

Effect of Fetal Undernutrition and Postnatal Overfeeding on Rat Adipose Tissue and Organ Growth at Early Stages of Postnatal Development

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Summary

Intrauterine and perinatal life are critical periods for programming of cardiometabolic diseases. However, their relative role remains controversial. We aimed to assess, at weaning, sex-dependent alterations induced by fetal or postnatal nutritional interventions on key organs for metabolic and cardiovascular control. Fetal undernutrition was induced by dam food restriction (50 % from mid-gestation to delivery) returning to *ad libitum* throughout lactation (Maternal Undernutrition, MUN, 12 pups/litter). Postnatal overfeeding (POF) was induced by litter size reduction from normally fed dams (4 pups/litter). Compared to control, female and male MUN offspring exhibited: 1) low birth weight and accelerated growth, reaching similar weight and tibial length by weaning, 2) increased glycemia, liver and white fat weights; 3) increased ventricular weight and tendency to reduced kidney weight (males only). Female and male POF offspring showed: 1) accelerated growth; 2) increased glycemia, liver and white fat weights; 3) unchanged heart and kidney weights. In conclusion, postnatal accelerated growth, with or without fetal undernutrition, induces early alterations relevant for metabolic disease programming, while fetal undernutrition is required for heart abnormalities. The progression of cardiac alterations and their role on hypertension development needs to be evaluated. The similarities between sexes in pre-pubertal rats suggest a role of sex-hormones in female protection against programming.

Key words

Adipose tissue • Cardiometabolic programming • Catch-up growth • Maternal undernutrition • Postnatal overfeeding

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Introduction

Mother-offspring interactions during intrauterine life and perinatal suckling period are critical for the manifestation of several diseases in adulthood, particularly cardiometabolic diseases (CMD), process known as fetal or perinatal programming (Barker and Osmond 1986, Bateson *et al.* 2004, Gluckman *et al.* 2008). Among other influences it is well recognized the key role of fetal undernutrition, as demonstrated by a number of epidemiological studies (Heijmans *et al.* 2008, Huxley *et al.* 2007, Lumey *et al.* 2009, Ravelli *et al.* 1998). This is a relevant problem, since CMD are one of the leading causes of death and disability worldwide and because fetal undernutrition and low birth weight remain prevalent in many countries. In poorly developed regions it is mainly due to insufficient food access, while in

industrialized societies it is related to social and gender reasons, such as severe gestational dietary restriction to remain slim or the delay of first pregnancy, which increases the prevalence of feto-placental alterations, premature babies and multiple pregnancies (Norman 2010, Poston 2011). The above epidemiological data have been confirmed in animal models, which have been useful to gain insight in the underlying mechanisms linking nutritional alterations during perinatal life and the development of adult disease (Bateson *et al.* 2004). These studies have demonstrated in several species (rats, guinea pigs, mice, calf, sheep) that fetal undernutrition, due to nutrient restriction during pregnancy or uterine artery ligation, produce offspring with low birth weight and features of CMD in adult age (Langley-Evans *et al.* 1999, Nielsen *et al.* 2013, Ozaki *et al.* 2001).

Early postnatal feeding patterns also seem to be critical for adult health, as evidenced by the link between accelerated postnatal growth due to reduction of exclusive breast feeding time in humans and the development of hypertension, dyslipemia, insulin resistance and obesity (Arenz *et al.* 2004, Singhal and Lucas 2004). Similarly, in animal models postnatal overfeeding, induced by reduction of litter size at birth, has also been linked to elevated insulin (Habbout *et al.* 2013) and altered fat metabolism in adult life (Balonan and Sheng 2000).

While there is consensus regarding the effects of fetal and early postnatal nutrition patterns in adult CMD development, their relative contribution has not been clarified, particularly at young age. Some authors postulate that the causal link between fetal malnutrition and CMD is the postnatal catch-up growth period that follows *in utero* nutrient restriction rather than fetal programming *per se* (Corpeleijn *et al.* 2013, Kerkhof *et al.* 2012). This controversy might be related to the fact that the majority of the studies on CMD programming assess the long term consequences of an early intervention and they omit the study of the perinatal period. This issue has been put forward by Kunes and co-workers who suggested the key importance of the period where the organism is dependent on the mother, i.e. from fertilization to weaning, particularly in the context of hypertension development (Kunes *et al.* 2012). In addition, we consider relevant to gain knowledge on specific alterations taking place during perinatal critical windows of development, where there is still enough plasticity for a preventive intervention to counteract CMD programming. In order to gain insight in this

problem we studied, at weaning, growth of several tissues and organs key for cardiovascular and metabolic control in the offspring from rats exposed to fetal or perinatal nutritional interventions. Specifically, we compared the consequences of suboptimal fetal nutrition (induced by dam food restriction) and postnatal overfeeding (induced by reduction of litter size at birth from normally fed dams) on glycemia, heart, kidney and liver weights and fat accumulation in the offspring from Sprague Dawley rats.

On the other hand, perinatal programming seems to have a sex-dependent component, being the deleterious long-term cardiovascular and metabolic consequences less prominent in females (Howie *et al.* 2012, Ozaki *et al.* 2001). Female protection against hypertension programming has been described only when the fetal stress is mild, while severe interventions affect both males and females (Vehaskari and Woods 2005) and can be due to the advanced developmental maturation of females compared to males. Alternatively, it can be related to the later protection by estrogens. Therefore, we also aimed to assess in our rat models if sex-dependent differences can be evidenced at early stages of life, before sexual maturity, and the relative importance of intrauterine and postnatal life.

Materials and Methods

Experiments were performed in Sprague Dawley rats from the colony maintained at the Animal House facility of the Universidad Autónoma de Madrid. All experimental procedures conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1996), the Spanish legislation (RD 1201/2005) and they were approved by the Ethics Review Board of Universidad Autónoma de Madrid.

The rats were housed in buckets 36.5/21.5/18.5 cm (length/width/height) on aspen wood bedding, under controlled conditions of 22 °C temperature, relative humidity 40 % and 12/12 light/dark photoperiod. They were fed with breeding diet SAFE A03 (Safe Augy, France) containing 51.7 % carbohydrates, 21.4 % protein, 5.1 % lipids, 3.9 % fiber, 5.7 % minerals and 12.2 % humidity. Drinking water was filtered by UV, mechanical and chemical treatments and provided *ad libitum* in all cases. The animal health monitoring indicated that they were free from any pathogen that may interact with the parameters studied. The health and welfare of the animals

was monitored by staff at least once a day.

Maternal Undernutrition (MUN) model

12 week-old dams were used and day 1 of gestation was determined by observation of sperm in the vaginal smear. Firstly, we determined in a group of dams that the maximum intake of rat chow in pregnant rats was 24 grams/day. Dams were divided in two groups: one with *ad libitum* diet throughout pregnancy (Control group, C, n=12 dams) and the other with *ad libitum* diet during the first 10 days of gestation and 50 % of the usual daily intake from day 11 to the end of pregnancy (MUN, n=12 dams). After delivery, both C and MUN rats were given food *ad libitum* during suckling period and food intake of the dams was weighted every 2 days. The dams were weighted at day 1 of pregnancy, at day 10 and 1-2 days prior to delivery to calculate weight gain during the first and second half of gestation.

24 h after birth the pups were sexed and weighed individually and the litter was standardized to 12 individuals, 6 males and 6 females if possible (smaller litters were not used). The rats were also weighted at day 7 and at weaning, which was performed at the age of 21 days. At this age some of the animals were sacrificed to quantify glycemia, tibial length, as well as the weight of heart, kidney, liver and fat deposits; the rest of the offspring was maintained for other studies.

Postnatal Overfeeding (POF) model

We used 12 week-old dams fed *ad libitum* regular diet throughout gestation and suckling period with the litters reduced at birth to 4 pups, 2 males and 2 females (n=12 dams). The offspring was weighted at birth, at day 7 and at weaning (21 days).

Experimental protocols in the offspring at weaning

At day 21 after birth, the pups were separated from the dam and a blood sample was immediately taken from the tail to determine glycemia with a glucometer (Accu-check Aviva, Roche). Glycemia was always measured at 9:00 h and we did not perform a fasting period in order to avoid the maternal separation stress.

Then, the pups were weighted and euthanized in a CO₂ atmosphere, followed by dissection of tibia, liver, heart (ventricles) and kidney and the following fat deposits: mesenteric (whole mesentery except the intestines), peri-renal (along the dorsal abdominal cavity and surrounding both kidneys), gonadal (around the ovaries or epididymis), subcutaneous (surrounding the

skin of both lower limbs) and brown fat (from the interscapular region). Immediately after dissection, tibial length was measured with a digital caliper (Comecta, Nessler, Spain) and the organs and fat deposits were weighted using an analytical balance (Boeco, Germany). Organ weights were expressed as relative weights (organ weight/body weight).

Perivascular mesenteric adipocyte size was assessed by confocal microscopy (Gil-Ortega *et al.* 2010). Briefly, a small sample from mesenteric adipose tissue was fixed in 4 % paraformaldehyde. Thereafter, the tissue was mounted intact between a slide provided with a small well filled with mounting medium (Citifluor, Anamed, Spain) and a coverglass. The samples were visualized with a Leica TCS SP2 confocal system (Leica Microsystems®, Wetzlar, Germany) using the 488 nm/515 nm line, since adipocytes exhibit autofluorescence at this wavelength. Five random images were captured with a 40x objective. The area occupied by adipocytes and their number were quantified by Metamorph image analysis software (Universal Imaging Co., UK) and the mean adipocyte area was calculated.

Statistical analysis

Sample size was calculated as 12 rats per group. This calculation was performed based on those parameters which exhibited the highest variability, assuming a probability error of alpha type of 5 % (p<0.05) and potency of 80 %. Statistical analysis was performed with SPSS software (v 17.0). Data are expressed as mean ± SEM and statistical significance was analyzed with one-way ANOVA followed by a Newman-Keuls *post hoc* test. Statistical significant level was established at p<0.05.

Table 1. Effect of food restriction during gestation on dams body weight gain and on food intake during lactation.

	C (n=12)	MUN (n=12)
Δ Body weight (day G1-10)	36.7±4.4	35.4±3.7
Δ Body weight (day G11-21)	97.5±4.4	39.0±5.8*
Mean of food intake day P2	27.16±8.3	29.8±4.6
Mean of food intake day P10	44.1±6.3	60.4±3.4*
Mean of food intake day P21	51.6±7.8	68.2±5.7

C, control; MUN, maternal undernutrition; n, number of rats; * p<0.05 compared to control dams; G=gestation; P=postnatal

Results

Parameters measured in the dams

MUN dams exhibited similar growth increase during the first half of gestation (exposed to *ad libitum* diet) compared to control group, while they exhibited significantly lower body weight gain during the second half of pregnancy (when they were exposed to 50 % food reduction). MUN dams returned to *ad libitum* diet during lactation and during this period there was a tendency towards a larger food intake compared to controls, reaching statistical difference only by mid lactation (day 10) (Table 1).

Offspring growth parameters

At birth, offspring from MUN rats had significantly smaller body weight compared to C group in both males and females (36 % smaller). Birth body weight of POF offspring did not differ from C rats (Fig. 1A). At postnatal day 7, MUN offspring still exhibited significantly lower body weight compared to their sex-matched controls, but had accelerated growth. Thus, males were now only 14 % smaller and females 11 % smaller compared to controls. During the first week of life POF offspring exhibited a marked acceleration in growth, being body weight 57 % and 63 % larger in males and females respectively, compared to control rats (Fig. 1B).

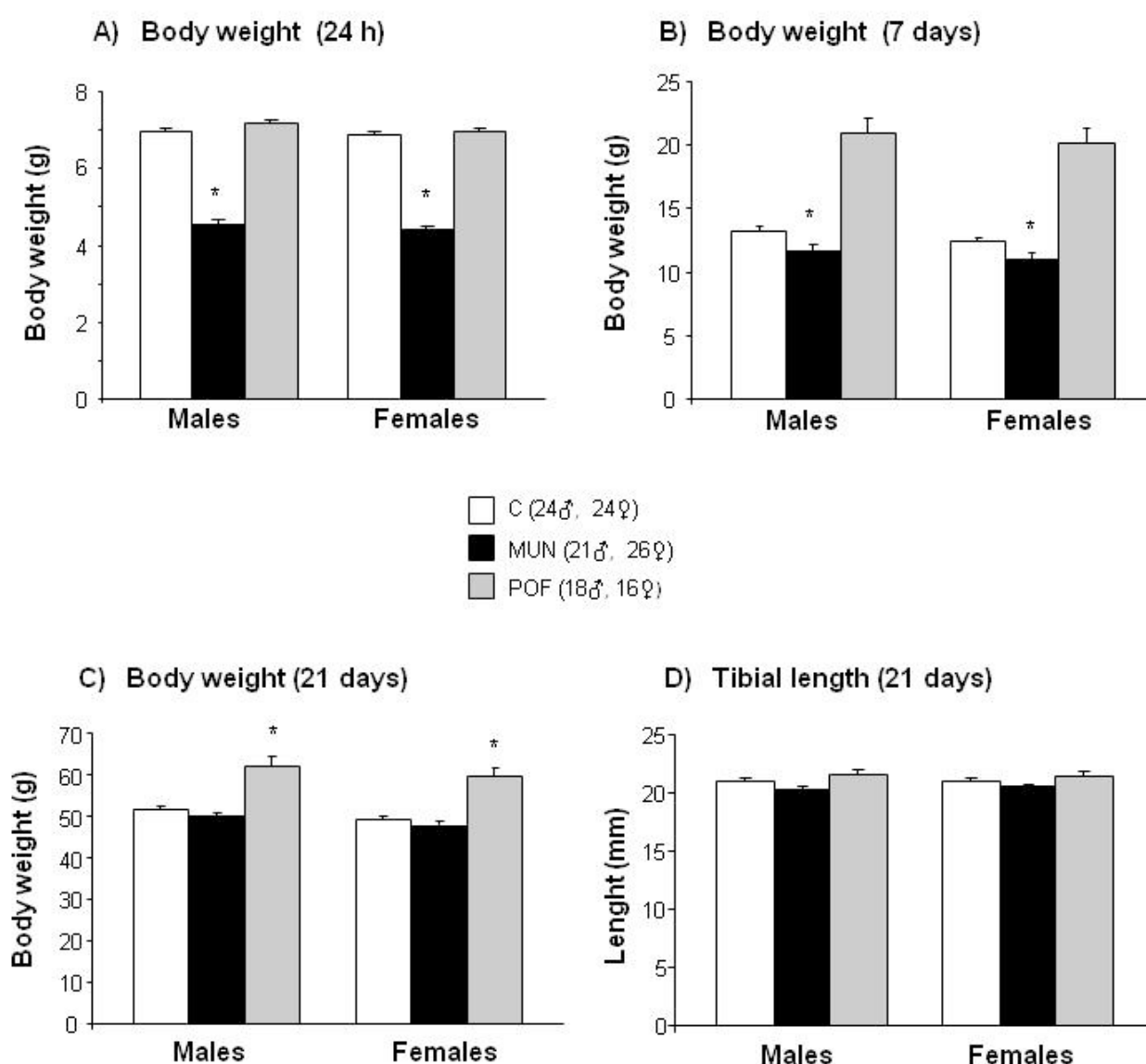


Fig. 1. Body weight at birth (A), at day 7 (B) and at day 21 (C) and tibial length at day 21 (D) in male and female MUN, C and POF offspring. C, rats with *ad libitum* diet in pregnancy and 12 rats/litter; MUN, rats exposed to 50 % of the normal daily intake during the second half of pregnancy and 12 rats/litter; POF, rats with *ad libitum* diet during pregnancy and 4 pups/litter. In parenthesis is shown the number of rats; * $p < 0.05$ when compared to C group.

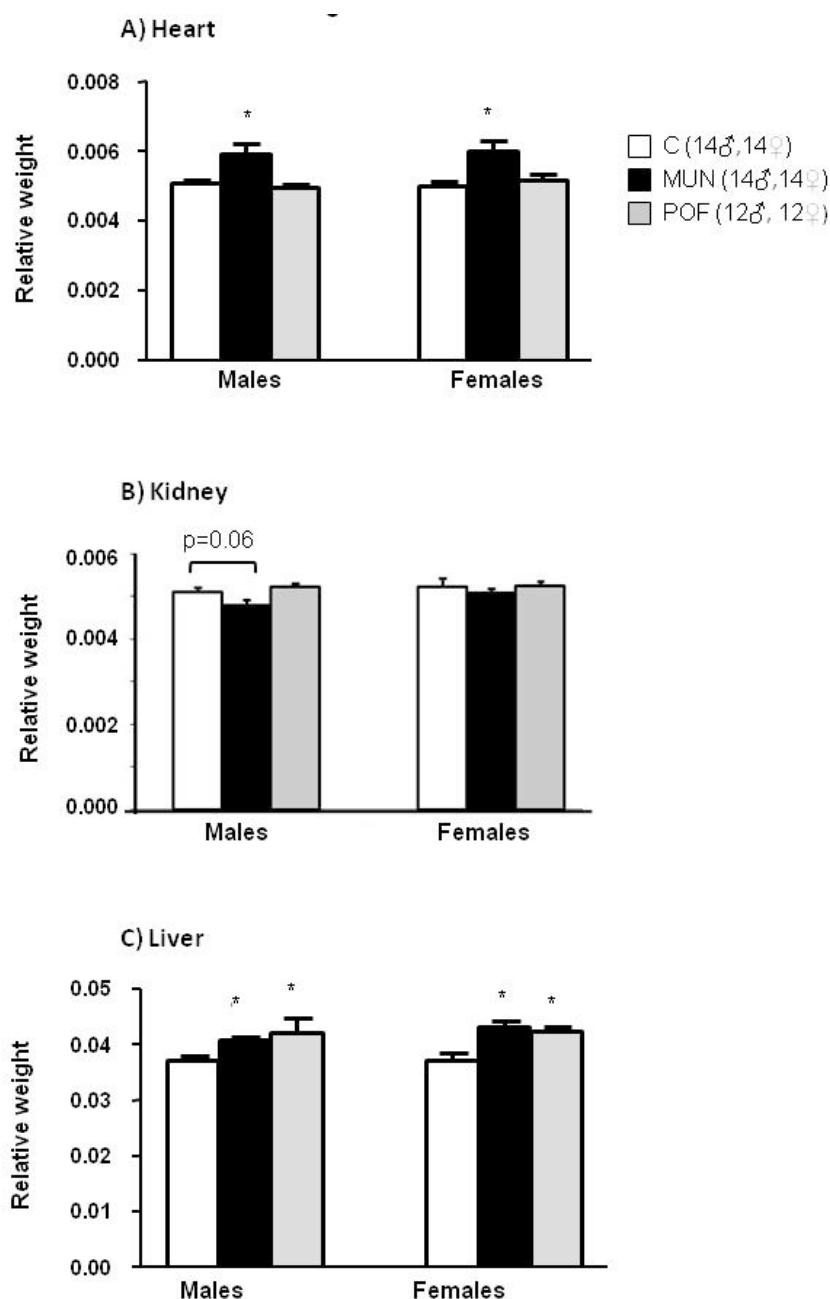


Fig. 2. Heart (A) and kidney (B) and liver (C) weight relative to body weight in male and female MUN, C and POF offspring. C, rats with *ad libitum* diet in pregnancy and 12 rats/litter; MUN, rats exposed to 50 % of the normal daily intake during the second half of pregnancy and 12 rats/litter; POF, rats with *ad libitum* diet during pregnancy and 4 pups/litter. In parenthesis is shown the number of rats; * $p < 0.05$ when compared to C group.

At day 21, body weight was not significantly different in male and female MUN offspring compared to their sex-matched C rats. At this age body weight of POF rats was 20 % and 17 % larger in males and females, respectively, compared to C offspring (Fig. 1C). At day 21 there were no significant differences in tibial length among experimental groups in either males or females (Fig. 1D).

There were no statistical differences between males and females within experimental groups at any of the ages studied.

Offspring organ weights and glycemia at day 21

Relative heart weight (ventricular weight) was significantly increased in MUN compared to control offspring in both males and females, while POF rats did not show differences in heart weight (Fig. 2A). MUN male offspring had a tendency towards smaller kidneys compared to controls, near statistical significance ($p=0.06$). This tendency was not observed in females. Relative kidney weight was not different between POF and control rats, either in males or females (Fig. 2B).

Relative liver weight was significantly elevated in both MUN and POF offspring compared to control

sex-matched rats. There were no statistical differences between males and females in any of the experimental groups (Fig. 2C).

Blood glucose was significantly elevated in both males and females from MUN and POF rats compared to C sex-matched counterparts (Table 2).

Table 2. Glycemia (mg/dl) in C, MUN and POF offspring at day 21.

	Control		MUN		POF	
	Males (n=12)	Females (n=11)	Males (n=12)	Females (n=12)	Males (n=11)	Females (n=12)
Glycemia	114.8±9.3	101.1±8.6	151.6±2.0*	148.8±3.2*	152.2±4.8*	159.2±5.2*

C, control; MUN, maternal undernutrition, POF, postnatal overfeeding; n, number of rats; * p<0.05 with respect to control

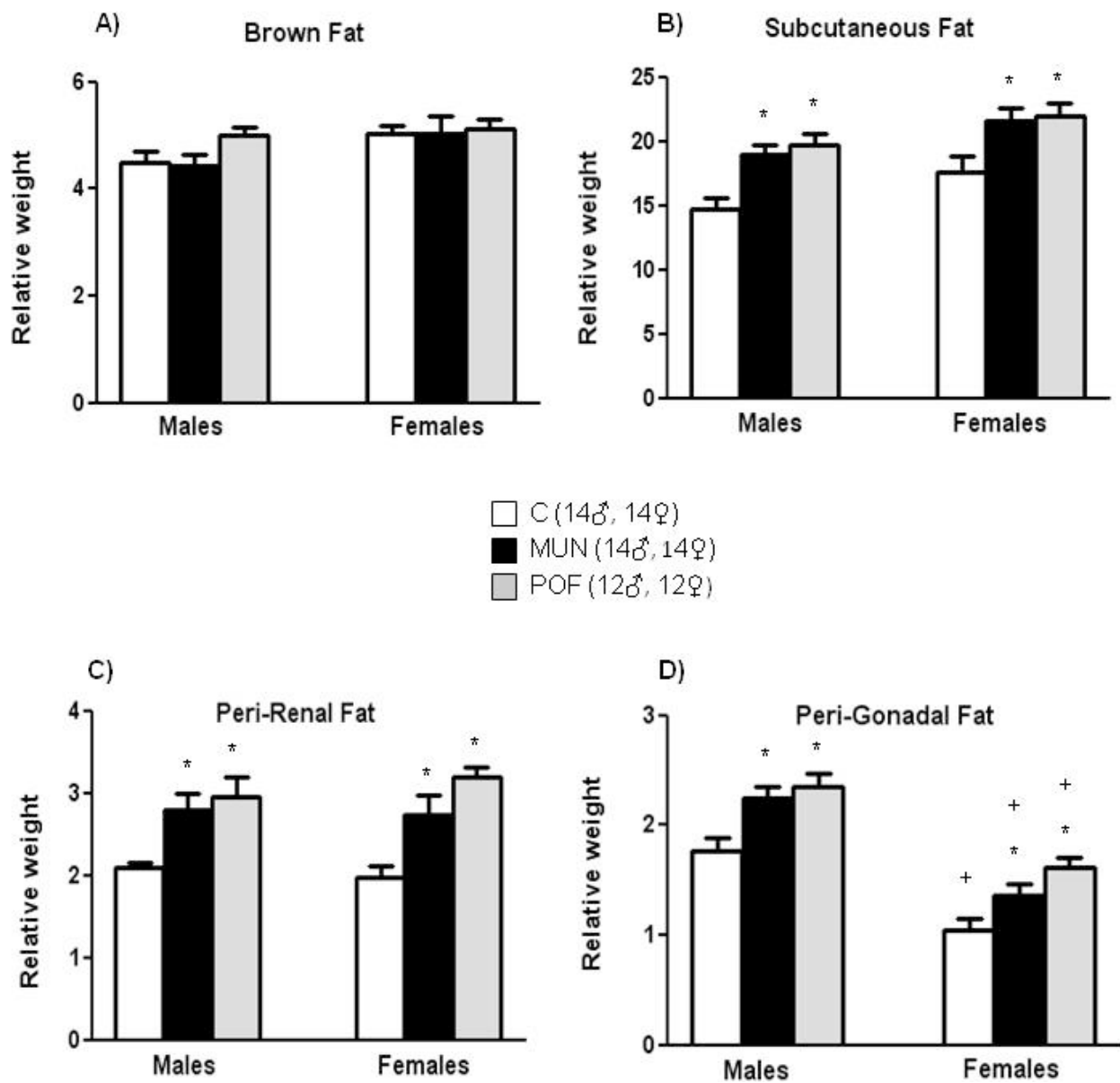


Fig. 3. Brown (A), subcutaneous (B), peri-renal (C) and peri-gonadal (D) adipose tissue weight relative to body weight in male and female MUN, C and POF offspring. C, rats with *ad libitum* diet in pregnancy and 12 rats/litter; MUN, rats exposed to 50 % of the normal daily intake during the second half of pregnancy and 12 rats/litter; POF, rats with *ad libitum* diet during pregnancy and 4 pups/litter. In parenthesis is shown the number of rats; * p<0.05 when compared to C group; + p<0.05 when compared to male rats.

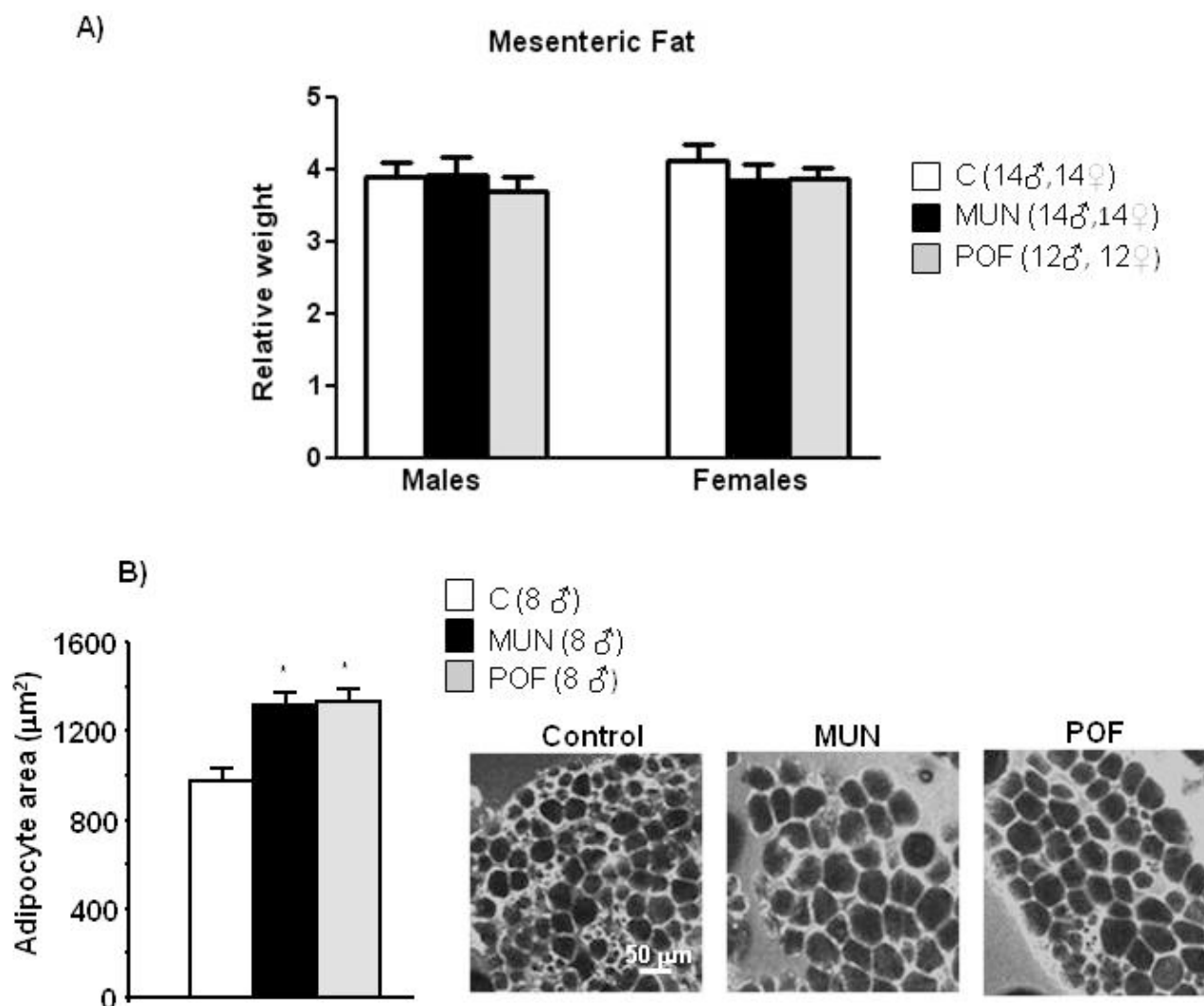


Fig. 4. (A) Mesenteric adipose tissue weight relative to body weight in male and female MUN, C and POF offspring. **(B)** Mean adipocyte area and representative confocal images from mesenteric perivascular fat obtained from male MUN, C and POF offspring. C, rats with *ad libitum* diet in pregnancy and 12 rats/litter; MUN, rats exposed to 50 % caloric intake during pregnancy and 12 rats/litter; POF, rats with *ad libitum* diet during pregnancy and 4 pups/litter. In parenthesis is shown the number of rats; * $p < 0.05$ when compared to C group.

Adipose tissue content

Male MUN rats exhibited similar brown adipose tissue content compared to male C rats. POF males tended to have larger brown tissue deposits, but did not reach statistical significance ($p = 0.07$). In female rats brown tissue content was not statistically different among the experimental groups (Fig. 3A).

Subcutaneous fat was significantly higher in both male and female MUN and POF rats compared to their respective sex-matched controls. There was no statistical difference between sexes in any of the experimental groups (Fig. 3B).

Peri-renal fat was significantly larger in MUN and POF rats compared to C in both male and female rats with no significant difference between sexes in any

experimental group (Fig. 3C). Peri-gonadal fat was also larger in male and female MUN and POF rats compared to sex-matched controls. Male rats had significantly larger peri-gonadal fat contents compared to female counterparts in all experimental groups (Fig. 3D). There were no differences between MUN and POF offspring in any of the visceral fat content.

Mesenteric fat weight was not statistically different among experimental groups (Fig. 4A). In order to assess possible differences in adipocyte size we obtained a sample from mesenteric perivascular fat in a group of male rats. Adipocyte size was significantly larger in MUN and POF male offspring compared to C rats and no differences between MUN and POF adipocytes were detected (Fig. 4B).

Discussion

Fetal undernutrition is a risk factor for development of cardiometabolic diseases and seems to have a sex-dependent component. However, it is not clear the relative role of fetal insult *per se* and postnatal catch-up growth. Moreover, there is also unclear whether female protection against fetal programming occurs during perinatal development or later in life due to the effects of estrogen protection. This controversy is likely due to the fact that most of the studies have been carried out in adult animals, when many factors already came into play. Therefore, we aimed to address these questions through a study during critical windows of postnatal development. Since maternal nutritional status has been proposed to be the major programming influence on the developing fetus, we compared the alterations induced in the offspring of rats exposed to maternal undernutrition (MUN), a model of fetal programming, with those induced by postnatal overfeeding (POF) without prior fetal stress.

The main findings from our study are the similarities at weaning between MUN and POF models in terms of hyperglycemia, liver hypertrophy and fat accumulation. These data suggest that accelerated growth during the perinatal life might lead to key changes for later metabolic disease programming, independently of the existence of a prior fetal stress. Moreover, in the long run, the increased early fat accumulation -particularly visceral fat- might also contribute to cardiovascular damage. On the other hand, early heart hypertrophy and a mild kidney reduction were present exclusively in MUN offspring, suggesting that fetal stress might have a higher impact on hypertension development and cardiovascular disease programming, compared to nutritional interventions during postnatal life. Additionally, our study demonstrates that there are no major sex differences before sexual maturity, suggesting that the female protection against fetal programming might be related to the effects of sex hormones, rather to advanced organ maturation. We cannot rule out, however, sex-differences at molecular or biochemical level not assessed in the present study.

Selection of animal models

Intrauterine growth retardation may be induced experimentally by several means. We used a rat model of maternal undernutrition, in which dams are food restricted during the second half of gestation, for several

reasons. Firstly, this intervention induces low birth weight and development of hypertension (Khorram *et al.* 2007, Vehaskari and Woods 2005) and obesity (Desai *et al.* 2005) in adult rats. Secondly, the last half of gestation has been shown to be critical for rat nephrogenesis (Paixao and Alexander 2013) and for CMD development in humans (Barker 1995, Barker *et al.* 1993).

To assess the role of postnatal accelerated growth we used a model of postnatal overfeeding induced by litter size reduction, since it has been demonstrated that the amount of milk intake is inversely proportional to litter size (Babický *et al.* 1973, Balonan and Sheng 2000). We avoided cross-fostering of newborn pups to dams with normal food intake during pregnancy – another common method to determine the relative importance of pregnancy or suckling period in disease programming – since there is evidence that cross-fostering itself can alter cardiovascular and metabolic function in rodents (Matthews *et al.* 2011).

We studied the animals at weaning. Physiological weaning is the time when the young first start taking food other than maternal milk, and has been described to be initiated by the age of 15 days (Babický *et al.* 1973). However, the end of weaning period, when the animals are separated from the mother, varies among different breeders from day 19 to 28 of age. We are aware that comparison of different weaning ages might lead to confounding results (Kunes *et al.* 2012) making difficult the interpretation of our data in comparison to others. Therefore, we weaned the rats at the age of 21 days to match the majority of studies in the field of nutritional influences of fetal and perinatal programming of CMD using the rat as experimental model (Balonan and Sheng 2000, Ozaki *et al.* 2001, Khorram *et al.* 2007, Ojeda *et al.* 2007, Moreira *et al.* 2009, Suzuki *et al.* 2010, Howie *et al.* 2012).

Role of fetal undernutrition versus catch-up growth in programming of metabolic alterations

Catch-up growth refers to accelerated gain in height, weight, or both, in postnatal life. This can be brought by artificial overfeeding or by a compensatory mechanism in offspring exposed to fetal suboptimal nutrition to balance intrauterine growth impairment. Some authors postulate that this compensatory growth after *in utero* nutrient restriction is the causal link between fetal malnutrition and CMD disease development later in life, rather than fetal programming *per se* (Singhal and Lucas 2004). We found that dietary

restriction during the second half of pregnancy induced a smaller weight gain in the dams and a subsequent reduction of offspring birth weight. During lactation MUN dams increase their food intake, in an effort to recover body weight, as previously described (Howie *et al.* 2012). We did not find differences in dam food intake in the last period of lactation, likely due to the initiation of solid food eating by the offspring after day 15 (Babický *et al.* 1973).

MUN offspring accelerated their growth along postnatal period and, by the age of 21 days, they reached the same body weight and tibial length as their sex-matched controls. These results are in agreement with catch-up growth reported in other rodent models of intrauterine growth retardation where the animals reached the same (Ozaki *et al.* 2001) or even larger body weight than controls (Desai *et al.* 2005) at 21 days of age. The increased rate of postnatal growth in the latter study compared to ours might be related either to the lower number of animals per litter (8 rats compared to 12 in the present study) or to cross-fostering, which has been demonstrated to increase appetite (Matthews *et al.* 2011).

Postnatal overfeeding, induced by reduction of litter size to 4 rats, without prior fetal insult, also led to accelerated growth along postnatal development. This is due to the increase in milk intake which has been demonstrated to be inversely proportional to litter size (Balonan and Sheng 2000). It has been demonstrated that growth rates of rats during suckling period is maximum during the first week of life and gradually decreases reaching a minimum at day 17 (Babický *et al.* 1973). Our data indicate that this the first week of life, when the animals depend solely on milk, also seems to be important in the process of growth acceleration. Thus, by day 7, both MUN and POF offspring multiplied by 3 their body weight, compared to controls which only doubled weight. Growth acceleration during the perinatal period was associated with excess adipose tissue accumulation, and this alteration was independent of a prior fetal undernutrition. This excess fat has been demonstrated to remain elevated in adult rats (Howie *et al.* 2012, Suzuki *et al.* 2010), but it can be prevented by a reduction of mother intake during lactation (Howie *et al.* 2012). The present data indicate the key influence of suckling period for early fat accumulation and support the importance to avoid postnatal growth acceleration through breastfeeding patterns to reduce the risk of CMD programming in humans (Singhal 2006).

Increased total fat content has been previously

reported at an early age by DEXA scanning (Desai *et al.* 2005, Howie *et al.* 2012). The present study adds information showing that increased adiposity in MUN and POF rats was located not only in subcutaneous tissue, but also around the viscera. The lack of differences in mesenteric fat weight was probably due to inaccuracy of quantification, since it was weighted together with the vascular system. We confirmed the increase in mesenteric fat content MUN and POF by the larger adipocyte size in both models. In addition to metabolic consequences, this early perivascular fat accumulation might be relevant as cardiovascular risk factor later in life. We have demonstrated that in initial stages of obesity it might be beneficial (Gil-Ortega *et al.* 2010); but in the long run excessive perivascular fat undergoes important structural and functional changes contributing to endothelial dysfunction and cardiovascular disease development (Fernández-Alfonso *et al.* 2013, Gil-Ortega *et al.* 2014).

The similarities between MUN and POF offspring indicate that, although the increased efficiency to store fat can be programmed during fetal life, through early induction of adipogenic transcription factors (Desai and Ross 2011), the postnatal period is also critical and an excess intake during suckling period produces the same effects. This is further supported by the similar amount of fat found in MUN fetus compared to controls (Nguyen *et al.* 2010).

We also found identical patterns of liver hypertrophy and hyperglycemia in MUN and POF offspring. Previous reports demonstrate liver enlargement and altered enzymatic systems in adult male rats exposed to fetal undernutrition, which are abolished if postnatal catch-up growth does not take place (Howie *et al.* 2012). Moreover, hyperglycemia has been previously found in overfed animals together with an increased fatty acid synthase and lipogenic activities in adipose tissues at weaning (Balonan and Sheng 2000). All these data support the important role of postnatal feeding patterns, independently of fetal stress, in the development of metabolic alterations.

Role of fetal undernutrition versus catch-up growth in programming of cardiovascular disease

Fetal nutrient restriction in rats has been implicated in the development of cardiovascular alterations, particularly hypertension (Langley-Evans 1999, Khorram *et al.* 2007). Therefore, we explored whether heart or kidney, key organs for cardiovascular control were affected at an early age and the role of

catch-up growth.

A reduction in kidney mass and nephron number has been postulated as a potential mechanism implicated in fetal programming of hypertension (Nuyt 2008) and the last 2 weeks of gestation seem to be a crucial period whereby undernutrition can compromise nephrogenesis in the rat (Paixao and Alexander 2013). In our MUN model females did not show kidney differences and male offspring only evidenced a tendency to have smaller kidneys, near statistical significance, and much milder compared to the effects produced by maternal protein restriction (Langley-Evans *et al.* 1999) or uterine artery ligation (Vehaskari *et al.* 2001). It is possible that the severity of fetal stress induced by global nutrient restriction is smaller compared to other interventions. It is also possible that other alterations, not determined in the present study, such as nephron number or function might be present in our MUN model.

Increased ventricular weight at weaning was only observed in MUN, but not in POF offspring, suggesting that it is programmed *in utero*. The importance of the fetal period for heart growth is evidenced by the observed increased insulin-like growth factor gene expression in hearts of lamb fetus exposed to intrauterine growth retardation (Wang *et al.* 2011). During fetal period there is extensive hyperplasia in the rat heart which continues until postnatal day 5; thereafter the heart enlarges mainly by cell hypertrophy (Zicha and Kunes 1999). We did not test whether the observed MUN heart enlargement at the age of 21 days was due to initial hyperplasia, later cell hypertrophy or both. We have evidence that by day 7, hearts from MUN offspring already tend to be larger, but did not reach statistical significance until weaning (Arribas *et al.* 2012), suggesting that the postnatal catch-up growth period plays a role. On the other hand, postnatal catch-up growth without a prior fetal stress does not seem to initiate heart hypertrophy, as shown by the lack of effect in POF rats. This is in contrast with the data of Moreira *et al.* (2009) who found that postnatal overfeeding induced heart hypertrophy in Wistar rats. This discrepancy can be explained by differences in the growth rates of Wistar and Sprague Dawley rats or by the fact that only overweight animals were selected in their work. We would like also to point out at the possible differences, between ours and other studies depending on the time of rat weaning, which might be important for organ development (Zicha and Kunes 1999). We also have evidence that MUN hearts

exhibit an early increase in collagen content (Arribas *et al.* 2012), which has been also observed in adult offspring from rats exposed to protein deficiency in pregnancy (Kawamura *et al.* 2007). The progression of cardiac alterations in MUN offspring and their role on hypertension development needs to be further evaluated.

Sex-dependent effects

Fetal programming in animal models has been shown to exhibit a sex dependent pattern, being females protected (Grigore *et al.* 2008). Thus, moderate global dietary restriction during gestation in the rat leads to hypertension which is more pronounced and develops earlier in male offspring (Ozaki *et al.* 2001) and liver and cholesterol alterations are found only in male rats (Howie *et al.* 2012). Whether this sexual dimorphism is related to perinatal programming itself or to the later protection of female sex hormones (Grigore *et al.* 2008, Jones *et al.* 2012) is still under debate. Our data showing identical alterations in MUN and POF males and females before sexual maturity support a later protective role of female sex hormones, as previously suggested. For example the fact that hypertension induced in the offspring of rats exposed to placental insufficiency is normalized in females after puberty (Alexander 2003, Jones *et al.* 2012). Moreover, blood pressure is significantly increased following ovariectomy in adult female growth restricted offspring (Ojeda *et al.* 2007). Taken all together, the present data showing that males and females exposed to undernutrition during fetal life, exhibit similar catch-up growth and organ alterations prior to sexual maturity, support the role of female hormones in the later protection against cardiometabolic programming. However, we cannot rule out other early differences, between males and females, for example at molecular or biochemical level, which were not studied in the present work and could also participate in the development of the sex-dependent cardiovascular and metabolic phenotype in adult life.

Conflict of Interest

There is no conflict of interest.

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