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Tissue cell stress response to obesity and its interaction with late gestational diet

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Abstract

Intra-uterine growth restriction in late pregnancy can contribute to adverse long term metabolic health in the offspring. We utilised an animal (sheep) model of maternal dietary manipulation in late pregnancy, combined with exposure of the offspring to a low activity, obesogenic environment after weaning, to characterise the effects on glucose homeostasis. Dizygotic twin-pregnant sheep were either fed to 60% of requirements (nutrient restriction (R)) or fed ad libitum (~ 140% of requirements (A)) from 110 days gestation until term (~147d). After weaning (~3 months of age), their offspring were kept in either a standard (in order to remain lean) or low activity, obesogenic environment. R mothers gained less weight and produced smaller offspring. As adults, obese offspring were heavier and fatter with reduced glucose tolerance, irrespective of maternal diet. Molecular markers of stress and autophagy in liver and adipose tissue were increased with obesity, with gene expression of hepatic *Grp78* and of omental *Atf6*, *Grp78* and *Edem1* only being increased in R offspring. In conclusion, the adverse effect of juvenile onset obesity on insulin responsive tissues can be amplified by previous exposure to a suboptimal nutritional environment in utero, thereby contributing to earlier onset of insulin resistance.

Introduction

Obesity and the associated metabolic syndrome pose an increasing burden on contemporary society. Low-grade inflammation, in conjunction with obesity, is a primary mechanism in the development of insulin resistance and cardiovascular disease (Adabimohazab *et al.* 2016). There is increasing evidence from both human and animal studies that the risk for these diseases can be enhanced by a suboptimal perinatal environment (de Rooij *et al.* 2007; Sartori *et al.* 2016). *In utero* development can be influenced through several factors, including placental insufficiency or maternal undernutrition, through reduced availability of oxygen, nutrients and hormones to the fetus. If maternal food intake is suboptimal in late pregnancy, coincident with maximal fetal energy requirements and absolute growth rate, intra-uterine growth restriction (IUGR) occurs leading to reduced birth weight (Mumbare *et al.* 2012), which has been linked to a range of non-communicable diseases in adults (Barker 1997).

Most organs and cells are regularly exposed to stimuli with the potential to cause cellular damage or cell death. These normally originate from within the cell, including misfolding of proteins, accumulation of metabolites including free fatty acids (FFA), energy deficit and activation of inflammatory pathways, which the cell responds to through a number of pathways (Fulda *et al.* 2010). The magnitude of cellular responses are dependent on several factors including the type and severity of insult, cell type and its adaptive capacity (Fulda *et al.* 2010). Cell stress response pathways are innate cellular mechanisms limiting or reversing the effect of metabolic challenges, and play a significant role in the physiological and pathological processes of development, ageing and disease (Schröder & Kaufman 2005). These pathways include the unfolded protein response (UPR) and autophagy, which are activated in response to both

61 nutritional deprivation and obesity (Nuñez *et al.* 2013). If those mechanisms do not sufficiently
62 limit the effect of an insult, cell death is activated through apoptosis, autophagy or necrosis.
63 Glucose-related protein (GRP)78 and endoplasmic reticulum (ER) stress degradation enhancer
64 molecule (EDEM) are markers for the UPR as they both bind to malfolded proteins, a process
65 enhanced through activation transcription factor (ATF)6, which reflects the amount of malfolded
66 protein within the ER (Yoshida *et al.* 2003). A second ER membrane-bound protein that
67 responds to stress is PRKR-like ER kinase (PERK), which induces activation transcription factor
68 (ATF)4 (B'chir *et al.* 2013), which then initiates the formation of the autophagosome if ER stress
69 exceeds the pro-survival processing capacity of UPR. This includes the molecules autophagy-
70 related gene 12 (ATG12) and Beclin 1 (Ohsumi 2001).
71
72 Obesity promotes the cell stress response in a range of organs including visceral adipose tissue
73 and liver, but whether these adaptations can be programmed *in* utero is unknown. Previous
74 studies have focussed on fat surrounding either the kidneys or heart (Sharkey *et al.* 2009a; Ojha
75 *et al.* 2015), but the extent to which other depots may be nutritionally programmed has not been
76 extensively investigated. One of the largest fat depots in adult sheep is the omental depot (Arana
77 *et al.* 2008) and has been suggested to be sensitive to nutritional programming. For example, in
78 an ovine surgical model of IUGR (i.e. carunclectomy), phosphorylation of omental AMPK was
79 reduced in offspring as measured 21 days after birth, consistent with increased postnatal weight
80 gain (Lie *et al.* 2013). Whilst, a bovine nutritional model of IUGR (i.e. consumption of a low
81 protein diet from mid-gestation), omental adipose tissue sampled from adult offspring exhibited
82 lower gene expression of insulin-like growth factor receptor 1 and 2 (*Igf1r* and *Igf2r*) and *Igf2*
83 whereas *Leptin* gene expression was raised (Micke *et al.* 2011), showing that the omental

adipose tissue is sensitive to long-term programming of adipocyte proliferation. Leptin is primarily produced in adipose tissue (Trayhurn *et al.* 1998) and stimulates hepatic oxidation of fatty acids through activation of AMPK (Minokoshi *et al.* 2001), in excess can contribute to liver disease (Zain *et al.* 2013), the extent of which will be determined both by plasma leptin concentration and the hepatic sensitivity mediated by the leptin receptor (Zain *et al.* 2013).

In the present study we hypothesised that juvenile onset obesity causes cell stress and inflammation responses in adipose tissue and liver. We hypothesised further that the effect is enhanced by *in utero* exposure to maternal nutrient restriction. We utilized a sheep model of nutritionally induced IUGR as compared to animals who were fed in excess in late pregnancy. This was followed by obesity induced by maintenance in an environment of restricted physical activity, and were compared to offspring with unrestricted activity, that remained lean. We have previously reported that adult glucose tolerance was lower in IUGR offspring as compared to offspring of mothers who were fed to requirements throughout pregnancy when exposed to an obesogenic environment after weaning (Dellschaft *et al.* 2015). In the current study we compared maternal over- and undernutrition in late pregnancy and whether offspring metabolic health was further influenced by obesity. In young adulthood, all animals were assessed for glucose tolerance, together with the metabolic and inflammatory characteristics of omental fat and liver.

Materials and Methods

Animals and experimental design

All animal procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 with approval from the Local Ethics Committee of the University of Nottingham. In brief, 19 Bluefaced Leicester cross Swaledale twin bearing sheep (*ovis aries*) were individually housed at 100 days of gestation (dGA) and, at day 110 dGA, randomly allocated to the experimental groups (for study overview, see Supplementary Figure 1). They included a calorically restricted group (R, n=9; 0.28 MJ/kg.$BW^{0.75}$ at 110 days gestation, increasing to 0.43 MJ/kg.$BW^{0.75}$ at dGA 130), receiving 60% of nutritionally required feed based on their body weight, and a group fed *ad libitum* (A, n=10; equal to approximately 140% nutritionally required feed, 0.64 MJ/kg.$BW^{0.75}$ at 110 days gestation, increasing to 1.01 MJ/kg.$BW^{0.75}$ at dGA 130). All sheep were individually weighed once a week prior to feeding in order that their total food requirements could be adjusted. All pregnancies continued normally until term (~145 ± 1 days) and produced heterozygous twins. Twins were raised by their mothers who were fed to 100% requirements during lactation and weaned at 3 months of age. After weaning, half of the offspring, i.e. one twin per mother, were kept in a low activity environment until 17 months of age in order to promote obesity (O, 6 animals on 19 m^2, fed *ad libitum* on straw nuts and a micronutrient supplement; RO, n=7, 2 males and 5 females; AO, n=10, 7 males, 3 females), the other half were kept in a normal physical activity environment, in order to remain lean (L, 6 animals on 1125 m^2, *ad libitum* access to grass and a micronutrient supplement; RL, n=9, 5 males and 4 females; AL, n=9, 6 males and 3 females). Discrepancies between the total number (n) of mothers and offspring are due to the death of 4 offspring before the end of the study, a loss of 10% of the total population, a standard mortality rate in sheep studies (Berger 1997; Dwyer 2007).

The numbers of twin bearing mothers entered into the study for each nutritional group were expected to produce sufficient numbers of male and female offspring for each of the postnatal intervention groups. However due to the uneven distribution of male and females born to *ad libitum* fed mothers there were fewer female offspring available than anticipated. The resulting groups permit us to draw comparisons between animals with IUGR and offspring of mothers exposed to overnutrition in late pregnancy (R *vs*. A) and, within those with IUGR and maternal overnutrition, to investigate the effects of post-weaning environment (RO *vs.* RL and AO *vs.* AL).

Timing of samplings and *in vivo* challenges

Maternal blood sampling: At 130 dGA, jugular venous blood samples (5 ml) were collected from the ewes in the morning, prior to feeding. Venous blood was collected into heparinized and K^+EDTA coated tubes and the plasma was immediately separated by centrifugation (2500 g x 10 min at 4˚C) and stored at -80˚C until analysis.

Offspring blood sampling: Venous blood samples (prepared and stored under identical conditions as described above) were collected after an overnight fast (≥18h) at both 7 and 16 months of age. Jugular catheters were inserted by percutaneous venipuncture 1-2 days before sampling.

Determination of insulin sensitivity: Glucose tolerance tests (GTT) were undertaken on all offspring at 7 and 16 months of age in which jugular vein catheters had been previously inserted and the area under the curve (AUC) calculated. Animals were fasted overnight (≥18 h) and injected intravenously with 0.5 g/kg glucose. Glucose and insulin concentrations were measured in plasma samples before and at 10, 20, 30, 60, 90, and 120 minutes after the intravenous glucose (Gardner

148 *et al.* 2005). The homeostatic model assessment for insulin resistance (HOMA-IR) index was
149 calculated by multiplication of glucose (mmol/L) and insulin (μg/L) concentrations measured in
150 fasted plasma (Wallace *et al.* 2004).
151 **Determination of physical activity at 15 months of age:** The level of spontaneous physical
152 activity in adulthood in their respective environments was determined using uniaxial
153 accelerometers (Actiwatch; Linton Instrumentation, Diss, UK).
154 **Determination of body composition at 16 months of age:** Total body fat was determined when
155 the animal was sedated (intramuscular injection of 1.5 mg/kg ketamine with 0.1 mg/kg xylazine)
156 and scanned in a transverse position using a Lunar DPX-L (fast-detail whole body smartscan, GE
157 Healthcare, Little Chalfont, UK).
158 **Post mortem procedures and tissue collection:** At 17 months of age, all offspring were
159 euthanased by electrical stunning and exsanguination after an overnight fast. The entire liver and
160 omental, pericardial and perirenal adipose tissue were dissected, weighed, and representative
161 subsections immediately flash frozen in liquid nitrogen. Samples were stored frozen at -80˚C until
162 analysis.
163
164 **Laboratory analysis**
165 **Plasma metabolites and hormones**
166 Plasma glucose was measured by colorimetric assays (Randox, Crumlin, UK). Insulin was assayed
167 using an ovine specific ELISA assay (Mercodia, Diagenics Ltd, Milton Keynes, UK). Leptin
168 (Delavaud *et al.* 2000) and cortisol (Dellschaft *et al.* 2015) were determined by a radio-
169 immunoassay.

Gene expression measurements

Representative samples of each tissue were homogenized and RNA isolated, using the RNeasy Plus mini kit (Qiagen, Hilden, Germany), quantified by Nanodrop (Thermo, Epsom, UK). An aliquot of 2 µg of RNA was reverse transcribed with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was amplified in a real-time thermocycler (Quantica, Techne, Burlington, NJ, USA) using a SYBR green system in Taq polymerase reaction mix (ABsolute blue QPCR SYBR green, Thermo Scientific, Epsom, UK). Specificity of primers was confirmed by sequencing PCR product (Supplementary Table 1). Liver and omental adipose tissue gene expression was assessed for the following pathways: a) inflammation: toll-like receptor 4 (*Tlr4*), 11β hydroxysteroid dehydrogenase 1 (*11bhsd1*) and Fas cell surface death receptor (*Fas*); b) autophagy: *Beclin1* and *Atg12*; c) UPR: *Edem1, Grp78, Atf4* and *Atf6*); d) energy sensing: 5' AMP-activated protein kinase (*Ampk*) and mammalian target of rapamycin (*Mtor*); and *Leptin* that was only measured in fat and the leptin receptor (*Obr*), measured in liver. Large ribosomal protein (*Rpo*) and tyrosine-3 monoxygenase/ tryptophan-3 monoxygenase activation protein (*Ywhaz*) showed a stable expression and the geometric means of their expression were used as a reference for the gene of interest in liver. *Rpo* and 60S ribosomal protein (*RP*) *L19* showed a stable expression and the geometric means of their expression were used as a reference for the gene of interest in omental adipose tissue. Gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Liver triglyceride (TG) quantification

Frozen liver (~150 mg) was homogenized in 2 ml 2:1 chloroform:methanol and agitated thoroughly for 20 minutes. Samples were filtered to remove debris, washing the filter and debris with a further 8 ml of chloroform to dissolve and collect any remaining lipids. Phases were

separated by adding 2 ml saline and centrifugation at 800g for 10 minutes. 2 ml of the chloroform phase were transferred and all liquid evaporated under nitrogen, then the remaining lipid re-dissolved in 100 µl tert-butanol with Triton X (60:40 v/v). TG were then determined with a colorimetric assay (Randox, as above).

Adipose tissue immunohistochemistry

Formaldehyde-fixed samples of omental adipose tissue were blocked in paraffin and sectioned to 6 µm. Slides were stained for GRP78 (SPA-826, Enzo Life Sciences, Exeter, UK; 1:200) and pJNK (SC6254, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:75) with a horseradish peroxidase – 3,3-diaminobenzidine (HRP-DAB) system on the Bondmax (Leica biosystems, Milton Keynes, UK), an automated slide processor. Stained slides were imaged with Nikon Eclipse 90i microscope with CCD high-speed colour camera (Micropublisher 3.3RTV; Qimaging, Surrey, BC, Canada) under constant conditions and analysed with Volocity 6 software (Improvision Ltd, Coventry, UK, see representative images in Supplemental Figure S2). Staining was digitally quantified using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA) after correcting all images for background staining by selecting brown pixels only, applying an appropriate threshold to exclude false-negative staining, and measuring the area stained per cell as well as adipocyte size, averaged for 500 cells per sample.

Statistical analysis

Statistical analysis of the data was performed using PASW® software (v 19, IBM, Chicago, USA). Kolmogorov-Smirnoff tests were performed on every parameter analyzed to determine the Gaussian distributions of the variables. Briefly, for the factorial study design the data was first interrogated with two-way analysis of variance (2-way ANOVA). Upon identification of a significant effect or interaction on the 2-way ANOVA, a hypothesis driven simple main effects

analysis was then performed for comparison between groups differing in only one factor (prenatal nutrient restriction or environment of rearing). Although the 2-way ANOVA is considered a robust test for analyses of data which is not normally distributed, non-parametric testing using Kruskal-Wallis test followed by Mann-Whitney was performed for any such data for confirmation of the ANOVA findings. All data is expressed as mean and standard error of the mean. If a variable did not have parametric distribution, the finding of an effect was confirmed by using a Kruskal-Wallis test followed by a Mann-Whitney test for the groups concerned. Correlation analysis was done by Pearson's test on parametric data.

Each variable was tested for difference determined by the sex of the animals. Body weight and fat mass are known to differ, in absolute scale, between male and female sheep (Bloor *et al.* 2013) thus sex-specific Z-score transformation was used prior to analyses.

Results

Mothers and offspring: As we have previously published (Dellschaft *et al.* 2015), R mothers gained less weight than those fed *ad* libitum (Figure 1). At 130 dGA fasted R mothers had significantly higher plasma NEFA concentrations but lower insulin and glucose concentrations, whereas triglyceride and cortisol concentrations were unaltered by maternal diet (Figure 2). R offspring were smaller at birth (4.07 ± 0.14 *vs*. 4.63 ± 0.16 kg, P=0.02) and remained so until 24 days of age. After weaning all animals were similar in weight and following exposure to reduced physical activity, plasma leptin was raised from 7 months of age, with body weight increasing by 15 months of age (Table 1). As expected, obese animals had a substantially lower mean activity as measured by accelerometer than L animals at 15 months of age. Obese animals were heavier, had more relative total and visceral fat mass as measured by DEXA and, at dissection, had heavier omental, pericardial and perirenal adipose depots than L animals. Maternal nutrition did not influence any of these measures of obesity with AO offspring having higher leptin than their lean counterparts.

Insulin sensitivity: At 7 months, peak plasma glucose was raised with obesity up to 60 minutes after glucose injection (Figure 3a), as was their AUC (Table 2). Basal insulin was similar between all groups but plateaued at a higher value after 60 minutes in AO animals (Figure 3a). HOMA-IR did not differ with either intervention at this age, but was higher in AO as compared to AL at 16 months of age. At this time point glucose concentrations were the same between all groups whereas insulin was higher in obese than lean groups (Figure 3b). This was reflected in the insulin AUC, which was raised with obesity (Table 2). At both time points, plasma NEFA (1.30 ± 0.08 mmol/l at 7 months; 0.43 ± 0.03 mmol/l at 16 months) and TG (0.17 ± 0.01 mg/dl at

7 months; 0.14 ± 0.01 mg/dl at 16 months) concentrations did not differ in the fasted state, showing that dyslipidaemia is not a programmed effect when comparing these pre- and postnatal interventions. Overall, glucose tolerance appeared to improve with age but was only accompanied with modified insulin sensitivity in lean but not obese animals (Figure 3).

Liver gene expression: Livers were heavier and had a higher lipid content in RO than in RL whereas the same effect could not be seen in A offspring (Table 3). Total liver TG was associated with liver weight in obese (r=0.662, $P<0.001$) but not in lean animals (r=0.364, $P=0.07$). Expression of *Beclin1* was higher in AO than in AL but much more strongly upregulated in RO as compared to RL and AO groups. *Atf4* expression showed an interaction between maternal and post weaning environment, and was downregulated with obesity in A but upregulated in R offspring, with a significantly higher expression in AL than in RL. *Atg12*, *Edem1* and *Grp78* were upregulated with obesity, with a more pronounced difference in *Atg12* and *Grp78* in R as opposed to A offspring whereas *Atf6*, *11bhsd1*, *Obr* and *Fas* were unchanged (Table 3).

Omental adipose tissue histology and gene expression: Adipocytes of obese offspring were significantly larger than those of lean animals (Figure 4) and GRP78 protein doubled whereas pJNK was unchanged (Table 4). Obesity upregulated gene expression of *Leptin*, *Tlr4*, *Cd68*, *Atf4*, *Atg12* and *Beclin1* in both A and R offspring (Table 4 and Figure 5). In contrast, expression of *Atf6*, *Grp78* and *Edem1* were increased with obesity in R but not A offspring (Figure 5), whilst *11bhsd1* and *Gcr* were unchanged.

Discussion

We have shown that the onset of insulin resistance can be induced in early adult life following the induction of obesity after weaning by restricting physical activity. This adaptation in insulin response to a glucose challenge with age occurred in conjunction with enhanced cell stress and inflammation responses in adipose tissue and liver. Prior exposure to suboptimal maternal nutrition through late pregnancy induced IUGR but only resulted in a subtle amplification of these long-term effects as compared to maternal overnutrition in late pregnancy. This is not unexpected given the extended time span required in large mammals to observe the adverse effects of a compromised *in utero* environment (Symonds *et al.* 2016). In addition, the magnitude of response can be modified by gender (Bloor *et al.* 2013) but due to an unexpected imbalance of the number of males and females reaching adulthood we could not examine this aspect further.

Glucose tolerance was diminished by obesity but was not altered by prenatal intervention

Glucose metabolism was impaired in 7 month old offspring subjected to the obesogenic environment despite no difference in body weight, suggesting that physical inactivity resulted in morphological changes in muscles that act to improve glucose tolerance (Hollenbeck *et al.* 1985). Additionally, increased plasma leptin indicates greater fat mass (Considine *et al.* 1996). By 16 months of age, although glucose tolerance improved compared to 7 months, obese offspring demonstrated raised insulin secretion, suggesting reduced sensitivity, but without any further impact of prenatal diet. Studies in humans demonstrate that the development of obesity related peripheral insulin resistance is secondary to obesity from as early as 6-12 years of age (Yoshinaga *et al.* 2006). Late gestational nutrient restriction was predicted to reduce insulin

resistance as shown in adult offspring of mothers exposed to the Dutch famine during late gestation (Ravelli *et al.* 1998), as we have seen previously (Dellschaft *et al.* 2015). In this earlier study we compared obese offspring subjected to either 60 or 100% of total calculated ME requirements in late gestation, although this is less than the amount of food such animals would consume if allowed to feed *ad libitum* (Budge *et al.* 2000). In the present study, both groups had further undergone accelerated growth in early postnatal life by only allowing one twin offspring to stay with their mother and effectively feed more before weaning. These contrasting outcomes may be indicative of a U-shaped association between early growth and glucose tolerance in later life (Rich-Edwards *et al.* 1999), i.e. both low and high birth weight are associated with reduction in glucose tolerance, therefore minimising any differences between the groups discussed here.

From the Dutch Famine cohort studies (de Rooij *et al.* 2007) we would have expected an increased risk for dyslipidaemic profiles as well as insulin resistance in animals exposed to late gestational nutrient restriction but there was no indication of this. Lipid metabolism in ruminants is very different compared to humans (Nafikov & Beitz 2007). In ruminants, the liver contributes little to fatty acid synthesis whilst adipose tissue is the primary site for this (Vernon 1980). It is plausible that ruminants are more resistant to plasma lipid abnormalities with insulin resistance because of the relatively low contribution of the liver to triglyceride production. The absence of any differences in plasma triglycerides in previously published sheep studies, despite the presence of abnormal glucose-insulin homeostasis (Gardner *et al.* 2005) supports such a proposal.

IUGR exacerbates obesity-induced elevation of hepatic lipid content and autophagy gene expression

Raised hepatic TG content is indicative of impaired liver function that is enhanced in adult individuals born at a low weight (Nobili *et al.* 2007; Fraser *et al.* 2008) who are more likely to exhibit non-alcoholic fatty liver disease (NAFLD). This adaptation is in accord with that seen in obese offspring exposed to sub-optimal maternal nutrition between early and mid-gestation (Hyatt *et al.* 2011) without any change in birth weight. Gene markers of both autophagy (i.e. *Atf4* and *Atg12*) and ER stress (i.e. *Grp78*) were upregulated more strongly in IUGR offspring following obesity. When nutritionally manipulated offspring are subjected to an obesogenic environment comprising increased food intake and low activity, raised hepatic lipid was accompanied with enhanced gene expression of *Pparg* and *Pgc1a*, that is indicative of reduced beta-oxidation (Hyatt *et al.* 2011). Obesity enhances the expression of other markers of hepatic ER stress (Ozcan *et al.* 2004; Gregor *et al.* 2009), including *Edem1*. Activation of UPR in response to ER stress can induce autophagy through activation of *Atf4* through the *Perk* pathway (B'chir *et al.* 2013). Constitutive autophagy in hepatic cells normally promotes lipid disposal, thereby improving their metabolism, together with insulin sensitivity and cell survival (Singh *et al.* 2009; Yang *et al.* 2010). Plasma lipids would then be raised in conjunction with an unchanged or lower hepatic TG content but the absence of such an adaptation may be due to insufficient lipid disposal through autophagy. Raised expression of genes involved in autophagy with obesity can paradoxically be associated with impaired autophagic flux (Yang *et al.* 2010; González-Rodríguez *et al.* 2014) which then progresses to NAFLD (Amir & Czaja 2011). Such a defect in the process of autophagy would promote additional lipid deposition in the liver and

ultimately compromise hepatic function and exacerbate the adverse effect of insulin resistance with IUGR.

IUGR and omental adipose tissue size, autophagy-related gene expression and the ER stress response to obesity

The post weaning low physical environment induced a higher total and visceral adipose mass, with a three-fold heavier omental adipose depot and increased adipocyte size, suggesting hypertrophy. Inflammation of visceral fat could be the underlying reason for the higher risk of insulin resistance and metabolic syndrome risk seen with IUGR (de Rooij *et al.* 2007). However, we found that even though the omental depot had a higher gene expression of leptin and markers of infiltration by immune cells (e.g. *Cd68* and *Tlr4*) with obesity this was not influenced by IUGR. Omental fat only develops after birth in sheep (Bryden *et al.* 1972) as it is not detectable in late gestation fetus (M.E. Symonds, unpublished), which could explain its resistance to any programming effects during late pregnancy. In contrast, perirenal adipose tissue develops *in utero* and exposure to suboptimal maternal nutrition between mid to late gestation results in a higher inflammatory response in obese one year old offspring (Sharkey *et al.* 2009a, b). However, in the absence of larger sheep studies focusing on depot-specific differential gene expression at defined stages of development and growth a more precise explanation is unknown. Mechanisms of intrinsic cell stress response, such as autophagy, UPR and ER stress, could be more sensitive indicators of metabolic inflammation than markers of immune cells infiltrated into adipose tissue.

Gene expression of autophagic genes *Atg12* and *Beclin1* and of energy-sensing gene *Ampk* were increased with obesity, but not influenced by IUGR. In contrast, the UPR was induced with obesity in R but not A offspring. The three genes that were promoted in this pattern, *Atf6*, *Edem1* and *Grp78*, are regulated through the same transcriptional regulators, which are the ER stress response element II (ERSE-II) and UPR element (UPRE), that are both activated by ATF6 and IRE1 (Zhang & Kaufman 2004). Induction of ER stress causes inflammation and insulin resistance (Ozcan *et al.* 2004). GRP78 protein expression was also clearly upregulated with obesity, indicating greater UPR (Cnop *et al.* 2012) and concomitant ER (Sharkey *et al.* 2009b), but it did not display the same IUGR-dependent pattern as in gene expression, suggesting post-translational regulation.

Our gene expression findings are consistent with previous studies on perirenal adipose tissue in lean one year old offspring after late pregnancy nutrient restriction, which showed an increase of UPR genes in that depot (Sharkey *et al.* 2009b). The ER may be sensitive to nutritional programming as it can use considerable amounts of energy and it has been shown *in vitro* that hypoglycaemia causes UPR (Park *et al.* 2004; Yacoub Wasef *et al.* 2006). We hypothesised that IUGR could impact on adipocyte number in a depot specific manner, which could then fill up faster with obesity, causing the ER stress response, inflammation, cell death and ultimately insulin resistance. As discussed earlier this may not be the case for omental adipose tissue that only develops after birth. However, the mesodermal pre-adipocytes which give rise to omental fat after birth could be affected by late gestational nutrient restriction as the omentum undergoes rapid growth during this period, that ultimately leads to a lower threshold for ER stress related responses.

In conclusion, IUGR can contribute to an enhanced cellular response to juvenile onset obesity but by young adulthood this does not exacerbate the onset of insulin resistance. Future studies with larger samples sizes, allowing analysis of sex effects, and older offspring could elucidate the extent to which these offspring exhibit more adverse clinically relevant symptoms.

Declaration of Interest: The authors have nothing to disclose.

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Figure Titles

Figure 1: Maternal weight development. Mothers were either fed ad libitum (A, closed symbols) or nutrient restricted (R, open symbols) during the intervention period, 110 days gestational age until term at 145 days gestational age. *, P<0.05; **, P<0.01.

Figure 2: Maternal plasma metabolites and hormones as measured at 130d gestation: A, insulin; B, glucose; C, non-esterified fatty acids (NEFA); D, triglycerides; E, cortisol. Mothers were either fed ad libitum (A, closed bars) or nutrient restricted (R, open bars) during the intervention period, 110 days gestational age until term at 145 days gestational age. *, P<0.05; **, P<0.01.

Figure 3: Offspring plasma glucose and insulin concentrations during a glucose tolerance test performed at 7 (A and B) and 16 months of age (C and D). Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). *, P<0.05 between AL and AO; #, P<0.05 between RL and RO.

Figure 4: Offspring average size of omental adipocytes at 17 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). **, P<0.01.

Figure 5: Expression of genes involved in ER stress and autophagic responses as measured in omental adipose tissue at 17 months of age, expressed relative to the RL group. Mothers were

either fed ad libitum (A, closed bars) or nutrient restricted (R, open bars) during the intervention period, 110 days gestational age until term at 145 days gestational age. After weaning offspring were either kept in a normal environment where animals remained lean or were kept in an environment which restricted their physical activity, causing animals to become obese. *, $P<0.05$; **, $P<0.01$.

Table Titles

Table 1: Offspring weight characteristics throughout the course of the study. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). Body weight was expressed after sex-specific z-score transformation or as absolute body weight. Measures of fat mass at 16 months are derived from DEXA (see Methods). The effects of maternal nutrition (prenatal) and of the activity level (post weaning) were determined by 2-way ANOVA #, $P<0.05$ for difference within the maternal group, i.e. between lean and obese offspring, as determined by simple main effects analysis.

Table 2: Glucose and insulin area under the curve (AUC) and homeostatic model assessment for insulin resistance (HOMA-IR) as determined during intravenous glucose tolerance tests at 7 and 16 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). The effects of maternal nutrition (prenatal) and of the activity level (post weaning) were determined by 2-way ANOVA. #, $P<0.05$ for difference within the maternal group, i.e. between lean and obese offspring, as determined by simple main effects analysis.

Table 3: Offspring hepatic weight, lipid content and gene expression at 17 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). The effects of maternal nutrition (prenatal) and of the activity

615 level (post weaning) as well as the interaction between the two factors were determined by 2-
616 way ANOVA. #, P<0.05 for difference within the maternal group, i.e. between lean and obese
617 offspring; *, P<0.05 for difference within the post weaning group, i.e. between A and R
618 offspring, both as determined by simple main effects analysis, P<0.05. There were no significant
619 interactions found in these variables.
620
621 Table 4: Protein (GRP78 and pJNK) and gene expression in offspring omental adipose tissue at
622 17 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad
623 libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an
624 environment restricting their physical activity (obese). The effects of maternal nutrition
625 (prenatal) and of the activity level (post weaning) were determined by 2-way ANOVA. #, P<0.05
626 for difference within the maternal group, i.e. between lean and obese offspring, as determined by
627 simple main effects analysis.

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Supplementary Information

Figure S1: Overview of the groups included in this study.

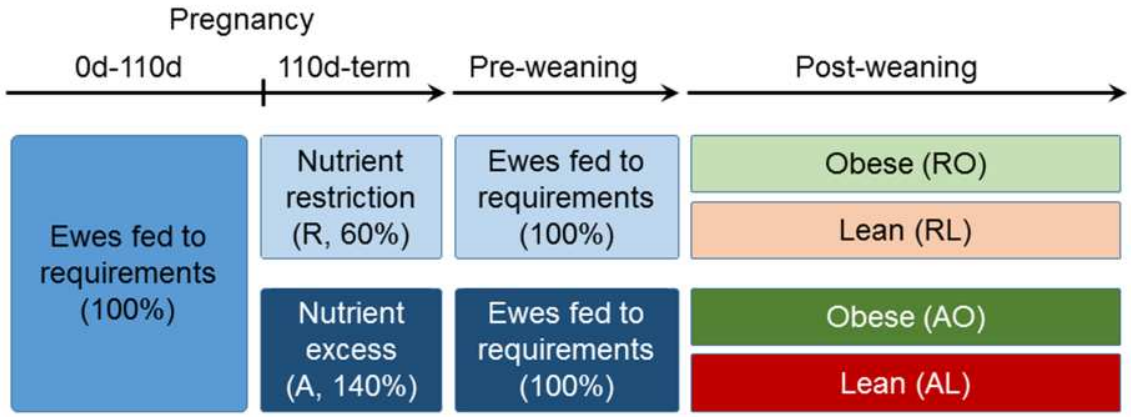


Table S1: Primer forward and reverse sequences and product length.

Pathway	Gene	Accession number	Sequence	Product size (kb)
Inflammation	*Tlr4*	NM_001135930.1	TGCTGGCTGCAAAAAGTATG	148
			CCCTGTAGTGAAGGCAGAGC	
	11bhsd1	NM_001009395	AGCATTGTGGTCGTCTCCT	127
			CCTTGGTCGCCTCATATTCC	
	Fas	NM_001123003	CGGGATCTGGGTTCACTTGTC	165
			AACAGGTGCTCACGATATAGGC	
Autophagy	*Beclin1*	NM_001033627	CCAGGAGGAAGAGGCTAACT	116
			AAGCTGTTGGCACTTTCTGT	
	Atg12	NM_001076982	CATTCTGCTAAAGGCTGTAGGA	127
			GTTCTGAAGCCACAAGTTTAAGG	
Unfolded protein response	*Edem1*	NM_001103092	GTCTGGAAAAGTACACAAAAGTCA	123
			AGCAGATACAGGTATTTACAGGTC	
	Grp78	NM_001075148	TGAAACTGTGGGAGGTGTCA	170
			TCGAAAGTTCCCAGAAGGTG	
	Atf4	NM_001142518	AGATGACCTGGAAACCATGC	189
			AGGGGGAAGAGGTTGCAAGA	

	Atf6	AY942654.1	AACCAGTCCTTGCTGTTGCT	223
			CTTCTTCTTGCGGGACTGAC	
Energy sensing	*Ampk*	NM_001112816	GCTGGATTTTGAATGGAAGG	157
			CAGCACCTCATCATCAATGC	
	Mtor	NM_001145455	GCCTTCCGACCTTCTGCCTTC	97
			CCGCTGTCCGTTCCTTCTCC	
Leptin	*Leptin*	NM_173928.2	GGGTCACTGGTTTGGACTTCA	97
			ACTGGCGAGGCTCTGTTGGTA	
	Obr	NM_001009763	TGAAACCACTGCCTCCATCC	131
			TCCACTTAAACCATAGCGAATCTG	
Reference	*Rpo*	NM_001012682.1	CAACCCTGAAGTGCTTGACAT	226
			AGGCAGATGGATCAGCCA	
	Ywhaz	NM_174814.2	TGTAGGAGCCCGTAGGTCATCT	100
			TTCTCTGTATTCTCGAGCCATCT	
	Rpl19	Xm_012141899	CAACTCCCGCCAGCAGAT	75
			CCGGGAATGGACAGTCACA	

Figure S2: Representative images demonstrating distribution of staining for GRP78 (A, B) and pJNK (C, D) in adipose tissue from lean (A, C) and obese (B, D) animals at 17 months of age (brown DAB staining in perinuclear areas). Bars represent 50 µm.

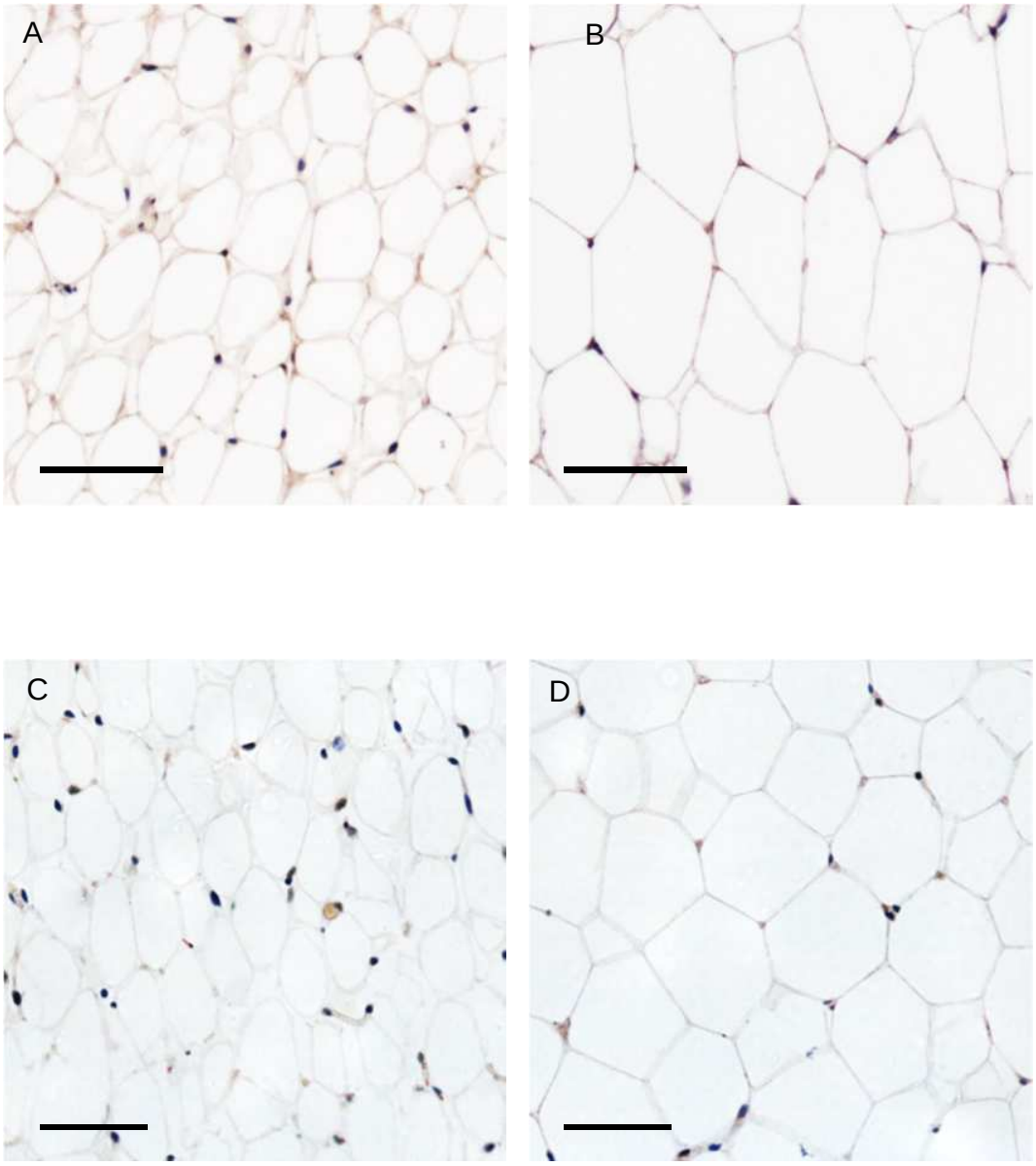


Table 1

Variable	Maternal Nutrition	Post weaning		Effect Prenatal	Effect Post weaning	Interaction
		Lean	**Obese**	*P* value	*P* value	*P* value
Body weight 3 months (z-score)	A	0.66±0.43	-0.03±0.23	NS	NS	0.045
	R	-0.73±0.28*	0.01±0.31			
Body weight 7 months (z-score)	A	0.05±0.42	-0.11±0.28	NS	NS	NS
	R	-0.10±0.36	0.27±0.38			
Plasma leptin 7 months (ng/ml)	A	1.18±0.12	1.98±0.19#	NS	<0.01	NS
	R	1.55±0.26	1.98±0.15			
Body weight 9 months (z-score)	A	0.28±0.44	-0.01±0.22	NS	NS	NS
	R	-0.07±0.31	-0.26±0.41			
15 months						
Body weight (z-score)	A	-0.67±0.20	0.68±0.16#	NS	<0.001	NS
	R	-0.97±0.12	0.98±0.29#			
Mean activity (counts)	A	471±67	150± 13#	NS	<0.001	NS
	R	536±69	74±33#			
16 months						
Body weight (kg)	A	59.6±3.2	75.6±4.5#	NS	<0.001	NS

	R	52.7±1.3	70.1±5.7#			
Body weight (z-score)	A	-0.59±0.19	0.68±0.16#	NS	<0.001	NS
	R	-1.00±0.14	0.98±0.29#			
Total fat mass (kg)	A	4.4±0.6	10.3±1.1#	NS	<0.001	NS
	R	3.9±0.5	9.9±0.7#			
Relative fat mass (%)	A	7.3±0.7	13.6±1.2#	NS	<0.001	NS
	R	7.5±0.9	14.4±1.0#			
Visceral fat mass (%)	A	14.8±0.7	26.1±1.3#	NS	<0.001	NS
	R	13.4±1.2	28.1±1.8#			
Total lean mass (kg)	A	55.2±2.8	65.3±4.0#	NS	<0.01	NS
	R	48.7±1.3	60.1±5.4			
Plasma leptin (ng/ml)	A	2.6±0.3	4.5±0.4#	NS	<0.01	NS
	R	3.1±0.2	3.8±0.5			
17 months						
Body weight (kg)	A	56.9±3.3	74.7±3.9#	NS	<0.001	NS
	R	51.3±1.7	69.6±5.0#			
Body weight (z-score)	A	-0.67±0.20	0.72±0.13#	NS	<0.001	NS
	R	-0.97±0.12	0.90±0.33#			
Omental fat mass (kg)	A	313±65	1526±163#	NS	<0.001	NS

	R	224±56	1655±151#			
Pericardial fat mass (kg)	A	66±10	101±9#	NS	<0.001	NS
	R	59±16	116±14#			
Perirenal fat mass (kg)	A	279±43	1003±119#	NS	<0.001	NS
	R	252±37	1014±104#			

Table 2

Variable	Maternal Nutrition	Post weaning		Effect Prenatal	Effect Post weaning	Interaction
		Lean	**Obese**	*P* value	*P* value	*P* value
7 months						
AUC glucose (mmol/l)	A	1370±67	1593±61#	NS	<0.01	NS
	R	1377±64	1549±44#			
AUC insulin (µg/l)	A	49.2±7.0	51.6±6.2	NS	NS	NS
	R	43.1±5.2	56.8±7.9			
HOMA-IR	A	5.57±0.2	5.54±0.6	NS	NS	NS
	R	6.89±1.8	6.07±0.4			
16 months						
AUC glucose (mmol/l)	A	1047±57	1031±38	NS	NS	NS
	R	1074±44	1136±74			
AUC insulin (µg/l)	A	31.7±14.8	67.7±9.1#	NS	<0.001	NS
	R	15.7±3.9	49.8±14.5#			
HOMA-IR	A	2.8±0.1	4.3±0.4#	NS	<0.001	NS
	R	3.0±0.1	3.6±0.3			

Table 3

Variable	Maternal Nutrition	Post weaning		Effect Prenatal	Effect Post weaning	Interaction
		Lean	Obese	*P* value	*P* value	*P* value
Liver weight (g)	A	649±26	716±34	NS	<0.01	NS
	R	600±23[b]	755±59[a]			
Relative liver weight (g per kg body weight)	A	11.6±0.6[a]	9.6±0.2[b]	NS	<0.01	NS
	R	11.7±0.4	10.9±0.4[*]			
Liver triglyceride (mg/g)	A	32.2±6.6	37.0±6.1	NS	0.04	NS
	R	28.6±3.78[b]	48.3±7.8[a]			
Total liver triglyceride content (g)	A	21.2±0.44[b]	27.0±0.55[a]	NS	0.02	NS
	R	17.0±0.26[b]	37.8±0.85[a]			
Beclin1 mRNA (arbitrary units)	A	0.98±0.05[b]	1.15±0.07[a]	0.034	0.002	0.042
	R	1.00±0.07[b]	1.67±0.24[a*]			
ATF4 mRNA (arbitrary units)	A	1.31±0.06	1.12±0.05	NS	NS	0.01
	R	1.00±0.03[*]	1.22±0.13			
ATG12 mRNA (arbitrary units)	A	0.89±0.05[b]	1.13±0.06[a]	NS	0.005	NS
	R	1.00±0.10[b]	1.40±0.14[a]			
	A	1.03±0.01	1.06±0.18	NS	0.02	NS

EDEM1 mRNA (arbitrary units)	R	1.00±0.03	1.08±0.04			
GRP78 mRNA (arbitrary units)	A	1.16±0.08	1.27±0.09	NS	0.04	NS
	R	1.00±0.09[b]	1.40±0.19[a]			
ATF6 mRNA (arbitrary units)	A	1.13±0.10	1.06±0.05	NS	NS	NS
	R	1.00±0.04	0.92±0.15			

Table 3: Offspring hepatic weight, lipid content and gene expression at 17 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). The effects of maternal nutrition (prenatal) and of the activity level (post weaning) as well as the interaction between the two factors were determined by 2-way ANOVA. Differing superscripts indicate a difference within the maternal group, i.e. between lean and obese, and asterisk indicates a difference within the post weaning group, i.e. between A and R offspring, both as determined by simple main effects analysis, $P<0.05$. There were no significant interactions found in these variables.

Table 4

Variable	Maternal Nutrition	Post Weaning		Effect Prenatal	Effect Post Weaning	Interaction
		Lean	**Obese**	*P* value	*P* value	*P value*
GRP 78 (μm^2/cell)	A	35.9±21.6	79.8±24.2	NS	0.02	NS
	R	30.4±11.0	70.9±21.8			
pJNK (μm^2/cell)	A	29.6±12.7	53.1±6.3	NS	NS	NS
	R	55.5±17.5	44.2±12.7			
Leptin mRNA (arbitrary units)	A	1.46±0.33[b]	7.89±2.20[a]	NS	<0.001	NS
	R	1.00±0.19[b]	8.02±1.99[a]			
TLR4 mRNA (arbitrary units)	A	1.08±0.57	1.44±0.21	NS	0.01	NS
	R	1.00±0.12[b]	1.33±0.13[a]			
CD68 mRNA (arbitrary units)	A	1.54±0.27[b]	5.46±1.5[a]	NS	<0.001	NS
	R	1.00±0.11[b]	4.79±2.41[a]			

Table 4: Protein (GRP78 and pJNK) and gene expression (leptin and TLR4) in offspring omental adipose tissue. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). The effects of maternal nutrition (prenatal) and of the activity level (post weaning) were determined by 2-way

ANOVA and differing superscripts indicate a difference within the maternal group, as determined by simple main effects analysis. There were no significant interactions found in these variables.

Figure 1

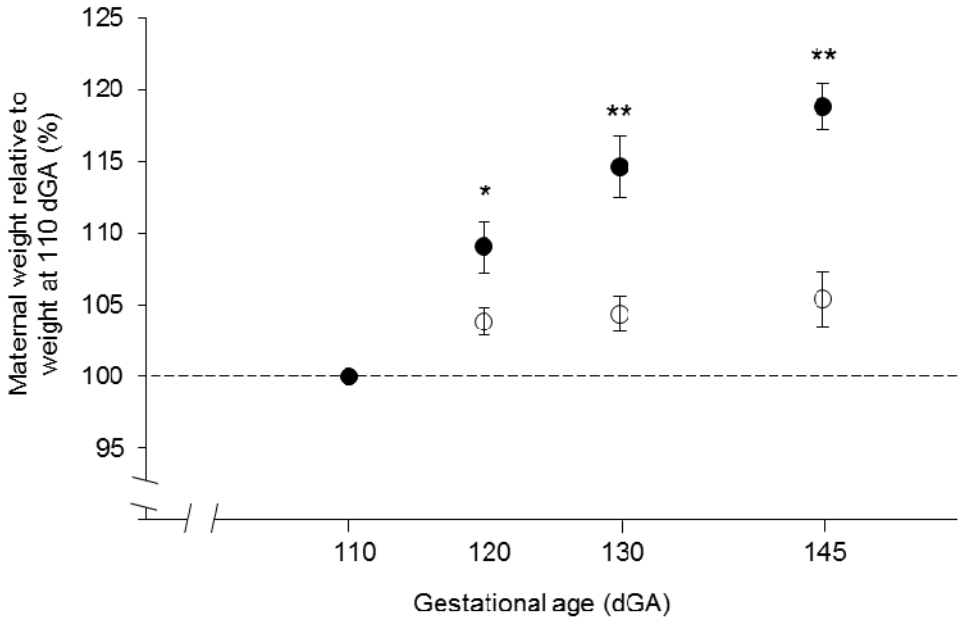
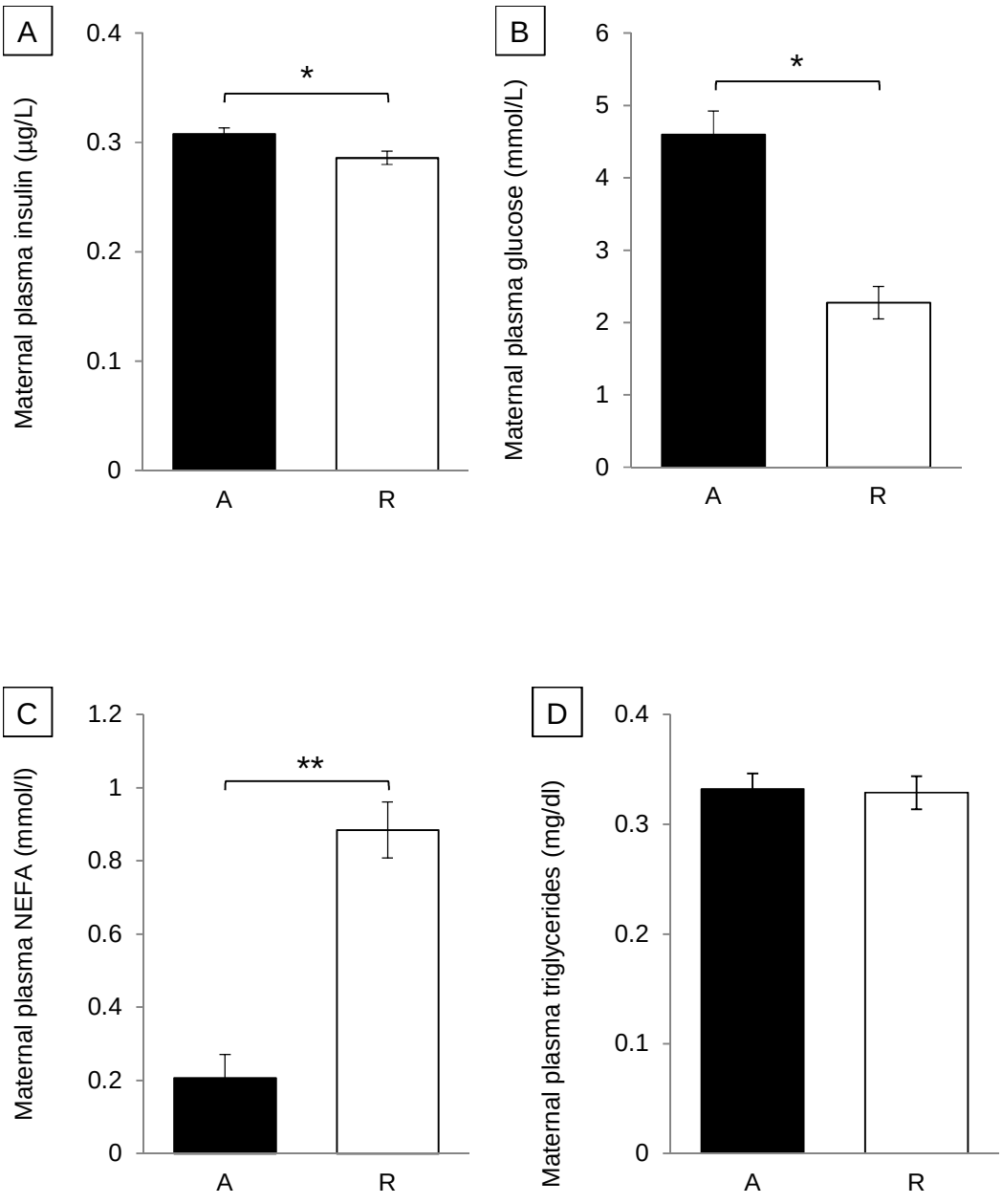


Figure 1: Maternal weight development. Mothers were either fed *ad libitum* (A, closed symbols) or nutrient restricted (R, open symbols) during the intervention period, 110 days gestational age until term at 145 days gestational age. *, $P<0.05$; **, $P<0.01$.

Figure 2


A
Maternal plasma insulin (µg/L)
0.4
0.3
0.2
0.1
0
*
A
R
B
Maternal plasma glucose (mmol/L)
6
5
4
3
2
1
0
*
A
R
C
Maternal plasma NEFA (mmol/l)
1.2
1
0.8
0.6
0.4
0.2
0
**
A
R
D
Maternal plasma triglycerides (mg/dl)
0.4
0.3
0.2
0.1
0
A
R

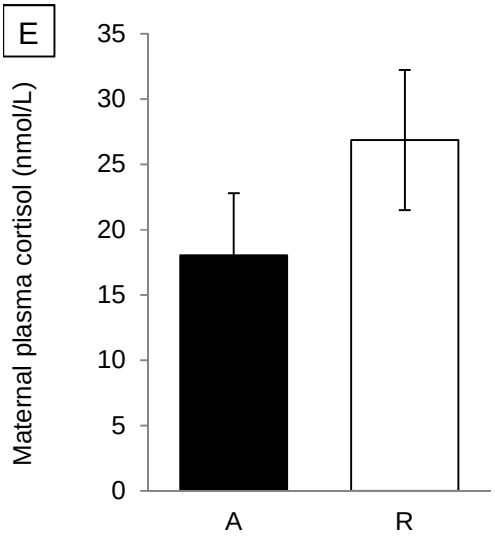
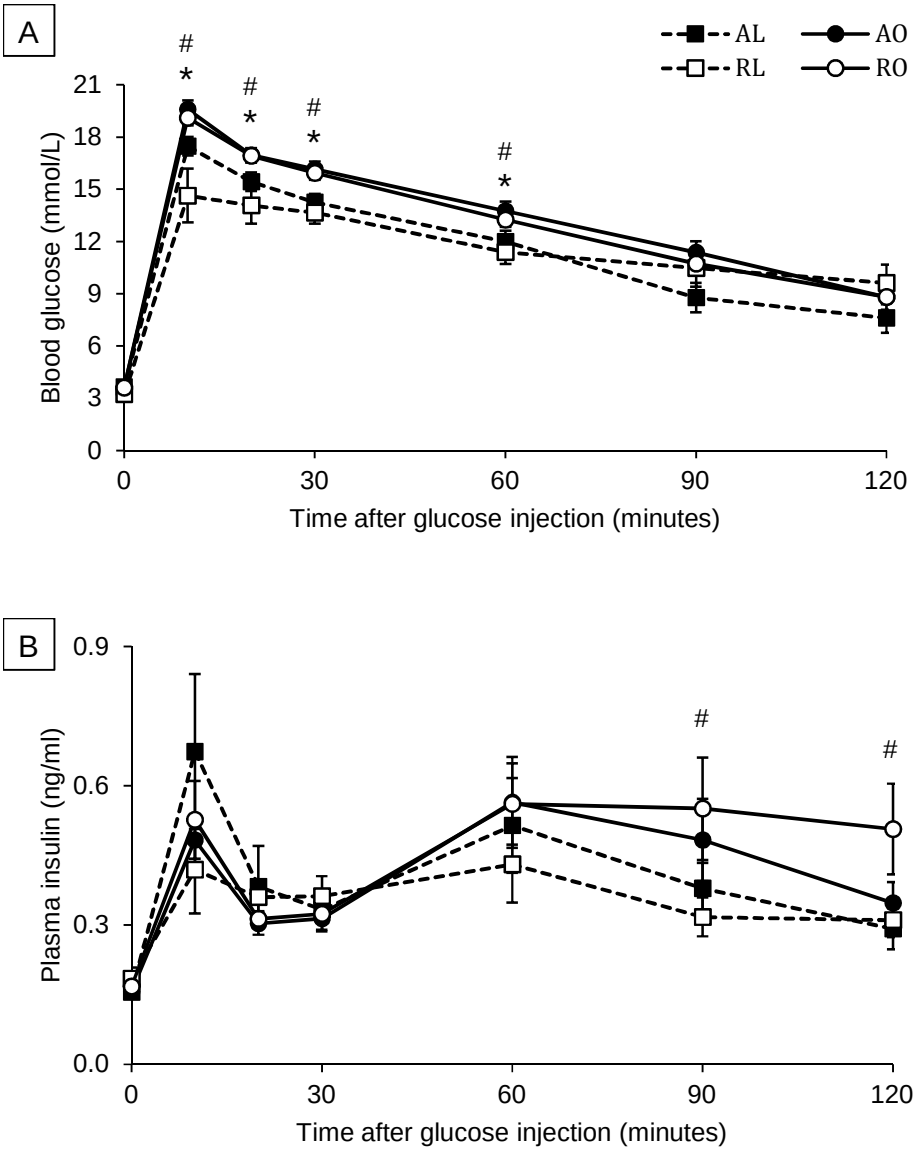


Figure 2: Maternal plasma metabolites and hormones as measured at 130d gestation: A, insulin; B, glucose; C, non-esterified fatty acids (NEFA); D, triglycerides; E, cortisol. Mothers were either fed *ad libitum* (A, closed bars) or nutrient restricted (R, open bars) during the intervention period, 110 days gestational age until term at 145 days gestational age. *, $P<0.05$; **, $P<0.01$.

Figure 3


A
AL
AO
RL
RO
Blood glucose (mmol/L)
Time after glucose injection (minutes)
B
Plasma insulin (ng/ml)
Time after glucose injection (minutes)

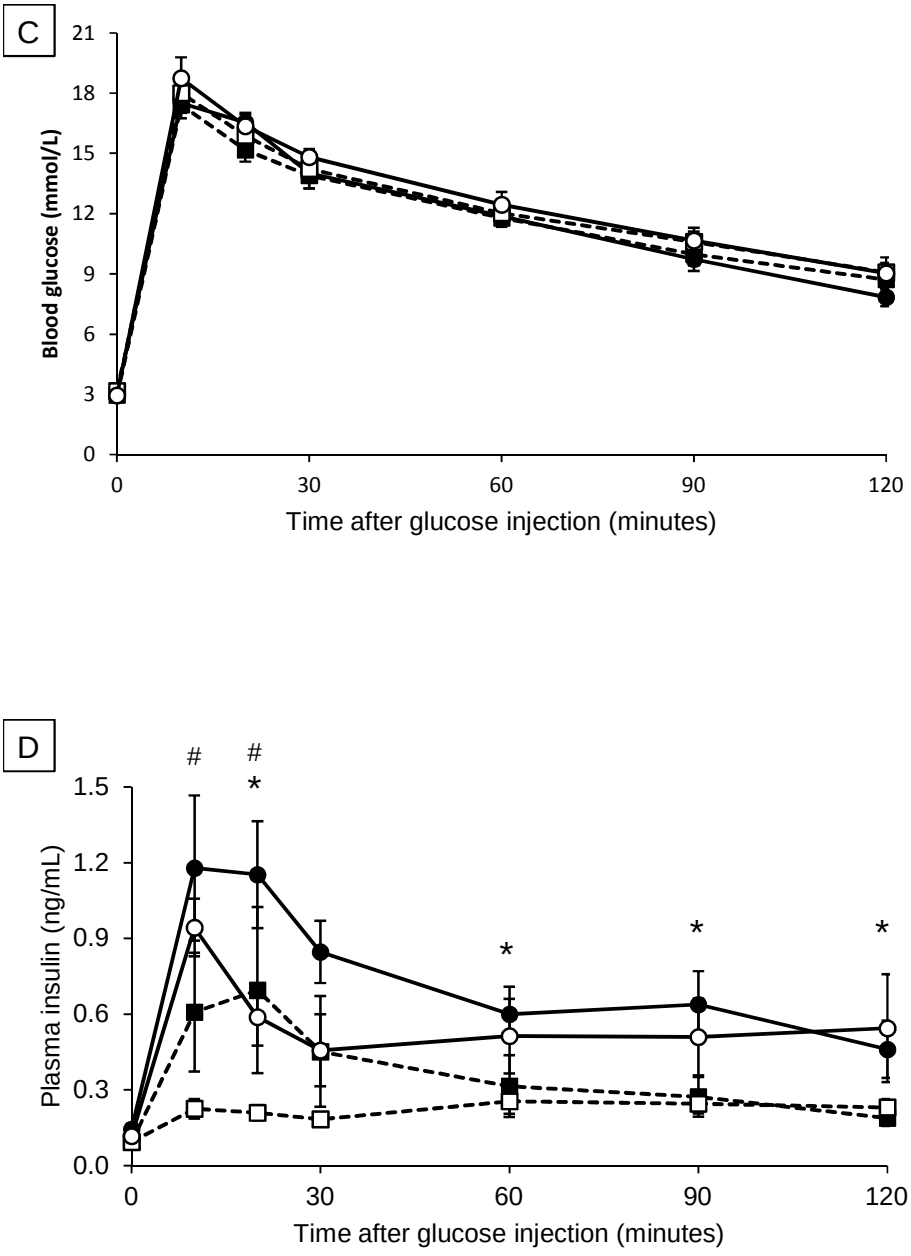


Figure 3: Offspring plasma glucose and insulin concentrations during a glucose tolerance test performed at 7 (A and B) and 16 months of age (C and D). Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). *, $P<0.05$ between AL and AO; #, $P<0.05$ between RL and RO.

Figure 4

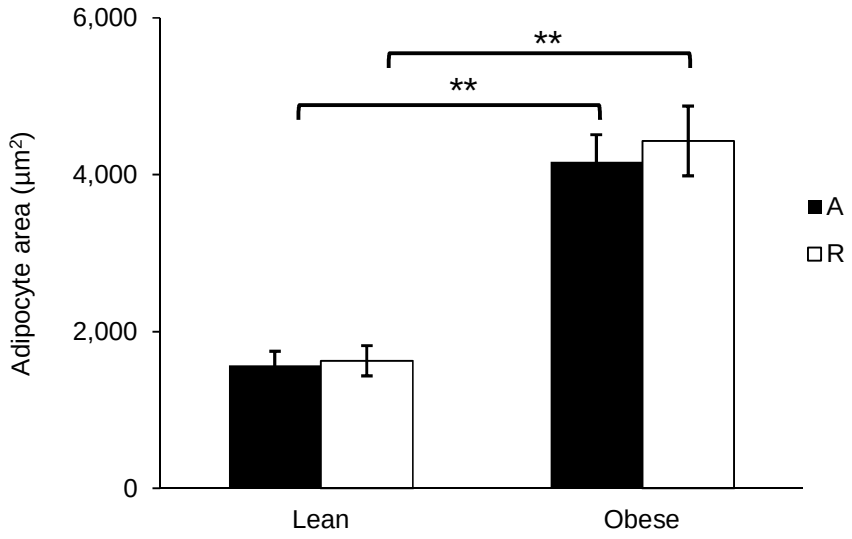
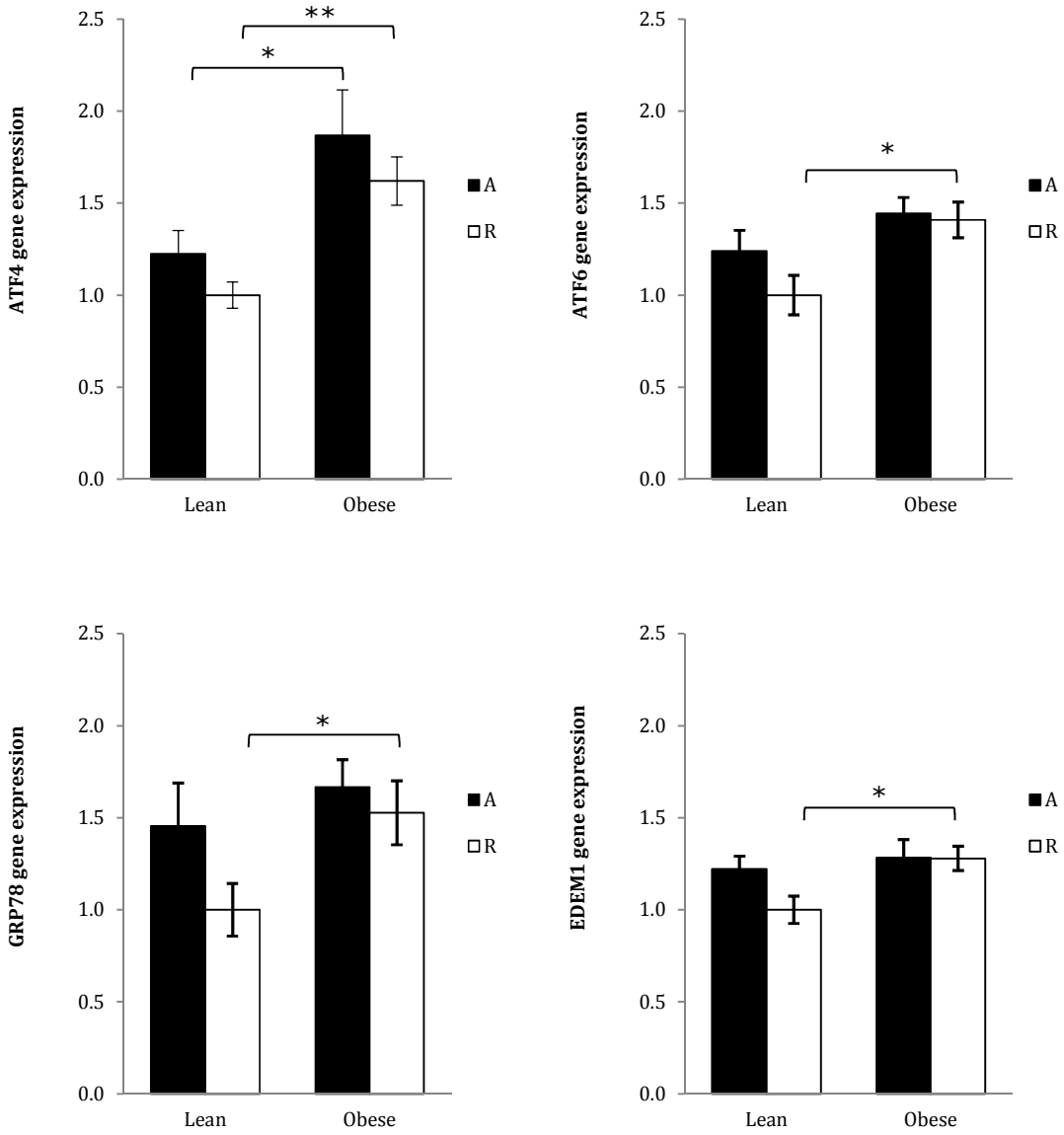


Figure 4: Offspring average size of omental adipocytes at 17 months of age. Sheep were either subjected to maternal nutrient restriction (R) or maternal ad libitum feeding (A) in late pregnancy and were kept in either a normal environment (lean) or an environment restricting their physical activity (obese). **, $P<0.01$.

Figure 5


ATF4 gene expression
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Lean
Obese
A
R
ATF6 gene expression
*
GRP78 gene expression
EDEM1 gene expression

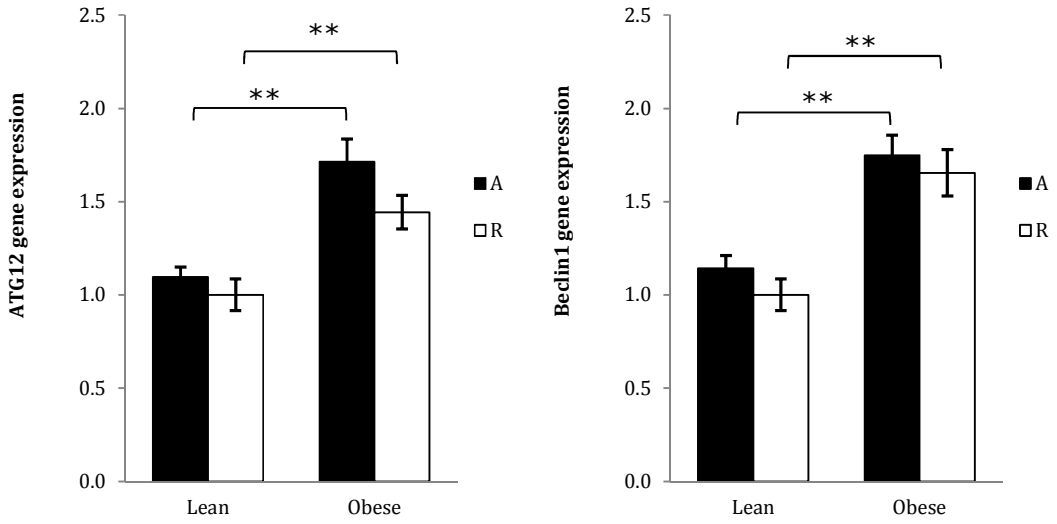


Figure 5: Expression of genes involved in ER stress and autophagic responses as measured in omental adipose tissue at 17 months of age, expressed relative to the RL group. Mothers were either fed *ad libitum* (A, closed bars) or nutrient restricted (R, open bars) during the intervention period, 110 days gestational age until term at 145 days gestational age. After weaning offspring were either kept in a normal environment where animals remained lean or were kept in an environment which restricted their physical activity, causing animals to become obese. *, $P<0.05$; **, $P<0.01$.