Maternal nutrient restriction between early-to-mid gestation and its impact upon appetite regulation following juvenile obesity

S.P. Sébert, M.A. Hyatt, L.L.Y. Chan, N. Patel, R. C. Bell[1], D. Keisler[2], T. Stephenson, H. Budge, M.E. Symonds and D.S. Gardner[3]

Centre for Reproduction and Early Life, Institute for Clinical Research, University Hospital, Nottingham, NG7 2UH United Kingdom, [1]Alberta Institute of Human Nutrition, University of Alberta, Edmonton, AB, Canada, T6G 0H3, [2]Department of Animal Sciences, University of Missouri, Columbia, Missouri 65201, USA and [3]School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, LE12 5RD United Kingdom

Abbreviated title: Programming of appetite - outcome with obesity

Corresponding author: Professor Michael E. Symonds or Dr David S Gardner; Academic Division of Child Health, School of Human Development, University Hospital, Nottingham NG7 2UH, United Kingdom. Tel: 44 115 823 0610; Fax: 44 115 823 0626; E-mail: michael.symonds@nottingham.ac.uk or david.gardner@nottingham.ac.uk

DISCLOSURE STATEMENT: The authors have nothing to disclose

Abstract:

The impact of maternal nutrient restriction during early-to-mid gestation, a period coinciding with early fetal brain development, on appetite regulation and energy balance in the offspring following juvenile obesity was examined. Pregnant sheep were either fed to fully meet their nutritional requirements throughout gestation or 50% of this amount between 30-80 days gestation. Following weaning, offspring were either made obese through exposure to a sedentary obesogenic environment or remained lean. Maternal nutrient restriction had no effect on birth weight or subsequent growth. At one week of age, only, gene expression for neuropeptide Y in the hypothalamus was reduced in nutrient restricted offspring. By 1 year of age, all obese animals had raised plasma leptin, non-esterified fatty acids and insulin, with the latter effect amplified in nutrient restricted offspring. Fasting plasma glucose, triglycerides and cortisol were unaffected by obesity. The entrained reduction in physical activity that led to obesity persisted when all animals were maintained within individual pens. Obese nutrient restricted offspring, however, exhibited reduced daily food intake and were, therefore, no longer in positive "energy balance". This adaptation was accompanied by elevated hypothalamic gene expression for the melanocortin-4 and insulin receptors, AMP-activated kinase and acetyl CoA carboxylase α. In conclusion, nutrient restriction specifically targeted over the period of early fetal brain development, contributes to a profoundly different adaptation in energy balance following juvenile obesity. The extent to which this adaptive response may benefit the offspring or result in an exacerbated risk for type II diabetes remains to be established.

Introduction

The incidence of obesity (BMI >30 kg/m^2) in the UK population and worldwide has increased markedly over the past decade (1, 2). Many epidemiological and prospective clinical studies in humans, together with experimental findings in animals, have demonstrated that modifications of the nutritional environment during fetal development can have a substantial impact on later health and disease, including the onset of obesity (3, 4). The extent to which, modifications in the maternal diet during early fetal development may contribute to changes in energy balance so as to modulate the effect of obesity remains unknown. Increasing maternal food intake to *ad libitum* during late pregnancy in sheep results in an upregulation in gene expression of pro-opiomelanocortin (POMC) at 30 days of postnatal age (5) but this adaptation is not accompanied by any persistent change in food intake. Indeed, it has no effect on the orexigenic neuropeptides neuropeptide Y (NPY) and agouti related peptide (AgRP) nor on the leptin receptor (ObR) (5) that normally mediates the effects of leptin on food intake (6). An increase in maternal food intake in late gestation was, therefore, unlikely to be associated with additional stimulation of energy expenditure through regulation of melanocortin receptors type 3 and 4 (MC3/4 R) (5). Conversely, severe maternal undernutrition in rats that is sufficient to induce intra-uterine growth retardation results in a reduction in physical activity in the resulting offspring, although they remain smaller than controls (7). To date, however, there are no studies that have examined the extent by which appetite regulation may be reset in the adult following exposure to nutrient restriction *in utero*, when this is followed by exposure to an obesogenic environment after birth.

Insulin has an important role in appetite regulation as it crosses the blood brain barrier in direct proportion to its circulating concentration (8) where it has a direct effect on a range of appetite pathways. These are mediated through the insulin receptor (IR) and the IR substrate type 2 (9) resulting in impaired gene expression and activity of NPY within the arcuate (ARC) and the paraventricular (PVN) nuclei of the hypothalamus (10). In the adult, plasma insulin concentration can be programmed by changes in the maternal diet during pregnancy (11) although it is has yet to be determined whether this impacts on appetite regulation or energy balance.

Other factors that are important in the regulation of energy balance include AMP-activated kinase (AMPK) and its co-factor acetyl CoA carboxylase (ACC)α (12, 13) that are both responsive to leptin (12). In addition, glucose transporter (GLUT)-1 regulates glucose transport in appetite responsive neurons (14) and the peroxisome proliferator-activated receptor gamma co-activator (PGC1)α determines fatty acid metabolism in the brain and may control energy expenditure through promoting physical activity (15). It remains unknown, however, whether the abundance of these key hypothalamic genes are modulated following obesity or whether the magnitude of response is reset in offspring exposed to maternal nutrient restriction during early development.

We have previously established in the sheep that maternal nutrient restriction, targeted over the period of the maximal placental growth (i.e. 30-80 days; term ~145 days), results in offspring with increased adiposity at birth and elevated tissue glucocorticoid sensitivity (16). In this regard, enhanced mRNA abundance, for both the glucocorticoid receptor (GR) and 11ß-hydroxysteroid dehydrogenase type 1, persists for up to 6 months of age (17), although fat mass is unaffected when the offspring are reared in an outdoor "free-living" environment. The period between early (30 days) to mid (80 days) gestation in the sheep represents a critical time for fetal organogenesis including brain development. This is likely to include the development of the appetite control network and energy sensing pathways in the hypothalamus (18, 19), although the extent to which such development may extend into, and beyond, the postnatal period is currently unknown. Maternal nutrient restriction between early-to-mid gestation reduces the size of the fetal brain (20) and is associated with behavioral

adaptations in the resulting adult (21). In contrast to the effects on glucocorticoid action in peripheral tissues, no effects are seen in the hypothalamus or pituitary in the newborn (22).

Dysregulation of energy balance is one of the main features of obesity (23). No study to date however, has assessed its long term outcomes in adults born to nutrient restricted mothers following adolescent-onset obesity. We therefore examined the hypothalamic consequences in the resulting offspring both in the immediate postnatal period as well as at 1 year of age following exposure to obesogenic environment after weaning. In particular, we aimed to examine whether the previously established beneficial effects on the kidney with regard to protection against glomerulerosclerosis that occur when previously nutrient restricted offspring become obese (24) could extend to the regulation of energy balance.

Material and methods
Animals, diets and experimental design
All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and approved by the local ethics committee of the University of Nottingham. At day 30 of gestation, 22 twin-bearing Welsh Mountain sheep were randomly allocated to receive either a control (C; 7MJ/day: n=14) or nutrient restricted (NR; 50% of C, n=8) diet until day 80 of gestation. Thereafter, all sheep were fed 100% of their calculated metabolisable energy requirements to term (12–13 MJ/day near term) (25). Offspring were delivered spontaneously at term (~147 days). The ratio of females to males was (1:1) in the control and (2:1) in the NR group and all animals remained intact. Seven control and 8 of the nutrient restricted offspring were tissue sampled at 7 days of age. The remaining offspring (C n=14; NR, n=8) were reared by their mothers up to weaning (~10 weeks of age). During lactation all mothers were fed a diet of hay *ad libitum* together with a fixed amount of concentrate pellets sufficient to fully meet their own metabolisable requirements, together with those required to maintain lactation. From weaning to 12 months of age, 7 of the gestational control fed sheep and all (n=8) nutrient restricted offspring were exposed to an 'obesogenic' environment: i.e. group-housed in a barn at a stocking rate of 17 animals per 50m^2 with *ad libitum* access to hay and concentrate pellets, as described previously (24). The remaining 7 control offspring were reared in a non-obesogenic 'free-living' environment: i.e. out to pasture at a stocking rate of 17 animals per 3000 m^2 with free grazing allowance and a fixed amount of concentrate pellets offered (400 g/day). Seven day old offspring are thus classified with regard to the maternal environment: i.e. control or nutrient restricted. The remaining post weaning "free-living" offspring were defined as lean (L, n=7). Offspring raised in the obesogenic environment were defined as obese (O, n=7) or nutrient restricted obese (NRO, n=8) as previously described (24). The number of animals analyzed in this study differed from the total number of offspring born because some of the controls were used in an independent study.

Determination of relative energy balance
Physical activity
The level of spontaneous physical activity was determined by an uniaxial accelerometer (Actiwatch; Linton Instrumentation, Diss, UK). These probes were placed on collars around the neck of the animal for a 24 h period, with a measurement of activity being recorded 32 times per second. This resulted in 960 measurements per 30 seconds that were automatically summated into 30 second time bins. The resulting 2880 readings for the 24 h period (arbitrary accelerometry units) were then downloaded to specialist software (Actigraph) for imaging and analysis. Spontaneous physical activity was recorded in all offspring at 12 months of age when housed individually in similar UK Home Office designated floor pens (3m^2). This allows an estimation and comparison of energy expenditure related to physical activity between experimental groups (26).

Individual food intake
Also at 12 months of age when all offspring were housed individually daily food intake was recorded in order to assess appetite regulation. For each animal, daily energy intake was assessed through weighed intake and food refusal i.e. sheep were offered sufficient energy for the 24 h period based on a mix of low (hay; 8.9 MJ/kg) and high (concentrate pellets; 12.6 MJ/kg)) energy dense food.

Determination of the ratio of energy intake to physical activity
From the all the individual values of food intake and physical activity we calculated relative values taking the average lean group as a reference (100%):
Then, we determined a ratio of food intake to physical activity:

(1): $\dfrac{\text{Food intake (\%)}}{\text{Physical activity (\%)}}$

Therefore:
A relative ratio can be determined:

(2): $\left[\textbf{(1)} \middle/ \dfrac{\text{Food intake}_{\text{Lean}}(\%)}{\text{Physical activity}_{\text{Lean}}(\%)} \right]$ -1

Thus, the average relative ratio for lean offspring is considered to be equal to 0. A positive value represents net gain in energy, whereas a negative value represents a net loss.

Plasma measurements

Morning (~ 0800 h) fasted blood samples (5 ml) were collected from the jugular vein into lithium heparin and K^+EDTA tubes at 12 months of age. Plasma was recovered by centrifugation (3000 rpm x 10 min at 4°C) and kept frozen at -20°C until analysis. Plasma leptin, ACTH (Diasorin, Slough, UK) and cortisol (Coat-a-Count, Euro DPC, Caernarfon, UK) were measured by radioimmunoassay as previously described (27, 28). Plasma insulin was assessed by ELISA assay (Mercodia, Sheep insulin ELISA kit, UK). Plasma glucose (Randox GPO-PAP, Randox, Crumlin UK), non-esterified fatty acid (NEFA) (wako NEFA-c Kit, Wako Chemicals Gmbh, Neuss, Germany) and triglyceride (TG) (Randox GOD-PAP, Randox, Crumlin UK) were assessed by spectrophotometic methods.

Determination of insulin sensitivity

An intravenous insulin tolerance test was used to assess insulin-dependent glucose uptake in adult sheep at 1 year of age. Briefly, after an overnight fast, 0.75 IU.kg^{-1} human insulin (Novorapid®, Novo Nordisk, Crawley, UK) was administrated as a rapid intravenous (jugular vein) bolus at time 0. Venous blood samples were collected into K^+-EDTA treated blood tubes every 2 minutes for 16 minutes. The percentage (%) of decline per minute (K_{itt}, in % glucose decline/kg/min) in plasma glucose from time 0 min was taken to represent peripheral tissue insulin sensitivity.

Post mortem analysis

All the animals were euthanized in the morning between 0900 and 1100 h. The 1 year old offspring were euthanized as previously described after an overnight fast (24). Seven day old offspring could not be subjected to a similar period of food withdrawal as this would have meant prolonged physical separation from their mothers, as such, would have been unethical. To avoid any additional handling stress in these animals prior to euthanasia blood samples were not taken from these offspring. Each animal's brain was immediately recovered and the entire hypothalamus dissected and snap frozen in liquid nitrogen, before being stored at -80°C. The hypothalamus was dissected using the rostral border of the optic chiasm and the caudal border of the mamillary bodies as landmarks on the ventral surface and the lateral hypothalamic sulci as the lateral boundaries. In addition, all visceral fat depots were dissected and weighed.

Gene expression

The mRNA expression for each candidate gene was determined in the entire hypothalamus as previously established in both sheep (29) and rats (30).

Total RNA from the entire hypothalamus was extracted using Qiazol® lysis reagent and RNeasy® extraction kit (Qiagen Ltd., Crawley, UK). Total RNA samples were DNAse treated (Promega RQ1 endonuclease, Promega Ltd, Southampton, UK) and reverse transcribed (Superscript® II reverse transcriptase, Invitrogen Ltd., Pasley, UK). cDNA was amplified using a real-time thermocycler (Quantica®, Techne incorporated, Barloword Scientific Ltd, Stone, UK) using Quantitect® SYBR green PCR kit (Qiagen Ltd., Crawley, UK) (31). Gene expression data (mRNA) were determined for: ACCα , AgRP, AMPK, GR, GLUT-1, INS-R, MC3/4-R, NPY and its type 1 receptor (NPY1R), PGC1α , POMC and the Ob-R. Primer sequences and reaction conditions for each gene examined are detailed in Table 1. Prior to real-time PCR, the PCR product of each primer set was gel extracted (QIAquick Gel Extraction Kit, Qiagen Ltd., Crawley, UK) and sequenced in order to confirm the specificity of the product and then to develop the necessary standards for real-time PCR analysis. 18S rRNA was used as a housekeeping gene and results calculated using the $2^{-\Delta CT}$ method (32). Gene expressions from 7 days old control offspring were used as a relative reference for all analyses.

Statistical Analyses

Data were analysed using SPSS v14.0 for Windows (SPSS Inc., Chicago, USA). Data

were normally distributed and were, therefore, analysed by using one-way ANOVA with Bonferroni *Post-Hoc* test. The effect of postnatal age on hypothalamic gene expression between C (7 days old) and L (1 year old) offspring were assessed by unpaired Student *t* test. Data are reported as mean ± S.E.M, a *P* value < 0.05 was taken to represent a statistically significant difference.

Results
Offspring morphometry
At birth, and at 7 days of age, no morphometric differences were observed between control and nutrient restricted offspring (Table 2A). There were no differences in growth rates between groups until after 3 months of age and exposure to the post-weaning obesogenic environment. By 1 year of age, all obese animals were heavier and fatter than those maintained in a "free-living" environment (Table 2B). In addition, the obese group were significantly longer (*i.e.* increased crown-to-rump length) and taller (*i.e.* increased shoulder height) than lean animals.

"Energy balance" at 1 year of age
The average daily caloric intake for obese and lean animals was similar at 1 year of age, but this was reduced in the nutrient restricted obese offspring (Figure 1). Total physical activity in all obese animals was ~50% lower than in the lean group. This reflected the difference in activity between those animals kept in a "free-living" environment as opposed to being maintained within an obesogenic environment (24). As a consequence relative ratio of daily energy intake to physical activity was significantly greater in obese compared to lean animals. However, the positive effect of obesity on this ratio was no longer apparent in obese offspring born to nutrient restricted mothers (Figure 1C).

Metabolic, endocrinological and molecular adaptations
Plasma metabolites and hormone concentrations
At 1 year of age, after 9 months exposure to an obesogenic environment, basal plasma glucose, TG, cortisol and ACTH were all similar in obese and lean animals (Table 3). Plasma leptin and NEFA were both significantly raised with obesity, irrespective of maternal diet. In addition, although plasma insulin was increased in all obese animals, this adaptation was amplified in offspring born to nutrient restricted mothers.
Insulin sensitivity
The decline of plasma glucose (K_{itt}, %/min/kg) following IVTT was lower in obese relative to lean animals (Table 3) but

prenatal nutrient restriction did not exacerbate this effect

Hypothalamic gene expression
Seven days after birth, the only hypothalamic gene examined that was decreased by maternal nutrition was NPY (Table 4). Between 7 days and 1 year of age, gene expression for all orexigenic factors increased as did mRNA abundance for the anorexigenic factors POMC and MC3R, although none were affected by the prenatal nutritional environment. In contrast, gene expression for NPY1R, GR and MC4R remained unchanged with age, but the latter was significantly upregulated in obese offspring born to nutrient restricted mothers.

Gene expression for the metabolic and energy sensing factors IR, PGC1α and ACCα all increased with age, whereas GLUT-1 and AMPK were unchanged. Obesity resulted in upregulation of IR and PGC1α gene expression, adaptations that were accompanied with increased mRNA abundance for both AMPK and ACCα, but only in the nutrient restricted group.

To further evaluate the magnitude of leptin resistance within the hypothalamus for each animal, plasma leptin concentration was expressed relative to mRNA abundance of AgRP and NPY. Following this analysis it was apparent that both were significantly elevated by obesity (Figure 2).

Discussion

We have shown that, in sheep, exposure to an obesogenic environment i.e. a simple reduction in the available living-space results in pronounced obesity, by one year of age. Importantly this reduction in activity persists when the animal is moved to a more confined living environment although food intake does not necessarily adapt accordingly. The obese phenotype we observed in this study is in accord with that seen in humans namely, raised plasma leptin and NEFA (33, 34). Furthermore, the lack of any change in gene expression for NPY or AgRP within the hypothalamus emphasises that all obese animals were leptin resistant (35). Interestingly, offspring born to nutrient restricted mothers exhibited an appreciable reduction in daily food intake when individually housed that was not seen in those obese animals born to control fed mothers. This adaptation was accompanied by raised plasma insulin that may have been the trigger for the adaptive response seen in the hypothalamus of those offspring born to nutrient restricted mothers.

Resetting of appetite control by early-to-mid gestational nutrient restriction?

In an environment with restrained physical activity, offspring born to nutrient restricted mothers maintained their mean daily food intake in accord with estimated metabolic demands with the result that they were in neutral energy balance. Obese offspring born to control fed mothers, however, failed to adapt in the same way. Unexpectedly, this period of energy restriction of the mother and fetus was not related to any major differences in gene expression within the hypothalamus as measured soon after birth. At this age, the only significant difference related to the prenatal environment was a downregulation in NPY in the nutrient restricted offspring. This transient adaptation was not, however, associated with any difference in early postnatal growth rate. The only other study during fetal development in which maternal nutrition had an effect on NPY was following a 50% reduction in food intake during the final month of gestation where fetal plasma glucose and insulin decreased (29). In accord with our findings, changes in NPY mRNA abundance around the time of birth

were not accompanied by any major changes in postnatal and juvenile growth (36, 37). In the present study, the materno-fetal endocrine environment may have been modified after 80 days of gestation following restoration of the maternal diet to the same amount as controls. For example, this results in a rise in maternal plasma cortisol, thus restoring its concentration to the same as in control fed mothers (16) with no effect, however, on maternal plasma glucose concentrations (16). Taken together, these findings support the hypothesis that the NPY/AgRP system is sensitive to changes in energy supply (5) although the primary factors regulating fetal NPY gene expression between mid and late gestation remain to be determined.

It should be noted that the expression of a majority of hypothalamic genes examined were much lower at 1 week compared with 1 year of age. Notable exceptions were the anorexogenic factors MC4 and GR. Interestingly, MC3R was found to be 100 times lower at 1 week, than at 1 year of age. These findings are in accord with those in the rat in which MC4R increases in the prenatal period, whereas MC3R expression, only appears near to the time of weaning (38). The ontogeny of GR gene expression we observed in the hypothalamus is in accord with that seen in the liver between birth and early adulthood (39). Relatively low expression of genes involved in the regulation of the appetite within the hypothalamus of the newborn suggests that the central control of appetite is much less important in the pre- and early postnatal period when the drive to feed is crucial to survival. In this regard, during early lactation, the interaction between the offspring and its mother and/or the competition for food with its siblings (40) may well have a much greater impact on feeding than hypothalamic signaling alone. This would explain why differences seen in hypothalamic gene expression of appetite controllers in the early postnatal period are not related to persistent differences in milk intake (5).

The long term programming of energy balance following obesity – the influence of

hypothalamic energy sensing and insulin signaling

One particularly interesting outcome of our study was that obese offspring born to nutrient restricted mothers were better adapted to maintain a neutral "energy balance" after they had become obese. This was despite the fact that they were markedly leptin resistant and would not, therefore, be predicted to show any difference in food intake compared to the obese offspring born to control fed mothers. Nevertheless, the control of food intake is a complex process based on the integration of a range of signals (41). It has been suggested that the energy sensing pathway and insulin responsiveness coordinate with leptin to regulate appetite and energy expenditure. For example, AMPK and ACCα have been found to directly interact with leptin (12) and adiponectin (42) to control hypothalamic NPY and POMC neuronal activity, in conjunction with the regulation of energy sensing (43). In addition, insulin acting through its hypothalamic receptor induces an anorexigenic signal (8, 10). It could be hypothesized that a combination of elevated plasma insulin in conjunction with up-regulation of the IR and of the energy sensing system (i.e. AMPK and ACCα) act synergistically to promote satiation. As a consequence, offspring born to nutrient restricted mothers showed a pronounced reduction in food intake as measured when individually housed and were able to maintain a neutral "energy balance". A response of this type would be further amplified by the increased gene expression of MC4R that would be predicted to reduce food intake and stimulate energy expenditure (41).

To date, the mechanisms underlying developmental programming of appetite and insulin homeostasis remain unclear. In our study we observed a long term effect of maternal nutrient restriction, resulting in elevated plasma insulin concentrations. Interestingly, a similar up-regulation of resting plasma insulin has been found in nutrient restricted rats when habituated onto a high energy diet (44). Surprisingly, this hyperinsulinemia was abolished when offspring were treated with leptin in the early post-natal period (44) that in the rat coincides with maturation of the hypothalamic-pituitary axis. One question that arises is whether the hyperinsulinemia observed in our offspring born to mothers nutrient restricted at a much earlier time of brain development could be related to low leptin concentrations in these offspring. Unfortunately, we could not measure plasma leptin in the neonatal period, although we found no difference in fat mass at one week of age and would thus not expect any difference in plasma leptin as this is the main determinant at this age of life (45). Nevertheless, one of the main functions of leptin is to reduce NPY gene transcription (46, 47). Thus, the down-regulation of NPY mRNA abundance we observed in the nutrient restricted offspring at 1 week of age could be mediated by a reduction in plasma leptin.

The thrifty phenotype hypothesis supports a link between an adverse fetal environment and the risk for adult metabolic diseases (48, 49). We nonetheless shown that, in our nutrient restricted obese offspring, maternal nutrient restriction protected against the development of glomerulosclerosis associated with obesity (24). Similarly, in this present study we demonstrate an adaptation that may promote improved regulation of food intake with obesity. However, the absolute level of obesity and insulin resistance remained similar between all animals. Furthermore, more importantly, the obese offspring born to nutrient restricted mothers developed hyperinsulinemia a well recognized major risk for type II diabetes (50).

One of the limitations our study is the use of real-time RT PCR technology on the entire hypothalamus in order to assess the expression of a large number of genes involved in appetite regulation. The advantage of this procedure is that it enabled us to obtain quantitative information with regard to the effect of age, maternal diet and obesity. It is important, however, to note that the hypothalamus is not a homogeneous entity but includes many nuclei that may exhibit differential regulation within some of the genes we examined (5). Gene expression on the entire hypothalamus has previously been used to study appetite

regulation in both rats (30) and sheep (29) but of course cannot provide detailed information on site specific regulations. So although our results substantiate current knowledge in appetite regulation, future work of this type needs to look at changes within specific nuclei of the hypothalamus.

In conclusion, we have nonetheless shown that, although maternal nutrient restriction targeted over the period of early fetal brain development has very little, if any, direct effect on appetite regulation early in postnatal life, it can have a significant impact in the obese adult. The potential mechanisms allowing better control of food intake with obesity remain to be fully determined as to whether it highlights a protective adaptation or an indirect consequence of hyperinsulinemia, but our model may enable the design of new therapeutic strategies against the major dysfunctional outcomes of obesity.

References

1. **Budge H, Gnanalingham MG, Gardner DS, Mostyn A, Stephenson T, Symonds ME** 2005 Maternal nutritional programming of fetal adipose tissue development: long-term consequences for later obesity. Birth Defects Res C Embryo Today 75:193-9

2. **Jebb SA, Rennie KL, Cole TJ** 2004 Prevalence of overweight and obesity among young people in Great Britain. Public Health Nutr 7:461-5

3. **Symonds ME, Stephenson T, Gardner DS, Budge H** 2007 Long-term effects of nutritional programming of the embryo and fetus: mechanisms and critical windows. Reprod Fertil Dev 19:53-63

4. **McMillen IC, Robinson JS** 2005 Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 85:571-633

5. **Muhlhausler BS, Adam CL, Findlay PA, Duffield JA, McMillen IC** 2006 Increased maternal nutrition alters development of the appetite-regulating network in the brain. Faseb J 20:1257-9

6. **Banks WA, DiPalma CR, Farrell CL** 1999 Impaired transport of leptin across the blood-brain barrier in obesity. Peptides 20:1341-5

7. **Vickers MH, Breier BH, McCarthy D, Gluckman PD** 2003 Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. Am J Physiol 285:R271-3

8. **Baura GD, Foster DM, Porte D, Jr., Kahn SE, Bergman RN, Cobelli C, Schwartz MW** 1993 Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. J Clin Invest 92:1824-30

9. **Masaki T, Chiba S, Noguchi H, Yasuda T, Tobe K, Suzuki R, Kadowaki T, Yoshimatsu H** 2004 Obesity in insulin receptor substrate-2-deficient mice: disrupted control of arcuate nucleus neuropeptides. Obes Res 12:878-85

10. **Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M** 2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 87:2988

11. **Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, Ozanne SE** 2005 Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. Am J Physiol Regul Integr Comp Physiol 288:R368-73

12. **Gao S, Kinzig KP, Aja S, Scott KA, Keung W, Kelly S, Strynadka K, Chohnan S, Smith WW, Tamashiro KL, Ladenheim EE, Ronnett GV, Tu Y, Birnbaum MJ, Lopaschuk GD, Moran TH** 2007 Leptin activates hypothalamic acetyl-CoA carboxylase to inhibit food intake. Proc Natl Acad Sci U S A 104:17358-63

13. **Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birnbaum MJ, Stuck BJ, Kahn BB** 2004 AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-74

14. **Tang W, Richards EM, Raizada MK, Sumners C** 1995 Angiotensin II increases glucose uptake and glucose transporter-1 mRNA levels in astroglia. Am J Physiol 268:E384-90

15. **Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich**

CF, Kelly DP** 2005 PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol 3:e101

16. **Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T, Symonds ME** 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. Endocrinology 144:3575-85

17. **Gnanalingham MG, Mostyn A, Symonds ME, Stephenson T** 2005 Ontogeny and nutritional programming of adiposity in sheep: potential role of glucocorticoid action and uncoupling protein-2. Am J Physiol Regul Integr Comp Physiol 289:R1407-15

18. **Muhlhausler BS, McMillen IC, Rouzaud G, Findlay PA, Marrocco EM, Rhind SM, Adam CL** 2004 Appetite regulatory neuropeptides are expressed in the sheep hypothalamus before birth. J Neuroendocrinol 16:502-7

19. **Adam CL, Findlay PA, Chanet A, Aitken RP, Milne JS, Wallace JM** 2008 Expression of energy balance regulatory genes in the developing ovine fetal hypothalamus at midgestation and the influence of hyperglycemia. Am J Physiol Regul Integr Comp Physiol 294:R1895-900

20. **Clarke L, Heasman L, Juniper DT, Symonds ME** 1998 Maternal nutrition in early-mid gestation and placental size in sheep. Br J Nutr 79:359-64

21. **Erhard HW, Boissy A, Rae MT, Rhind SM** 2004 Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. Behav Brain Res 151:25-35

22. **Whorwood CB, Firth KM, Budge H, Symonds ME** 2001 Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. Endocrinology 142:2854-64

23. **Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B** 1998 A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392:398-401

24. **Williams PJ, Kurlak LO, Perkins AC, Budge H, Stephenson T, Keisler D, Symonds ME, Gardner DS** 2007 Hypertension and impaired renal function accompany juvenile obesity: the effect of prenatal diet. Kidney Int 72:279-89

25. **Agricultural and Food Research Council** 1993 Technical Committee on Responses to Nutrients. Report No. 9. In: Technical Committee on Responses to Nutrients. Report No. 9. CAB International, Wallingford, UK; pp 812-815

26. **Crouter SE, Schneider PL, Bassett DR, Jr.** 2005 Spring-levered versus piezo-electric pedometer accuracy in overweight and obese adults. Med Sci Sports Exerc 37:1673-9

27. **Gardner DS, Von Bon BMW, Dandrea J, Goddard PJ, May SF, Wilson V, Stephenson T, Symonds ME** 2006 Effect of periconceptual undernutrition and gender on hypothalamic-pituitary-adrenal axis function in young adult sheep. J Endocrinol 190:203-212

28. **Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A, Kann G** 2000 Effect of sheep nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA. J Endocrinol 165:519-526

29. **Warnes K, Morris MJ, Symonds ME, Phillips ID, Owens JA, McMillen IC** 1998 Effects of gestation, cortisol and maternal undernutrition on hypothalamic neuropeptide Y mRNA levels in the sheep fetus. J Neuroendocrinol 10:51-57

30. **Teske JA, Levine AS, Kuskowski M, Levine JA, Kotz CM** 2006 Elevated hypothalamic orexin signaling, sensitivity to orexin A, and spontaneous physical activity in obesity-resistant rats. Am J Physiol Regul Integr Comp Physiol 291:R889-99

31. **Mostyn A, Sebert SP, Litten JC, Perkins KS, Laws J, Symonds ME, Clarke L** 2006 Influence of porcine genotype on the abundance of thyroid hormones and leptin in sow milk and its impact on growth, metabolism and expression of key adipose tissue genes in the offspring. J Endocrinol 190:631-639

32. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2T Method. Methods 25:402-408

33. **Boden G** 1996 Fatty acids and insulin resistance. Diabetes Care 19:394-5

34. **Boden G** 1998 Free fatty acids (FFA), a link between obesity and insulin resistance. Front Biosci 3:d169-75

35. **Askari H, Liu J, Dagogo-Jack S** 2005 Energy adaptation to glucocorticoid-induced hyperleptinemia in human beings. Metabolism 54:876-80

36. **Hyatt MA, Budge H, Walker D, Stephenson T, Symonds ME** 2007 Effects of maternal parity and late gestational nutrition on mRNA abundance for growth factors in the liver of postnatal sheep. Am J Physiol Regul Integr Comp Physiol 292:R1934-42

37. **Gardner DS, Tingey K, Van Bon BW, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T, Symonds ME** 2005 Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. Am J Physiol Regul Integr Comp Physiol 289:R947-54

38. **Kistler-Heer V, Lauber ME, Lichtensteiger W** 1998 Different developmental patterns of melanocortin MC3 and MC4 receptor mRNA: predominance of Mc4 in fetal rat nervous system. J Neuroendocrinol 10:133-46

39. **Hyatt MA, Budge H, Walker D, Stephenson T, Symonds ME** 2007 Ontogeny and nutritional programming of the hepatic growth hormone-insulin-like growth factor-prolactin axis in the sheep. Endocrinology 148:4754-60

40. **Johnson P** 1979 Comparative aspects of of the control of breathing. In: von Euler C, Lagercrantz H (eds) Central nervous control mechanisms in breathing. Pergamon Press, Oxford

41. **Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW** 2006 Central nervous system control of food intake and body weight. Nature 443:289-95

42. **Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, Kozono H, Takamoto I, Okamoto S, Shiuchi T, Suzuki R, Satoh H, Tsuchida A, Moroi M, Sugi K, Noda T, Ebinuma H, Ueta Y, Kondo T, Araki E, Ezaki O, Nagai R, Tobe K, Terauchi Y, Ueki K, Minokoshi Y, Kadowaki T** 2007 Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 6:55-68

43. **Claret M, Smith MA, Batterham RL, Selman C, Choudhury AI, Fryer LG, Clements M, Al-Qassab H, Heffron H, Xu AW, Speakman JR, Barsh GS, Viollet B, Vaulont S, Ashford ML, Carling D, Withers DJ** 2007 AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. J Clin Invest 117:2325-36

44. **Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, Breier BH, Harris M** 2005 Neonatal leptin treatment reverses developmental programming. Endocrinology 146:4211-4216

45. **Bispham J, Budge H, Mostyn A, Dandrea J, Clarke L, Keisler DH, Symonds ME, Stephenson T** 2002 Ambient temperature, maternal dexamethasone, and postnatal ontogeny of leptin in the neonatal lamb. Pediatr Res 52:85-90

46. **Korner J, Savontaus E, Chua SC, Jr., Leibel RL, Wardlaw SL** 2001 Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. J Neuroendocrinol 13:959-66

47. **Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, Prunkard DE, Porte D, Jr., Woods SC, Seeley RJ, Weigle DS** 1996 Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes 45:531-5

48. **Hales CN, Barker DJP** 1992 Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35:595-601

49. **Hales CN, Barker DJ** 2001 The thrifty phenotype hypothesis. Br Med Bull 60:5-20

50. **Eckel RH, Grundy SM, Zimmet PZ** 2005 The metabolic syndrome. Lancet 365:1415-28

Table and Figure legends

Table 1: Summary of ovine specific oligonucleotide primers for hypothalamic genes determined using real-time PCR.

Table 2: Offspring morphometry at (A) birth, 7 days and (B) 1 year of age in lean (L) and obese (O) animals born to mothers that were either fed a control diet throughout pregnancy or nutrient restricted (NR) between early to mid gestation. Values are mean concentrations ± SEM.

Table 3: Mean plasma concentrations of hormones and metabolites and insulin sensitivity at 1 year of age in lean (L) and obese (O) animals born to mothers that were either fed a control diet throughout pregnancy or nutrient restricted (NR) between early to mid gestation. Values are mean concentrations ± SEM

Table 4: Mean hypothalamic gene expression in 7 days and 1 year old offspring in lean (L) and obese (O) animals born to mothers that were either fed a control (C) diet throughout pregnancy or nutrient restricted (NR) between early to mid gestation. Values are mean ± SEM.

Figure 1: Mean (A) food intake, (B) physical activity and (C) ratio of A to B at 1 year of age in lean (L) and obese (O) animals born to mothers that were either fed a control diet throughout pregnancy or nutrient restricted (NR) between early to mid gestation. Values are mean ± SEM. Significant differences from between L and O groups: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and between O groups: # $P<0.05$.

Figure 2: Mean leptin sensitivity in lean (L) and obese (O) adult sheep that were born to mothers that were either fed a control diet throughout pregnancy or nutrient restricted (NR) between early to mid gestation. Comparison based on relative ratios of leptin with AgRP (A) and NPY (B). Values are mean ± SEM. Significant differences from between L and O groups: * $P<0.05$, ** $P<0.01$.

Table 1

Genes		Primer Sequences
AgRP	*Forward*	5'-TGA AGC GGA TAA TGG AGG AAC AG-3'
	Reverse	5'-GAG AGG GTC AAG TAG AGA TAG AGG-3'
MC3R	*Forward*	5'-CGG GTG CCT GAC TCC AAA CTC-3'
	Reverse	5'-GTT CTG GGT GTC CGT GCT CTG-3'
MC4R	*Forward*	5'-GGT GTC GGG CGT CTT GTT C-3'
	Reverse	5'-CGG TGA TGA GGC AGA TGA TGA C-3'
NPY	*Forward*	5'-TCA TCA CCA GGC AGA GAT ACG G-3'
	Reverse	5'-GAG CAA GTT TCC CAT CAC C-3'
NPY1R	*Forward*	5'-GTT GAA CCC CTT TGT GCA GT-3'
	Reverse	5'-TCA GGA AGG GCA GAG AAG AA-3'
Ob-R	*Forward*	5'-TGA AAC CAC TGC CTC CAT CC-3'
	Reverse	5'-TCC ACT TAA ACC ATA GCG AAT CTG-3'
POMC	*Forward*	5'-GCT GCT GGT CTT GCT GCT TC-3'
	Reverse	5'-CCT GAC ACT GGC TCG TCT CC-3'
GLUT-I	*Forward*	5'-GCA GGA GAT GAA GGA GGA GAG C-3'
	Reverse	5'-GCA GCA CCA CGG AAA TGA G-3'
IR	*Forward*	5'-CTG CAC CAT CAT CAA CGG AA-3'
	Reverse	5'-CGTAAC TTC CGG AAG AAG GA-3'
GR	*Forward*	5'-ACT GCC CCA AGT GAA AAC AGA-3'
	Reverse	5'-ATG AAC AGA AAT GGC AGA CAT TTT ATT-3'
AMPK	*Forward*	5'-GCT GGA TTT TGA ATG GAA GG-3'
	Reverse	5'-CAG CAC CTC ATC ATC AAT GC-3'
ACCα	*Forward*	5'-GCT ATG GAA GTC GGC TGT GGA AG-3'
	Reverse	5'-TCG TCA GGA AGA GGC GGA TGG-3'
PGC1α	*Forward*	5'-GTG TGT GTT TGC CTG GTT TG-3'
	Reverse	5'-GCC TGT GGT TGG TTG GTT AG-3'
18S	*Forward*	5'-GAT GCG GCG GCG TTA TTC C-3'
	Reverse	5'-CTC CTG GTG GTG CCC TTC C-3'

Table 2

A

	Age	**C** **(n=7)**	**NR** **(n=7)**
Body weight (kg)	Birth	3.29 ± 0.24	3.19 ± 0.15
	7 days old	4.44 ± 0.32	4.58 ± 0.25
Crown-rump-length (cm)	Birth	42.4 ± 1.0	43.1 ± 1.2
	7 days old	48.8 ± 1.2	48.1 ± 0.7
Fat mass (g)	7 days old	58.8 ± 8.5	65.7 ± 7.2
Fat mass (% body weight)	7 days old	1.3 ± 0.1	1.4 ± 0.09

B

	L **(n=7)**	**O** **(n=7)**	**NRO** **(n=8)**
Body weight (kg)	57.9 ± 3.7	90.6 ± 1.9***	88.7 ± 1.7**
Crown-rump-length (cm)	98.4 ± 1.2	110.0 ± 2.2**	104.3 ± 3.7
Height (cm)	73.6 ± 1.9	78.7 ± 1.9*	77.7 ± 2.1
Subcutaneous fat depth (mm)	5.7 ± 0.6	13.9 ± 1.3***	13.0 ± 1.0***
Visceral fat mass (kg)	1.62 ± 0.15	7.24 ± 0.35***	6.77 ± 0.45***
% Visceral Fat mass	2.5 ± 0.3	8.0 ± 0.3***	7.7 ± 0.6***

Significant differences with L group: * $P <0.05$; ** $P <0.01$; *** $P <0.001$.

Table 3

	L (n=7)	O (n=7)	NRO (n=8)
Glucose (mmol/l)	4.47 ± 0.58	5.77 ± 0.62	5.17 ± 0.51
Triglycerides (mmol/l)	0.17 ± 0.03	0.20 ± 0.05	0.21 ± 0.08
NEFA (mmol/l)	0.31 ± 0.05	0.64 ± 0.16*	0.56 ± 0.06*
Insulin (ng/ml)	0.56 ± 0.16	1.03 ± 0.15*	1.66 ± 0.29*[#]
Cortisol (nmol/l)	58.5 ± 8.9	76.6 ± 17.1	89.8 ± 14.8
Leptin (ng/ml)	3.0 ± 0.5	18.9 ± 1.4**	22.3 ± 1.5**
ACTH (mmol/l)	55.9 ± 8.2	60.3 ± 4.4	56.0 ± 6.1
K_{ITT} (% glucose decline/kg/min)	2.84 ± 0.26	2.13 ± 0.20*	2.19 ± 0.29

Significant differences with L group: * $P<0.05$; ** $P<0.01$; or between O and NRO groups [#] $P<0.05$.

Table 4

Gene		7 days old			1 year old			
		C (n=7)	NR (n=7)	*P* - 7 days	L (n=7)	O (n=7)	NRO (n=8)	*P - age*
Orexigenic factors	**AgRP**	1.0 ± 0.2	0.6 ± 0.1	*NS*	17 ± 5	34 ± 14	25 ± 7	*< 0.01*
	NPY	1.0 ± 0.2	0.5 ± 0.1	*< 0.05*	11 ± 3	6.5 ± 1.5	7.6 ± 0.7	*< 0.05*
	Ob-R	1.0 ± 0.3	0.6 ± 0.1	*NS*	2.5 ± 0.4	3.2 ± 1.6	2.0 ± 0.4	*< 0.05*
	NPY1R	1.0 ± 0.1	2.6 ± 0.7	*NS*	1.3 ± 0.2	1.6 ± 0.5	1.8 ± 0.4	*NS*
Anorexigenic factors	**POMC**	1.0 ± 0.2	1.0 ± 0.2	*NS*	2.6 ± 0.9	3.5 ± 1.2	8.0 ± 4.9	*< 0.05*
	MC3R	1.0 ± 0.2	0.7 ± 0.1	*NS*	135 ± 18	311 ± 155	197 ± 43	*< 0.01*
	MC4R	1.0 ± 0.2	0.8 ± 0.1	*NS*	0.7 ± 0.1	1.1 ± 0.3	1.5 ± 0.3 *	*NS*
	GR	1.0 ± 0.2	0.7 ± 0.1	*NS*	0.3 ± 0.1	0.4 ± 0.05	0.5 ± 0.1	*<0.05*
Metabolic and energy sensor factors	**GLUT-I**	1.0 ± 0.3	0.6 ± 0.1	*NS*	1.1 ± 0.1	1.3 ± 0.3	1.2 ± 0.2	*NS*
	IR	1.0 ± 0.1	0.9 ± 0.1	*NS*	2.7 ± 0.7	6.7 ± 1.1 *	5.7 ± 0.5 *	*< 0.05*
	AMPK	1.0 ± 0.1	1.2 ± 0.2	*NS*	1.7 ± 0.3	2.3 ± 0.2	3.0 ± 0.4 *	*NS*
	PGC1α	1.0 ± 0.1	1.0 ± 0.1	*NS*	3.6 ± 0.5	6.3 ± 0.8 *	7.6 ± 0.6 *	*< 0.05*
	ACCα	1.0 ± 0.1	1.0 ± 0.1	*NS*	3.3 ± 0.3	4.2 ± 0.5	5.2 ± 0.4 *	*< 0.05*

Significant differences from the L group at 1 year of age: * *P*<0.05.
P – age: represents age-related differences between 7 days old control (C) and 1 year old lean (L) offspring