121**:e146–9.
57. Williamson C, Gorelik J, Eaton BM, Lab M, de SM, Korchev Y. The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intra-uterine fetal death in obstetric cholestasis. *Clin Sci* 2001; **100**:363–369.
58. Hardy KJ, Hoffman NE, Mihaly G, Sewell RB, Smallwood RA. Bile acid metabolism in fetal sheep; perinatal changes in the bile acid pool. *J Physiol* 1980; **309**:1–11.
59. Herrera E, Amusquivar E, López-Soldado I, Ortega H. Maternal lipid metabolism and placental lipid transfer. *Horm Res* 2006; **65**:59–64.
60. Bartels A, O'Donoghue K. Cholesterol in pregnancy: a review of knowns and unknowns. *Obstet Med* 2011; **4**:147–151.
61. Hulme CH, Nicolaou A, Murphy SA, Heazell AEP, Myers JE, Westwood M. The effect of high glucose on lipid metabolism in the human placenta. *Sci Rep* 2019; **9**:14114.
62. Lee YJ, Kim JW. Monoacylglycerol O-acyltransferase 1 (MGAT1) localizes to the ER and lipid droplets promoting triacylglycerol synthesis. *BMB Rep* 2017; **50**:367–372.
63. Anderson CM, Stahl A. SLC27 fatty acid transport proteins. *Mol Aspects Med* 2013; **34**:516–528.
64. Brett KE, Ferraro ZM, Yockell-Lelievre J, Gruslin A, Adamo KB. Maternal-fetal nutrient transport in pregnancy pathologies: the role of the placenta. *Int J Mol Sci* 2014; **15**:16153–16185.
65. Ma Y, Zhu MJ, Uthlaut AB, Nijland MJ, Nathanielsz PW, Hess BW, Ford SP. Upregulation of growth signaling and nutrient transporters in cotyledons of early to mid-gestational nutrient restricted ewes. *Placenta* 2011; **32**:255–263.
66. Vonnahme KA, Lemley CO, Caton JS, Meyer AM. Impacts of maternal nutrition on vascularity of nutrient transferring tissues during gestation and lactation. *Nutrients* 2015; **7**:3497–3523.
67. Owens JA, Falconer J, Robinson JS. Effect of restriction of placental growth on umbilical and uterine blood flows. *Am J Physiol* 1986; **250**:R427–R434.