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ORIGINAL ARTICLE

Prenatal programming of obesity in a swine model of leptin resistance: modulatory effects of controlled postnatal nutrition and exercise

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The main role of early nutritional programming in the current rise of obesity and associated diseases is well known. However, translational studies are mostly based in postnatal food excess and, thus, there is a paucity of information on the phenotype of individuals with prenatal deficiencies but adequate postnatal conditions. Thus, we assessed the effects of prenatal programming (comparing descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy) on gene expression, patterns of growth and fattening, metabolic status and puberty attainment of a swine model of obesity/leptin resistance with controlled postnatal nutrition and opportunity of exercise. Maternal restriction was related to changes in the relationships among gene expression of positive (insulin-like growth factors 1 and 2) and negative (myostatin) regulators of muscle growth, with negative correlations in gilts from restricted pregnancies and positive relationships in the control group. In spite of these differences, the patterns of growth and fattening and the metabolic features during juvenile growth were similar in control gilts and gilts from restricted pregnancies. Concomitantly, there was a lack of differences in the timing of puberty attainment. However, after reaching puberty and adulthood, females from restricted pregnancies were heavier and more corpulent than control gilts, though such increases in weight and size were not accompanied by increases in adiposity. In conclusion, in spite of changes in gene expression induced by developmental programming, the propensity for higher weight and adiposity of individuals exposed to prenatal malnutrition may be modulated by controlled food intake and opportunity of physical exercise during infant and juvenile development.

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Introduction

Obesity and associated disorders have been traditionally reported in people from developed countries. However, the most recent epidemiological studies indicate that their incidence is currently increasing at a high rate in individuals living in rapidly developing areas.[1] At the same time, although obesity is traditionally more prevalent in adults, the incidence of obesity and overweight is currently increasing at an alarming rate in childhood. The number of overweight children was over 42 million in 2010; around 35 million of these are living in developing countries (http://www.who.int/dietphysicalactivity/childhood/en/). These two facts indicate important changes in the prevalence, incidence and sociodemographic profile of the disease, pointing to childhood and youth from developing countries.

Young people from developing countries are characterized by having intrinsic ethnic features, by descending from ancestors adapted to food scarcity and by a current exposure to nutrients excess, mostly in the form of high caloric obesogenic diets. The consequences have been mainly studied in India, which is currently facing epidemics of obesity and diabetes.[2] Indian ethnicity is thought to have an adaptive *thrifty phenotype* for surviving in scarce food environment; a high percentage of Indian newborns are affected by intrauterine growth retardation (IUGR)[2–4] and the postnatal exposure of these children to diets abundant in amount and calories causes increased adiposity, insulin resistance (IR) and cardiometabolic risk as early as at eight to nine years of age.[4,5] The same increase in childhood obesity is being reported in other areas like Brazil,[6] China[7] and Middle East countries.[8,9]

These findings closely resemble data supporting the hypothesis of the Developmental Origin of Health and Disease (DOHaD),[10] which addresses that the interaction between genetic predisposition, nutrition of the conceptus during pregnancy and postnatal exposure to obesogenic environments (mainly inadequate nutrition and lack of physical activity) markedly determines juvenile growth, fitness/obesity and appearance of some metabolic diseases.

The study of potential interactions of DOHaD with the physiology and pathophysiology of a complex multi-factorial disease like obesity makes necessary the development of observational, mechanistic and interventional studies. Experimentation in humans is obviously limited by ethical issues and, therefore, research needs to be performed in animal models; specifically, for translational purposes, in mammalian species.[11] Most of the studies have been carried out in mice; however, the use of

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large animals (rabbit, sheep, pig) offers numerous profitable characteristics for translational studies.[12] The most amenable large animal model for obesity studies is the pig (proportional organ sizes, omnivorous habits, similar characteristics of lipoprotein metabolism and propensity to sedentary behaviour and obesity).[13–15] There is a swine breed, the Iberian pig, which is a well-recognized translational model for studies on obesity and associated diseases in humans.[16,17] The Iberian pig faces similar conditions to previously cited for humans living in developing countries. Specifically, the Iberian pig has an adaptive *thrifty phenotype* owing to a gene polymorphism for the leptin receptor, similar to the syndrome of leptin resistance described in human medicine[18–20] with effects on food intake, body weight and fat deposition.[21,22] Moreover, the Iberian pig has a background of exposure to harsh environments and food scarcity and a current availability of nutrients in excess.

Previous studies of our group[23–25] indicate that maternal undernutrition in Iberian swine induces IUGR and therefore lower birth weight and corpulence of the offspring; in a similar way to lean swine breeds. The results found in lean genotypes indicate that IUGR cannot be compensated during postnatal growth. The postnatal development after IUGR is driven by offspring sex, in the Iberian genotypes. In a similar way to lean swine breeds, IUGR in Iberian males cannot be compensated during postnatal growth; however, females compensate IUGR by occurrence of catch-up growth, as early as during the breast-feeding period.[23,25] Hence, at weaning, weight and size are similar in female piglets born to restricted and non-restricted sows. At adulthood, in case of exposure of these piglets to an obesogenic environment (*ad libitum* access to a high-fat diet and lack of physical activity) during their juvenile period, females born to restricted mothers are heavier and more fattened than females from control sows.[25]

At the present time, concomitantly with these results in the Iberian model, the incidence of overweight in girls from developing countries is currently increasing, for reaching and even exceeding rates reported for boys.[8,9,26] This is a very concerning issue since the health importance of overweight and obesity in girls is aggravated by their relationship with reproductive disorders; mainly, an earlier age of menarche.[27–29]

Currently, although in the middle of controversy,[30] the fight against obesity and associated disorders is based both on prevention (mainly, adequate lifestyle and nutrition)[6] and medical treatment (mainly insulin sensitizers like metformin,[31] although combined with controlled diet and exercise).[32] However, the available information on the links between DOHaD and obesity suggests that individuals with genetic predisposition to obesity, exposed to prenatal malnutrition and evidencing catch-up growth in the early postnatal stages are fated to develop obesity and metabolic diseases.[33–35] Experimental studies on DOHaD are mostly based in a mismatch between prenatal nutritional scarcity and postnatal food excess. However, there is limited information on juvenile and adult phenotype of individuals affected by prenatal programming but growing up in a non-obesogenic postnatal environment with adequate nutrition and physical activity. Thus, the main objective of the present experiment was to determine the effects of prenatal programming on gene expression, patterns of growth and fattening, metabolic status and reproductive features (puberty attainment) during juvenile and adult periods of Iberian pigs with controlled nutrition and opportunity of exercise.

Materials and methods

Animals and handling

The experimental work was carried out at the INIA Animal Unit, under Project License assessed and approved (report CEEA 2010/003) by the INIA Committee of Ethics in Animal Research. The INIA Animal Unit meets the requirements of the European Union for Scientific Procedure Establishments and management of animals was performed accordingly to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

The experiment involved 17 Iberian gilts (purebred Torbiscal strain) from 17 different litters born to purebred Torbiscal sows with similar phenotypes, body weights and age (three to four parities) but different nutritional management during pregnancy. A first group of gilts (control group, $n = 8$) was born to sows that were fed with a standard grain-based diet (13.0% of crude protein, 2.8% of fat and 3.00 Mcal/kg of metabolizable energy) fulfilling their daily maintenance requirements for pregnancy. The second group (restricted group, $n = 9$) received the same amount of food than control group until day 35 of gestation but only 50% of such quantity during the remaining last two-thirds of pregnancy. At birth, as a consequence of the nutritional management as previously described,[23,25] the mean body weight was significantly lower in the newborns from restricted sows than in the control group (1.38 ± 0.47 *v.* 1.61 ± 0.41 kg, $P < 0.05$); both male and female offspring were equally affected by nutritional restriction. On the other hand, there were no effects in litter size between control and restricted sows.

At birth, a gilt representative of the mean was selected and identified in each litter for further studies to be carried out during the juvenile period (from weaning at 28 days of age to adulthood at 290 days of age), but they remained with their mothers (i.e. there was no cross-fostering). From farrowing to weaning, all the sows were fed with the same amount (3 kg) of a standard grain-based food diet with 15.0% of crude protein, 3.1% of fat and 3.10 Mcal/kg metabolizable energy (i.e. the restricted sows were only restricted during pregnancy).

At weaning, all the gilts were housed, isolated from boars, in collective pens at the facilities of the INIA Animal Laboratory Unit (Madrid, Spain), with around 7 m^2 of surface per animal (between 7 and 12 folds, depending on age, the surface indicated by welfare regulations). During the first month after weaning, all the piglets were fed with a standard diet with mean values of 18% of crude protein, 4.5% of fat and 3.35 Mcal/kg of metabolizable

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energy. Afterwards, from 60 to 290 days of age, all the gilts were fed with a diet containing mean values of 15.1% of crude protein, 2.8% of fat and 3.08 Mcal/kg of metabolizable energy; the amount of food offered was re-calculated with age for fulfilling daily maintenance requirements.

Changes in growth, corpulence, adiposity and metabolic status were evaluated in all the gilts until they reached 290 days of age. Concomitantly, appearance of puberty was also studied by assessing changes in plasma progesterone concentrations. Finally, at 290 days of age, all the females were euthanized by the i.v. injection of a euthanasia solution (T-61; MSD AH, Boxmeer, The Netherlands). Immediately, a sample of longissimus dorsi was obtained at the level of the last rib for *ex vivo* evaluations of amount and composition of intramuscular fat and muscular gene expression. Samples were immediately divided into two portions and either vacuum packaged in individual bags and stored at −20°C until analyzed for fat composition or snap frozen in liquid nitrogen and, afterwards, stored at −80°C until analyzed for gene expression.

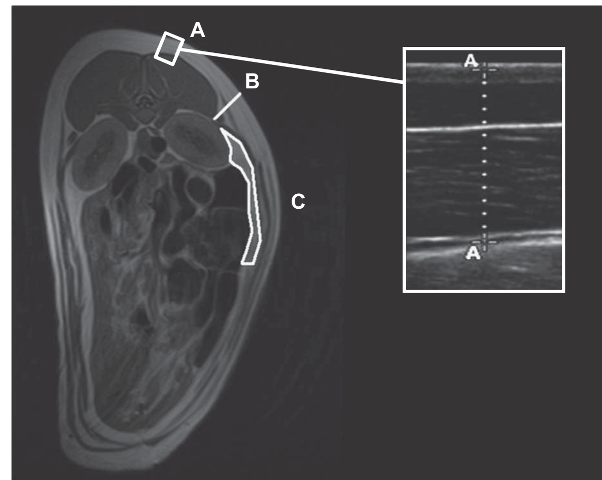

Fig. 1. Anatomical references for measuring subcutaneous and visceral fat depots by ultrasonography (*a*) and magnetic resonance imaging (*b*, *c*).

Evaluation of growth pattern and corpulence

Body weight and size (thoracic and abdominal circumferences, respectively, named as TC and AC, obtained with a measuring tape) were measured, monthly, from 28 to 290 days of age, in all the gilts. Data from weight and body sizes were used for determining two formulae for calculating body mass index (BMI).

The first formula (BMI1) was extrapolated from human clinical studies:

$$\text{Weight (kg)}/\text{Length}^2\,(\text{m}^2)$$

The second formula (BMI2) takes into account the trunk volume and, thus, incorporated values of TC and AC, respectively), indicative for the amount of total, visceral and subcutaneous fat in swine:[36–38]

$$\frac{\text{Weight (kg)}}{\pi/3 \times \text{Length} \times \left[(\text{TC}/2\pi)^2 + (\text{AC}/2\pi)^2 + (\text{TC}/2\pi \times \text{AC}/2\pi)\right]}$$

Evaluation of adiposity

Subcutaneous fat depth was evaluated monthly, from 60 days of age, by ultrasonography; a SonoSite S-Series equipped with a 5–8 MHz lineal array probe (SonoSite Inc., Bothell, WA, USA) was used. The probe was placed against the skin, in a point at the right side of the animal located at 4 cm from the midline and transversal to the head of the last rib as determined by palpation (Fig. 1a).

Magnetic resonance imaging (MRI) was used for assessing subcutaneous and visceral adiposity at three selected age points (120, 180 and 290 days of age), since MRI is highly adequate for visualization of both subcutaneous and visceral adipose tissue.[39] The gilts were anaesthetized with isofluorane vapours (IsoFlo; Laboratorios Esteve, Barcelona, Spain), after sedation with xylazine (Rompun; Bayer Ag, Leverkusen, Germany) and ketamine (Imalgène 1000; Merial, Lyon, France), for minimizing stress and breathing movements during scans. MRI scans were carried out at the UCM Veterinary Teaching Hospital, by means of a Panorama 0.23T scanner with a body/spine XL coil (Philips Medical Systems, Best, The Netherlands). Animals were placed in lateral recumbence. Images were obtained in the transverse plane using a T1 weighted turbo spin echo sequence, from the thoracic inlet through the cranial margin of the ilium, and analyzed in a dedicated workstation using the ViewForum R6.3V1L3 software package (Philips Medical Systems). Measurements of subcutaneous fat depots were based in the maximum length obtained tracing a line perpendicular to the kidney at the level of the first lumbar vertebra (Fig. 1b) whilst values for visceral depots were based on axial areas obtained at the level of the third lumbar vertebra (Fig. 1c).

Evaluation of metabolic status

Plasma indexes of carbohydrate and lipid metabolism were assessed monthly, from 120 to 290 days of age. Possible changes in insulin secretion were evaluated at 120, 210 and 290 days of age, whilst plasma concentrations of leptin were assessed at 120, 180 and 290 days of age. Blood samples were drawn, concurrently with body measures, by jugular venopuncture with 5 ml sterile heparin blood vacuum tubes (Vacutainer[TM] Systems Europe; Becton Dickinson, Meylan, France). Immediately after recovery, the blood was centrifuged at 1500 ***g*** for 15 min and the plasma was separated and stored into polypropylene vials at −20°C until assayed.

Glucose and fructosamine were measured with a clinical chemistry analyzer (Saturno 300 Plus; Crony Instruments s.r.l., Rome, Italy) whilst insulin was determined with a Porcine Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). The assay sensitivity was 0.26 IU/l; the intra-assay variation coefficient was 3.5%. Possible changes in beta cell function and IR were

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assessed by the homeostasis model assessment (HOMA), using the equations HOMA-IR = (FINS × FPG)/22.5 to assess IR[40] and HOMA-β = (20 × FINS)/(FPG-3.5) to assess beta cell function;[41] where FINS is the fasting plasma insulin concentration in IU/l and FPG the fasting plasma glucose concentration in mmol/l.

Triglycerides, total cholesterol, high-density lipoproteins cholesterol (HDL-c) and low-density lipoproteins cholesterol (LDL-c) were measured with the same analyzer (Saturno 300 Plus). Plasma HDL-c ratio and LDL-c ratio were calculated by dividing HDL-c and LDL-c concentrations, respectively, by total cholesterol; plasma LDL-c/HDL-c ratio was obtained by dividing LDL-c levels by HDL-c concentrations.

Concentrations of leptin were determined in a single analysis using the Multi-species Leptin RIA kit (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany). The assay sensitivity was 1.0 ng/ml; the intra-assay variation coefficient was 3.1%.

Evaluation of reproductive status

The criterion used for determining the occurrence of puberty, adapted from Flowers *et al.*,[42] was an increase in plasma progesterone levels above 2.0 ng/ml for at least two consecutive samples taken every 2 weeks; onset of puberty was identified with the first of these two samples. Blood samples were obtained and processed, every 2 weeks from 120 days of age, as previously described. Plasma progesterone concentrations were measured in a single analysis using an enzyme immunoassay kit (Demeditec Diagnostics GmbH) as described by Ueshiba *et al.*[43] and validated in sows by Gonzalez-Añover *et al.*;[44] assay sensitivity was 0.045 ng/ml and the intra-assay variation coefficient was 5%.

Evaluation of fat composition

Samples of longissimus dorsi were lyophilized by mixture with 100 ml of CH_3Cl:MeOH (2:1, v/v), filtering and addition of 0.9% NaCl solution. The resulting biphasic system was allowed to separate and the upper aqueous phase was eliminated whilst the lower phase was filtered through anhydrous sodium sulphate and collected. Finally, solvent was evaporated with a rotary evaporator under vacuum and further evaporated under nitrogen.[45]

Afterwards, the fatty acids (FA) were extracted and quantified using the one-step procedure described by Sukhija and Palmquist[46] for lyophilized samples, with pentadecanoic acid (C15:0; Sigma, Alcobendas, Madrid, Spain) being used as the internal standard. In brief, fat extracts were methylated in the presence of sulphuric acid and methylated FA were identified according to Rey *et al.*[47] using a gas chromatograph (Model HP6890; Hewlett Packard Co., Avondale, PA, USA) and a 30 × 0.32 × 0.25 mm cross-linked polyethylene glycol capillary column (Hewlett Packard Innowax). A temperature program of 170°C to 245°C was used. The injector and detector were maintained at 250°C. The carrier gas (helium) flow rate was 3 ml/min. From individual FA percentages, the saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) proportions were calculated. SFA is the result of C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0. MUFA is the result of C15:1 + C16:1n-9 + C17:1 + C18:1n-9 + C18:1n-7 + C20:1. PUFA is the result of C18:2n-6 + C18:3n-3 + C18:4n-3 + C20:3n-9 +C20:4n-6. Finally, the desaturation index (DI) is the ratio of MUFA to SFA.

Evaluation of muscular gene expression

From the results obtained by assessing differences in weight, corpulence and adiposity at adulthood, three key genes with known roles in muscular development were selected for mRNA quantification: *insulin-like growth factors 1* and *2* (*IGF-1* and *IGF-2*, respectively) and *myostatin* (*MSTN*).

Samples of *longisimus dorsi* (50–100 mg) stored at −80°C were used for total RNA extraction, by using RiboPure RNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA obtained was quantified using a NanoDrop equipment (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was assessed with an Agilent bioanalyzer device (Agilent Technologies, Palo Alto, CA, USA). The RNA Integrity Number values obtained for all the tissues sampled from carcasses were in the range 8.0–9.0, thus assuring their homogeneity and high quality. First-strand cDNA synthesis was carried out with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 μl containing 1 μg of total RNA and following the supplier's instructions.

The expression of three selected candidate genes was quantified by quantitative polymerase chain reaction (qPCR). Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Madison, WI, USA) from the available GenBank and/or Ensembl sequences, covering different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in Table 1. Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche). The qPCR reactions were prepared in a total volume of 20 μl containing 2.5 μl of cDNA (1/20 dilution), 10 μl of SYBR Green mix and 0.15 μM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C (15 s) and 60°C (1 min) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95°C (15 s) followed by 60°C (20 s) and ramp up to 95°C with acquired fluorescence during the ramp to 0.01°C/s. Data were analysed with LyghtCycler480 SW1.5 software (Roche). All points and samples were run in triplets as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. For each gene PCR efficiency was estimated by standard curve calculation using four points of cDNA serial dilutions. Values of PCR efficiency are indicated in Table 1. Mean Cp values were transformed to quantities using

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Table 1. *Gene selection and primer design for quantitative polymerase chain reaction gene expression quantification*

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Size (bp)	Efficiency (%)
IGF-1	tgcggagacaggggcttttatttc	ccttgggcatgtccgtgtgg	199	98
IGF-2	gccgctgctcgtgctgctcgtctt	gcttgccggcctgctgaa	151	95
MSTN	ccactccgggaactgattgat	gttgggcctttactactttattgt	211	90

the comparative Cp method, setting the highest relative quantities for each gene to 1 (Qty = 10 − ΔCp/slope) and employed for the statistical analyses of differential expression. Data normalization was carried out using the two most stable endogenous genes out of: *GAPDH, B2M, TBP* and *ACTB*. Endogenous genes stability measures (M) were calculated from Genorm software (http://medgen.ugent.be/jvdesomp/genorm/). Finally, the qPCR expression data normalization was performed using normalization factors calculated with the Genorm software (http://medgen.ugent.be/~jvdesomp/genorm/) from *GAPDH* and *B2M* expression values. Relative quantities were divided by the normalization factors that were the geometric means of the two reference genes quantities.

Statistical analyses

Data were analyzed using SPSS® 19.0 (IBM, New York, NY, USA). Effects of maternal diet on body weight, fat content and metabolic features were assessed by ANOVA for repeated measures (split-plot ANOVA), whilst changes over time were determined by Pearson correlation procedures. ANOVA, or a Kruskall–Wallis test if a Levene's test showed non-homogeneous variables, were used for discerning the effect of maternal diet on gene expression, adult phenotype, and age and weight at the onset of puberty and relative percentages within age and weight at puberty attainment; a Duncan *post-hoc* test was performed to contrast the differences among groups. Possible relationships among *IGF-1*, *IGF-2* and *MSTN* gene expression were assessed by Pearson correlation procedures. Statistical analysis of results expressed as percentages was performed after arc-sine transformation of the values for each individual percentage. Results were expressed as the mean ± S.E.M. and statistical significance was accepted from $P < 0.05$.

Results

Effects of maternal nutrition on growth patterns and adiposity of the offspring

The mean birth weight was significantly lower in the gilts selected from restricted sows than in the control group (1.29 ± 0.42 *v.* 1.57 ± 0.37 kg, $P < 0.005$). However, at weaning (28 days of age), the mean body weight was similar in gilts born to control and restricted sows (7.9 ± 0.5 *v.* 8.2 ± 0.3 kg).

Afterwards, the values for body weight, size and fatness increased over time in all the animals ($P < 0.0005$; Fig. 2). There were no significant differences in body weight between groups from 28 to 180 days of age. However, females born to restricted mothers showed a trend for a higher weight at 210 and 240 days of age and differences reached statistical significance for the last weighing at 290 days of age ($P < 0.05$). In the same way, the values calculated for the trunk volume were similar in both groups from 28 to 180 days of age, tended to be higher in the restricted group at 210 and 240 days of age and were significant at 290 days of age ($P < 0.05$). Thus, with similar changes in weight and body size, the values obtained for BMI1 and BMI2 were similar in both groups throughout the period of study.

The assessment of subcutaneous back-fat depth by both ultrasonography and MRI, as well as by measurement of visceral fat content by MRI showed no significant differences between groups throughout the period of study (Fig. 2c, 2d and 2e). In the same way, there was no difference in the percentage of intramuscular fat at adulthood (13.1 ± 0.8% in restricted females *v.* 12.1 ± 1.2% in control females).

Effects of maternal nutrition on metabolic features of the offspring

Assessment of plasma leptin concentrations showed a significant increase with age in both groups ($P < 0.0005$), without significant differences between them at any sampling (Fig. 3).

Screening of changes in plasma glucose showed no significant differences between groups and between samplings within groups (Fig. 4). Plasma glucose levels were maintained with a similar secretion of insulin and, thus, there were no differences in the HOMA-IR and HOMA-β indexes. On the other hand, the plasma concentrations of fructosamine remained in similar values in both groups between 120 and 210 days of age (Fig. 4). However, the values were significantly higher in the females from restricted pregnancies at 240 and 290 days of age ($P < 0.05$).

Analysis of parameters related to lipid metabolism (Fig. 5) showed, overall, a lack of remarkable differences between groups. There were higher but non-significant values of plasma triglycerides in gilts from restricted mothers at 120 and 180 days of age. On the other hand, triglycerides concentrations were significantly lower in these gilts at 290 days of age ($P < 0.05$). Assessment of total, LDL and HDL cholesterol concentrations showed significant differences between groups only at 150 (lower HDL-c in the restricted gilts, $P < 0.05$) and 180 days of age (higher total and LDL cholesterol in the restricted group, $P < 0.05$). Finally, there were no differences in the LDL-c, the HDL-c or the LDL-c/HDL-c ratios throughout the entire period of study.

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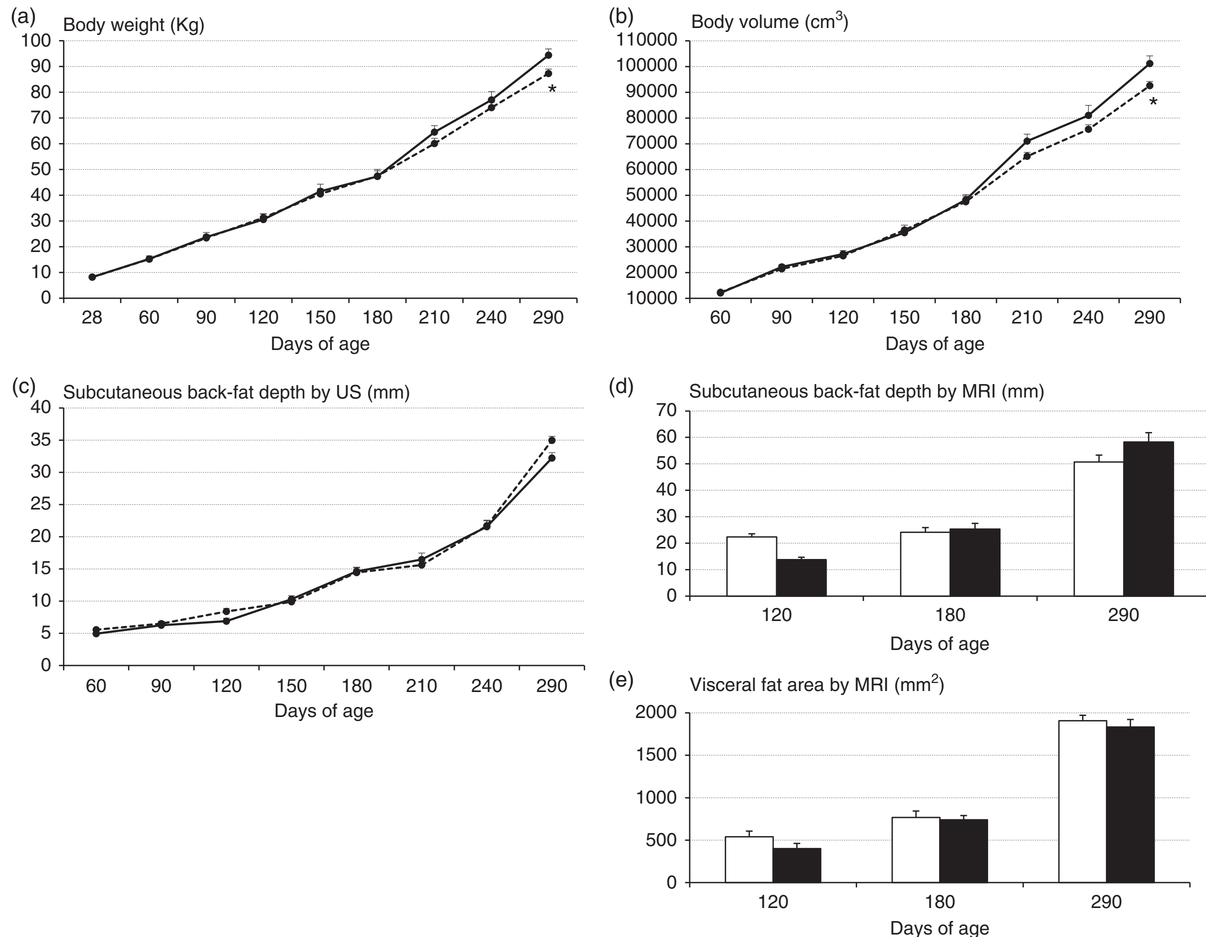

Fig. 2. Changes over time in mean values (± S.E.M.) for body weight (*a*), body volume (*b*), subcutaneous back-fat depth as determined by ultrasonography (*c*) and MRI (*d*), and area of visceral fat depot measured by MRI at the level of the third lumbar vertebra (*e*) in gilts descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy (white and black bars, respectively). Asterisks denote significant differences ($P < 0.05$). MRI, magnetic resonance imaging.

Effects of maternal nutrition and body composition on reproductive features of the offspring

The results found in the current study indicate a lack of differences between groups in the age, weight and fatness at puberty attainment (Fig. 6).

In the control group, the animals started their reproductive activity from 210 days of age and 41 kg of weight. Mean age and weight at puberty onset were 218.7 ± 5.2 days of age and 53.7 ± 3.1 kg, respectively, whilst back-fat depth was 16.4 ± 8.7 mm. Females that were born to mothers restricted during the pregnancy reached puberty from 210 days of age and 42 kg of weight; mean age at puberty onset was 210.7 ± 3.4 days of age, mean body weight was 53.8 ± 3.5 kg and back-fat depth was 14.4 ± 1.2 mm.

Effects of maternal nutrition on muscle gene expression

The three selected candidate genes (*IGF-1*, *IGF-2* and *MSTN*) showed similar expression in control gilts and gilts from restricted pregnancies (Fig. 2). However, the analysis of relationships among the expressions of three genes showed, in the control group, significant positive correlations between

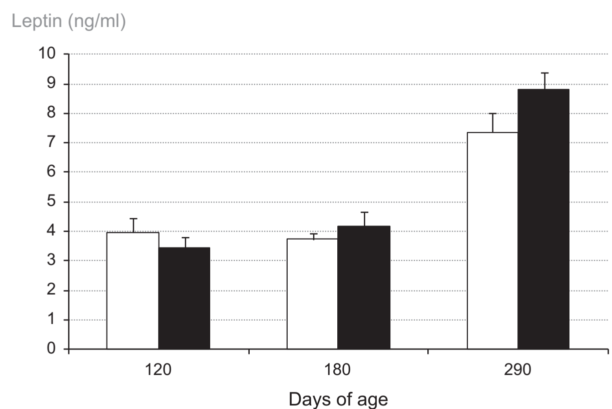

Fig. 3. Changes over time in mean values (ng/ml ± S.E.M.) for plasma leptin concentrations in gilts descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy (white and black bars, respectively).

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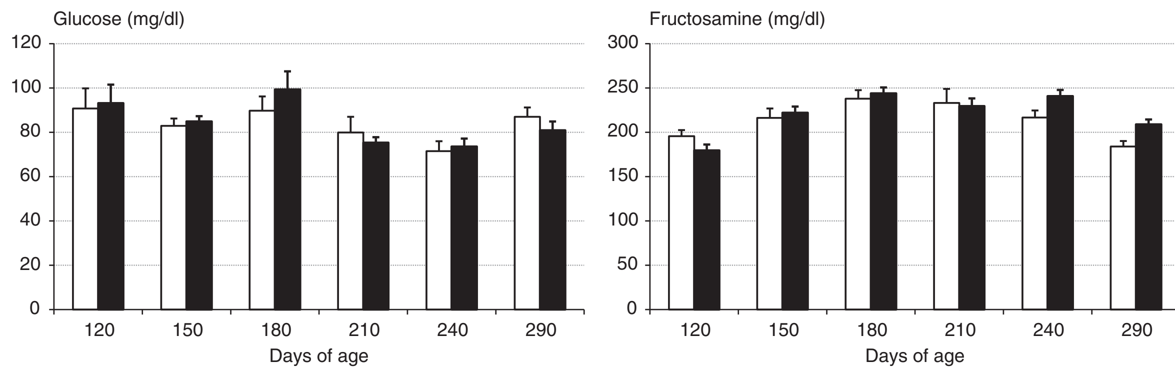

Fig. 4. Changes over time in mean values (mg/dl ± s.e.m.) for plasma glucose and fructosamine concentrations (left and right figures, respectively) in gilts descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy (white and black bars, respectively).

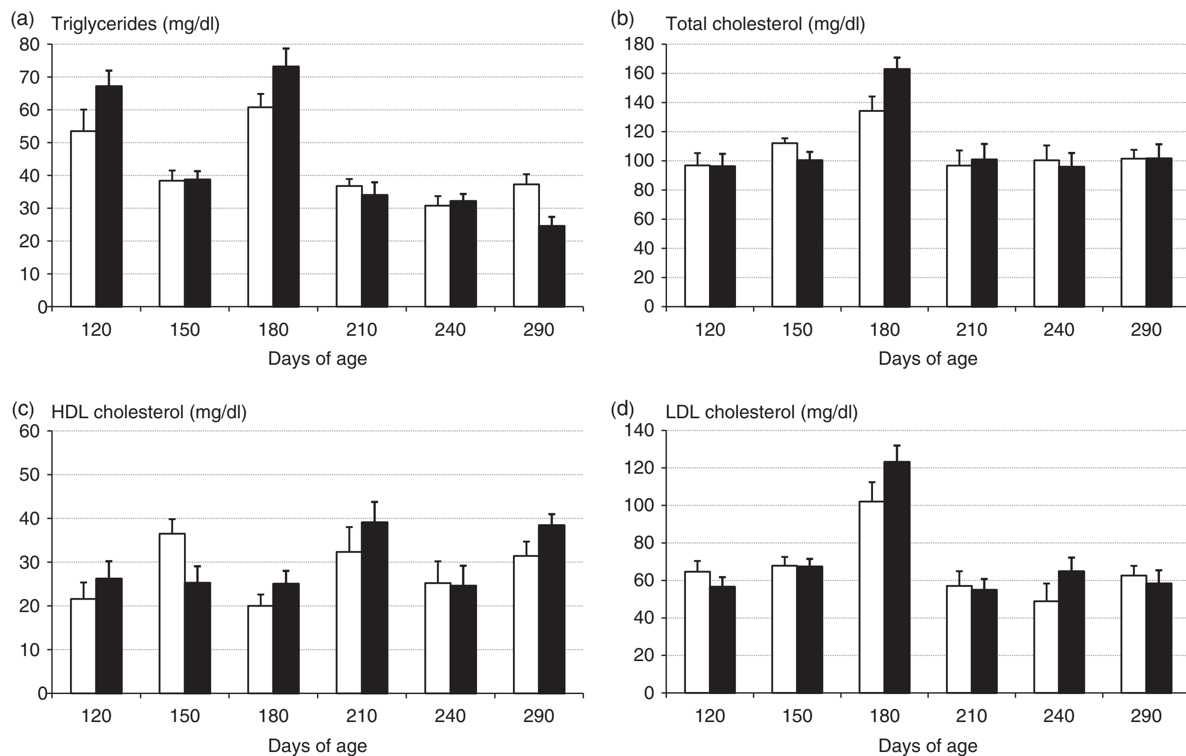

Fig. 5. Changes over time in mean values (mg/dl ± s.e.m.) for plasma concentrations of triglycerides (*a*), and total (*b*), HDL (*c*) and LDL cholesterol (*d*) in gilts descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy (white and black bars, respectively). HDL, high-density lipoproteins; LDL, low-density lipoproteins.

IGF-1 and *MSTN* ($r = 0.732$, $P < 0.05$) and between *IGF-2* and *MSTN* ($r = 0.743$, $P < 0.05$) in the control group. Conversely, the relationship was negative in the gilts from restricted pregnancies (*IGF-1*/*MSTN*: $r = -0.882$, $P < 0.01$; *IGF-2*/*MSTN*: $r = -0.907$, $P < 0.01$).

Effects of maternal nutrition on muscle gene expression

There were no differences in fat composition between the different maternal nutritional treatments. The proportion of SFA, MUFA and PUFA and the FA DI were similar in both groups, in spite of a higher content in C14:0 and C14:1 in the females from restricted pregnancies ($P < 0.05$).

Discussion

The results of the present study indicate that, in spite of changes in gene expression induced by developmental programming, the propensity for higher weight and adiposity of gilts exposed to prenatal malnutrition may be modulated by

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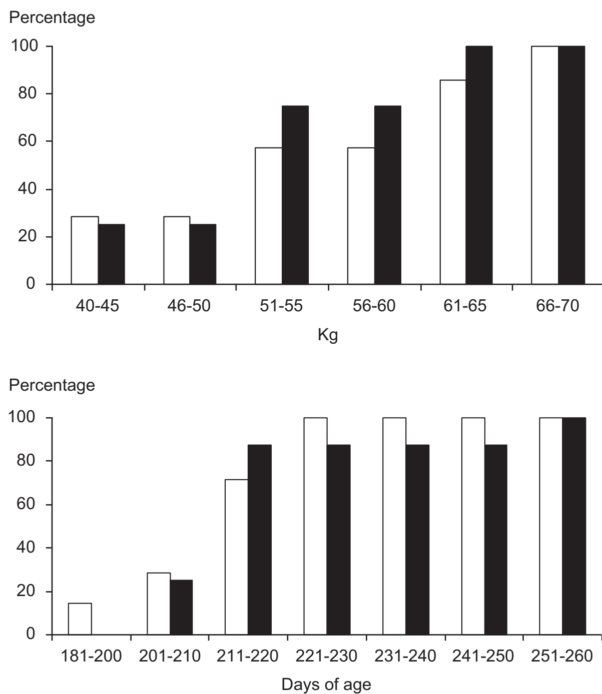

Fig. 6. Cumulative percentage of animals attaining puberty, overweight (upper panel) and age (lower panel), in gilts descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy (white and black bars, respectively).

controlled food intake and opportunity of physical exercise during infant and juvenile development.

The exposure of Iberian gilts to restrictions in maternal food intake during their prenatal development induces changes in the hypothalamic expression of genes, mainly in genes driving anorexigenic peptides.[25] After birth, such epigenetic changes induce both a higher anabolism and a higher voluntary feed intake and, thus, propensity to enhanced growth and adiposity. The results of the present study suggest that the function of some genes regulating postnatal muscle development (*IGF-1*, *IGF-2* and *MSTN*) may be modified as a consequence of prenatal nutritional programming. There were no differences in the absolute values of gene expression between groups but in the relationships between positive (IGF-1 and IGF-2) and negative (MSTN) regulators of growth and development of muscle mass. Previous literature[48,49] have reported that the relationship among IGF-1, IGF-2 and MSTN influences skeletal and cardiac muscle development, with MSTN as main actor.[50] Our results suggest some effects of prenatal programming on muscle development through the regulatory factors IGF-1, IGF-2 and MSTN (previously reported in rats).[51] These changes on the expression of genes that control muscle growth may have a prominent role in postnatal growth patterns of IUGR offspring; however, further studies are necessary for determining their role in the adult phenotype.

The gilts of the present study that were exposed to undernutrition during foetal development showed, in agreement with DOHaD hypothesis and our previous data,[23,25] lower birth weight but increased postnatal growth rate for reaching a body weight similar to control females at weaning.

Afterwards, in the current study, the patterns of growth and fattening during the infant (from weaning to 120 days of age) and juvenile periods (120–210 days of age) were similar in control gilts and gilts from restricted pregnancies. These results contrast to the results found in our previous study,[25] in which the progeny of control and restricted sows had controlled feeding during infant period and *ad libitum* access to obesogenic diets during the juvenile period. In this case, females from restricted sows were significantly heavier than control females both at 120 and 210 days of age ($P < 0.05$ and $P < 0.005$, respectively); at 210 days of age, females from restricted sows had a significantly higher adiposity than control females ($P < 0.01$). The comparison of both studies highlights the determinant role of postnatal environment in the development of juvenile obesity, as previously described in Iberian females that were not exposed to prenatal programming.[22] At the same time, in agreement with DOHaD hypothesis, the combined results of our experiments confirm that a strong inconsistency between pre- and postnatal nutritional levels (scarcity *v.* abundance) led to alterations in the growth of the offspring; stability between pre- and postnatal conditions fulfils the protective (adaptive) purpose of prenatal programming and, hence, prevents appearance of phenotypic changes. Thus, we can conclude that adequate nutritional levels during juvenile development may modulate the effects of maternal malnutrition during prenatal development.

However, there is an even more outstanding finding when comparing both experiments. The amount and composition of the diet offered from weaning to 120 days of age was exactly the same in our previous and current studies; the single difference was the space allowance per animal ($7\,m^2$ in the current experiment and $3\,m^2$ in the previous trial). Thus, our results indicate a significant influence of exercise on the anabolic trends of young individuals with controlled food intake, in agreement with previous experimental studies[52,53] and supports the development of appropriate exercise recommendations specific to this particular population.[54,55]

The lack of significant differences between groups in the patterns of growth and fatness during juvenile development was concomitant with a lack of differences in the timing of puberty attainment. These results are contradictory to previous reports in women with IUGR and low birth weight, who had a reduced age of pubertal onset and menarche.[56–59] However, it is controversial whether such advanced menarche is a direct consequence of prenatal malnutrition or an indirect consequence through the postnatal occurrence of obesity by catch-up growth.[60] Supports for the later hypothesis are based in the fact that menarche occurs earlier in obese girls, independently of prenatal origin.[27–29] Our current results reinforce argument in favour of a prominent causative role of juvenile obesity, since there were no differences in puberty onset between non-obese gilts from normal and restricted sows. Moreover, in a previous study comparing females with the same prenatal origin but

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differential postnatal feeding,[22] gilts eating obesogenic diets showed a higher fattening and, thus, a significant earlier puberty onset. Thus, the present study supports that adequate diet and exercise may be preventive strategies for precocious menarche.[59]

After reaching puberty and adulthood, females born in the current study to restricted sows showed a trend for a higher weight and corpulence, which was statistically significant at adulthood. However, such increases in weight and corpulence were not accompanied by increases in adiposity. Hence, although it is not possible to assure it in the conditions of the present study, it is possible to hypothesize that differences may be related to differences in muscle development, as suggested by the previously cited differences in the relationships among *IGF-1*, *IGF-2* and *MSTN* expression.

In summary, there were no differences in adiposity or in BMI between offspring from control and restricted pregnancies with controlled postnatal food intake and opportunity of physical exercise, either at adulthood or at younger stages. Moreover, there were no differences in their metabolic features. Preliminary unpublished studies of our group on the metabolic features of female Iberian piglets born to restricted sows and exposed to obesogenic diets during juvenile development indicate, as early as at 180 days of age, a higher adiposity and the existence of impairments of glucose regulation, with increased insulin secretion for maintaining adequate plasma glucose concentrations, in a similar way to humans.[61,62] In this way, the combination of previous and current data confirms that chronic consumption of high-fat diets results in rapid weight gain, decreased insulin sensitivity and impaired glucose homeostasis. On the other hand, our results support current hypothesis addressing that controlled food intake and exercise would prevent appearance of IR and other components of the metabolic syndrome (obesity, elevated fasting glucose and triglyceride levels, and dyslipidaemia).[63]

Finally, the analysis of fat composition at adulthood in the present study showed that females born to restricted pregnancies had a higher content in C14:0 and C14:1 than control females. However, the DI was similar in both groups. This index indicates the ratio between the substrate (e.g. C14:0) and the product (e.g. C14:1) of the activity of FA desaturases that catalyses the conversion of SFA to MUFA. The determination of the DI is currently being studied as a potential biomarker of metabolic risk,[64–66] since it is increased in obesity and associated metabolic disorders.[65–69] In this sense, in the preliminary study cited above, overfed pigs from restricted pregnancies showed a higher DI than control individuals. Overall, these results are giving further evidence on the positive effects of adequate food intake and exercise on growth, adiposity and metabolism.

In conclusion, the results of the present study indicate that the effects of prenatal programming by maternal food restriction on gene expression and postnatal patterns of growth and fattening of the offspring may be modulated by controlled food intake and opportunity of physical exercise during infant and juvenile development. Hence, our trial endorses, with experimental evidences, the current recommendations for avoiding childhood overweight and obesity. At the same time, our results encourage the necessity of further research on longitudinal assessments of changes over time in weight and adiposity of offspring from different maternal origins and postnatal environments.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Spanish Policy for Animal Protection RD1201/05) and has been approved by the institutional committee (INIA Scientific Ethic Committee).

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