Sex-dependent nutritional programming: fish oil intake during early pregnancy in rats reduces age-dependent insulin resistance in male, but not female, offspring

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Submitted 30 August 2012; accepted in final form 18 December 2012

Sardinha FL, Fernandes FS, Tavares do Carmo MG, Herrera E. Sex-dependent nutritional programming: fish oil intake during early pregnancy in rats reduces age-dependent insulin resistance in male, but not female, offspring. Am J Physiol Regul Integr Comp Physiol 304: R313–R320, 2013. First published December 19, 2012; doi:10.1152/ajpregu.00392.2012.—Prenatal and early postnatal nutritional status may predispose offspring to impaired glucose tolerance and changes in insulin sensitivity in adult life. The long-term consequences of changes in maternal dietary fatty acid composition were determined in rats. From day 1 until day 12 of pregnancy, rats were given isocaloric diets containing 9% nonvitamin fat based on soybean, olive, fish (FO), linseed, or palm oil. Thereafter, they were maintained on the standard diet; offspring were studied at different ages. Body weight at 4, 8, and 12 mo and lumbar adipose tissue and liver weights at 12 mo did not differ between females on the different diets, whereas in males the corresponding values were all lower in the offspring from the FO group compared with the other dietary groups. Plasma glucose concentrations (both basal and after an oral glucose load) did not change with sex or dietary group, but plasma insulin concentrations were lower in females than in males and, in males, were lowest in the FO group. Similar relations were found with both the homeostasis model assessment of insulin resistance and insulin sensitivity index. In conclusion, the intake of more n–3 fatty acids (FO diet) during early pregnancy reduced both fat accretion and age-related decline in insulin sensitivity in male offspring but not in females. It is proposed that the lower adiposity caused by the increased n–3 fatty acids during the intrauterine life was responsible of the lower insulin resistance in male offspring.

fetal programming; insulin sensitivity; oral glucose tolerance; aging; gender differences; dietary fatty acids

COMPELLING HUMAN EPIDEMIOLOGICAL DATA from different populations indicate that prenatal and early postnatal nutritional status may influence adult susceptibility to impaired glucose tolerance, cardiovascular disease, and obesity (21, 23, 34). In experimental animals it has also been shown that prenatal and early postnatal nutrition may program susceptibility in the offspring to later development of several chronic diseases including altered carbohydrate metabolism (8, 15, 25, 34). This effect has been shown in rats to be dependent on both the sex and time window of exposure (38, 39). Most of these studies have been designed to determine the long-term effects of low-protein diets or of changes in the total food intake, but very few studies have paid attention to the long-term metabolic effects of changes in dietary fatty acid composition. There is even a lack of robust animal data on the effects of increasing exposure to n–3 long chain polyunsaturated fatty acids (LCPUFA), during the perinatal period alone, on body fat mass in the offspring (26). To the best of our knowledge there are only two studies in which the exposure to increased n–3 LCPUFA was confined to the prenatal and lactation period (19, 27), whereas the first one showed that a diet rich in α-linolenic acid (18:3 n–3) decreased body and fat pad weights in offspring at 1 and 3 wk of age, the second one showed that a diet rich in docosahexaenoic acid (DHA, 22:6 n–3) increased the total percentage of body fat in offspring at 6 wk of age. However, there are no studies where the timing of intervention in rodents was confined to the prenatal stage. This is surprising given that maternal fatty acid status during pregnancy and lactation greatly influences newborn and infant status, as recently reviewed (14, 20).

Thus, to define more precisely the biological mechanisms by which a metabolic imprint of increased fetal n–3 LCPUFA exposure might occur, animal studies, in which dietary fatty acid intervention is confined to specific periods of pregnancy, are required. By giving different dietary fatty acids during the first half of pregnancy in rats, we recently determined the fatty acid composition of maternal adipose tissue and of maternal and fetal plasma at mid- and late pregnancy and found that during the first half of pregnancy maternal adipose tissue stores dietary-derived fatty acids, which are released into blood during late pregnancy enabling LCPUFA to become available to the fetus (10). We also found that the fatty acid profile of colostrum and milk in these same dams, as well as in plasma and the brain in their 2-day-old pups, was also influenced by the diet they had been eating during the first half of pregnancy (9).

The present study was designed to determine whether changes in maternal fatty acid status during this same period (i.e., the first half of pregnancy) had long-term consequences on adiposity and insulin sensitivity in the offspring. Sex-dependent differential responses to either n–3 fatty acid feeding during the perinatal period (16) or to a low maternal protein diet (38) have previously been reported. Therefore, special attention has been taken here to determine whether any potential consequence of the treatment differed between female and male offspring. For that reason we used the same experimental design as used previously (9, 10), in which female rats were given diets containing different proportions of fatty acids during just the first 12 days of pregnancy.

MATERIALS AND METHODS

Animals and experimental design. Female Sprague-Dawley rats were obtained from the animal quarters of the University San Pablo-CEU, Madrid. The experimental protocol was approved by the Ani-
nal Research Committee of the University San Pablo-CEU in Madrid, Spain. The rats were initially fed a standard nonpurified diet (B&K Universal, Barcelona, Spain) and mated when they were 3 mo old and all the litters were culled to 8 pups within 24 h of delivery by selecting the excess number of pups at random and removing them.

After being weaned (postnatal day 21), female and male pups from each experimental group were separated from each other and housed independently. SO, soy oil diet; OO, olive oil diet; FO, fish oil diet; LO, linseed oil diet; PO, palm oil diet plus 1% sunflower oil; and in the PO group the diet contained 8% sunflower oil plus 1% soybean oil; in the FO group the diet contained 9% soybean oil; in the LO group the diet contained 8% linseed oil plus 1% sunflower oil; and in the PO group the diet contained 8% palm oil plus 1% soybean oil.

Sunflower or soybean oil was added to some diets to meet the minimum requirement for essential fatty acids. The fatty acid content of these diets is shown in Table 1, and the experimental design is summarized in Fig. 2. The experimental diets were isoenergetic (4.1 kcal/g) and were stored at −20°C until use. Rats had free access to the assigned diet and tap water. Fresh experimental diets were provided ad libitum every 24 h. After 12 days on an experimental diet (day 12 of pregnancy), the experimental diets were withdrawn and all rats were given the standard pellet diet (B&K Universal). Pups were maintained with their mothers until weaning, and all litters were culled to 8 pups within 24 h of delivery by selecting the excess number of pups at random and removing them. After being weaned (postnatal day 21), female and male pups from each experimental group were separated from each other and housed in collective cages (3–4 per cage) and given the standard pellet diet until the end of the experiment.

Determination of composition of fatty acid in experimental diets. Nonadecenoic acid (19:1) (Sigma Chemical, St. Louis, MO) was added as internal standard to fresh aliquots of each diet, which were subjected to lipid extraction and purification (12). After the lipid extracts were reduced to dryness under vacuum and the residue was resuspended in methanol-toluene, lipids were simultaneously saponified and methylelated in the presence of acetyl chloride at 80°C for 2.5 h as previously described (2). Fatty acid methyl esters were separated and quantified on a Perkin-Elmer gas chromatograph (Autosystem, Norwalk, CT) with a flame ionization detector and a 20 m × 0.25 mm Omegawax capillary column. Nitrogen was used as carrier gas, and the fatty acid methyl esters were identified by comparison with purified standards (Sigma Chemical).

**Figure 2.** Design of the study. Rats were fed with the corresponding experimental diet during the first 12 days of pregnancy; thereafter, all rats were given the standard pellet diet. At 4 and 8 mo of age, all animals from each group were subjected to an oral glucose tolerance test (OGTT) that was performed as follows. Tests were conducted between 11:00 and 13:00 h after a 3-h fast. After tail blood was collected (time 0), rats received an oral load of 2 g of glucose/kg body wt, and blood was collected from the tail at 7.5, 15, 22.5, 30, and 60 min in tubes containing 1 g Na2-EDTA/I. Plasma was separated from fresh blood by centrifugation at 1,500 g for 15 min at 4°C and stored at −80°C until analyzed for glucose and insulin using commercial kits (Spinreact, Girona, Spain and Merckodia Rat Insulin ELISA AB, Upsala, Sweden, respectively). The tests were all carried out on samples from individual animals and never from pooled samples. Values of plasma glucose and insulin concentrations were used to determine their respective area under the curve (AUC), which was calculated according to the trapezoid method. The insulin sensitivity index (ISI) and the homeostasis model assessment of insulin resistance (HOMA-IR) values were calculated as previously described (24, 28) using the following equations:

\[
\text{ISI} = 10,000 \sqrt{\frac{\text{FPG} \times \text{FPI} \times \text{G} \times \text{I}}{2,430}}
\]

\[
\text{HOMA-IR} = \frac{(\text{FPG} \times \text{FPI})}{2,430}
\]

where FPG is fasting plasma glucose (expressed in mg/dl), FPI is fasting plasma insulin (expressed in μU/ml), and mean G and mean I are the mean glucose and mean insulin concentrations in the same units determined during the OGGT.

Rats were killed at 12 mo of age by decapitation while under CO2 anesthesia, and trunk blood was collected in receptacles containing 1 g Na2-EDTA/I. Plasma was separated from fresh blood and stored as
described above. Liver and lumbar adipose tissue (i.e., retroperitoneal adipose tissue) were quickly excised and weighed.

Statistical analysis. All analyses were conducted in SPSS (version 15.0, Chicago, IL). Results are expressed as means ± SE. Distributions of the studied variables were identified as normal after examination with the Shapiro-Wilk test; therefore, parametric analyses were applied. One-way analysis of variance (ANOVA) was initially used to compare the data from different diets, from male and female and for different periods of the OGTT. When treatment effects were significantly different (P < 0.05), Newman-Keuls simultaneous tests were used to establish statistical differences between individual dietary interventions. Statistical differences between two groups were analyzed by Student’s t-test. Statistical significance was set at P < 0.05.

RESULTS

Body and tissue weights. As previously reported (10), no difference was found in dams’ body weight and food intake at days 12 and 20 of gestation between the groups fed diets containing different fatty acid compositions during the first 12 days of pregnancy. Female offspring did not show any difference in their body weights at birth (9) or at weaning (data not shown) or at different ages up to 12 mo (Table 2). At this age, female offspring of the SO group showed a lumbar adipose tissue mass higher than those from the FO group, although the difference was no longer present when corrected for body weight. No difference between the groups was found either in absolute or in body-weight-corrected liver weights in female offspring.

Body weight of male offspring did not show any difference between dietary groups at birth as previously reported (9) and at weaning (data not shown) or at 4 mo of age (Table 2). At 8 mo, male offspring from the FO group had lower body weights than those of the OO and PO groups; at 12 mo they had lower body weights than those of OO, LO, and PO groups. At the time of being euthanized (i.e., 12 mo old), the weight of lumbar adipose tissue was lower in the FO group than in the others, the difference not being apparent when corrected for body weight, although when statistical differences were analyzed between pairs of groups it appeared that adipose tissue mass per 100 g body wt was significantly lower (P < 0.01) in the FO group compared with the SO, OO, or PO groups, although not to the LO group. Liver weight was lower in the FO group than in PO, and this difference also disappeared when corrected by body weight.

Basal plasma glucose and insulin values. Basal plasma concentrations of both glucose and insulin at 4, 8, and 12 mo of age are shown in Table 3. In female offspring plasma glucose levels tended to decrease with age, whereas insulin levels progressively increased with age in the five studied groups, with small differences between them. In male offspring plasma glucose was maintained at a stable value at all ages and in all the studied groups, with only small differences between them. However, insulin concentrations in male offspring increased with age in all the groups, and values were consistently higher than in the females at 8 and 12 mo. The exception was that the insulin concentrations of the rats of the FO group were lower than in the rats of the other groups and at 8 mo remained similar to the females’ values.

Oral glucose tolerance tests. The AUC for both glucose and insulin in response to an oral glucose load at 4 and 8 mo of age are shown in Table 4. The AUC of glucose were always in the
Table 3. Plasma glucose and insulin concentrations at different ages in offspring of dams that were given diets containing different fatty acids during the first 12 days of pregnancy

<table>
<thead>
<tr>
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<th>Female Offspring</th>
<th>Male Offspring</th>
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<tr>
<td></td>
<td>SO</td>
<td>OO</td>
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<tr>
<td>n</td>
<td>12</td>
<td>8</td>
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<tr>
<td>Glucose, mg/dl</td>
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<tr>
<td>4 mo</td>
<td>129 ± 10.9</td>
<td>130 ± 4.2</td>
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<td>8 mo</td>
<td>126 ± 2.1</td>
<td>129 ± 1.1</td>
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<tr>
<td>12 mo</td>
<td>127 ± 2.2</td>
<td>122 ± 4.3</td>
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<tr>
<td>Insulin, ng/l</td>
<td></td>
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<tr>
<td>4 mo</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.09</td>
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<tr>
<td>8 mo</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>12 mo</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
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Values are means ± SE. SO: Soy oil group; OO: Olive oil group; FO: Fish oil group; LO: Linseed oil group; PO: Palm oil group. Different lowercase letters in the same row mean statistical differences (P < 0.05) between dietary groups, either in female or male offspring. *Statistically significant difference between female and male offspring on the same day. Different uppercase letters in the same column mean statistical differences for the same dietary group between 4, 8, and 12 mo of age.

Table 4. Glucose tolerance tests at different ages in offspring of dams that were given diets containing different fatty acids during the first 12 days of pregnancy

<table>
<thead>
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<th>Female Offspring</th>
<th>Male Offspring</th>
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<td>SO</td>
<td>OO</td>
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<tr>
<td>n</td>
<td>12</td>
<td>8</td>
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<tr>
<td>AUC for glucose</td>
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<tr>
<td>4 mo</td>
<td>9,730 ± 0.9</td>
<td>8,930 ± 0.9</td>
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<tr>
<td>8 mo</td>
<td>9,575 ± 0.9</td>
<td>8,780 ± 0.9</td>
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<tr>
<td>AUC for insulin</td>
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<tr>
<td>4 mo</td>
<td>47.0 ± 4.8</td>
<td>47.8 ± 4.1</td>
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<tr>
<td>8 mo</td>
<td>75.3 ± 6.8</td>
<td>79.6 ± 6.2</td>
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</table>

Values are means ± SE. AUC, area under the curve; Different letters mean statistical difference (P < 0.05) between experimental groups of females or males. *Statistically significant difference between 4 and 8 mo of age in each group. **Statistically significant difference between females and males of the same age.
same range for female and male offspring, and values did not differ between the five groups in either sex except for higher values in females of the SO group at 8 mo and in males of the PO group at 8 mo. The AUC of insulin increased with age in both female and male offspring, and values were always lower in the females. In females no difference was found between the five groups at 4 or 8 mo of age. In males at 4 mo of age the AUC of insulin was not statistically different between the groups, but at 8 mo values in the FO group were lower than those in OO, LO, and PO groups, and those of SO group were lower than in both OO and PO groups.

These differences between the groups prompted us to estimate some indices of insulin sensitivity, and values are shown in Fig. 3. In females the HOMA-IR value progressively increased with age, although the change found in the OO group was not significant (Fig. 3A), whereas the ISI declined with age in all the groups (Fig. 3B); no difference between the values of the five groups for either variable were found in females of the same age.

In males, HOMA-IR followed the same tendency of progressively increasing with age (Fig. 3C) as observed in females, although the values were normally higher in the male offspring. In males the values of ISI were always lower than in females and they also decreased with age (Fig. 3D). When comparing these indices in the different groups of males, it can be seen that in the FO group at both 8 and 12 mo the HOMA-IR was lower than in the OO and PO groups, and the ISI was higher in the FO group than in the others.

**DISCUSSION**

The results presented here show, for the first time, that the intake by rats of a diet enriched with n–3 LCPUFA, such as in

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**Fig. 3.** Homeostasis model assessment of insulin resistance (HOMA-IR) and insulin sensitivity index (ISI) in offspring at different ages of dams that were given diets containing different fatty acids during the first 12 days of pregnancy. **A:** HOMA-IR values in female offspring; **B:** ISI values in female offspring; **C:** HOMA-IR values in male offspring; **D:** ISI values in male offspring. Different lowercase letters mean statistically significant difference (P < 0.05) between experimental groups of either female or male offspring. Different uppercase letters mean statistically significant difference for each group at different ages.
the FO-supplemented diet used here, during the first half of gestation only, delayed the progressive decline of insulin sensitivity with age in male but not female offspring. The differential sex response in the FO group occurred in parallel to the specific reduced age-related increases in body weight and lumbar adipose tissue weight found in males but not in the other groups. Thus our findings agree with the previously proposed age-related decline in insulin sensitivity being secondary to age-related changes in body composition in humans rather than a primary effect of the aging process (17). A previous report in rats has also related the decrease in insulin sensitivity with age in both sexes to adiposity (13). Although some changes in the studied variables were also found in some of the offspring of dams fed the SO, OO, LO, and PO diet, they were minor in comparison and inconsistent. The composition of all these diets was carefully controlled such that the only variable was the fatty acid composition; in that respect, each of the groups could be considered control of the others. This discussion will concentrate on the changes observed in the FO group.

Differences in dietary fatty acid composition during the first half of gestation influence not only the fatty acid profile in maternal circulation, and therefore the type of fatty acids reaching the embryo, but also the type of fatty acid being stored in maternal adipose tissue (10). These fatty acids in maternal adipose tissue are released during late pregnancy, reaching the fetus during late stages of gestation and can even be taken up by mammary gland and become available to the newborns via lactation as shown both in rats (9, 10) and in pigs (1). Thus, although the present findings show an important role of dietary fatty acids taken in during the first stages of pregnancy on the later health of the offspring, it is not possible to establish whether the effect should be ascribed to the fatty acids being taken up by the embryo during early stages of development (i.e., at the time of feeding) or to a longer term effect mediated by storage in adipose tissue and later release. It is notable, bearing in mind the obvious limitations of extrapolating findings from humans to rats, that studies in humans have reported evidence that fetal growth outcome is most vulnerable to maternal dietary inadequacies during early pregnancy (30, 37). A previous experiment with exactly the same design (9, 10) reported that the highest contents of eicosapentaenoic (EPA; 22:5, n–3) and docosahexaenoic (DHA; 22:6, n–3) acids in the plasma of the pregnant mothers at day 12 of pregnancy, of the 20-day fetuses and of the 2-day-old newborns were found in the group given the FO diet. It is therefore suggested that this observed elevation of LCPUFA could be responsible for the long-term effects on body and adipose tissue weights and insulin sensitivity in male offspring reported here.

Although not yet studied, the mechanism of such an effect could be epigenetic. As has been proposed (36), fatty acid nutrition during development could affect the establishment of epigenetic gene regulation by inducing (or repressing) transcription of specific genes during critical ontogenic periods, during which tissue-specific marks may be initiated. However, in 6-wk-old offspring of rats receiving omega-3 supplementation throughout pregnancy and lactation there was an increase in subcutaneous fat mass but no change in the expression of adipogenic/lipogenic genes was demonstrated (27). Nevertheless, studies on the potential epigenetic mechanism involved in our findings are warranted. Studies in humans on the effects of n–3 LCPUFA during the perinatal period on later body composition are very variable (29), but to our knowledge only one longitudinal study addressed the relationship between prenatal n–3 fatty acid intake and long-term adiposity at 3 years of age (7); higher maternal prenatal n–3 intake resulted in higher DHA + EPA concentrations in umbilical cord and were associated with lower adiposity and obesity in early childhood. In rats, a role for dietary fatty acids in the developmental establishment of epigenesis has been proposed (36). In fact, the offspring of dams given a lard-rich diet during pregnancy and lactation have been shown to develop symptoms of metabolic syndrome (3), and when the dams were fed the high-lard diet during the suckling period only, their adult offspring developed impaired glucose homeostasis (18). In contrast, the offspring of rat dams fed a high-fat diet rich in polyunsaturated fatty acids rather than saturated fatty acids did not become glucose intolerant (32). Although the evidence that fatty acid nutrition affects epigenetic gene regulation is limited, these reported findings taken together with our observations, using a diet rich in n–3 PUFA but with normal total fat intake, indicate that the quantity and type of fatty acid consumed by the mother during pregnancy and lactation can induce permanent changes in gene expression and metabolism in her offspring.

The mechanism by which the increased n–3 consumption during early pregnancy reduces the age-dependent increase of adipose tissue mass and the decline of insulin sensitivity in male but not in female offspring is unknown. Here we determined just the lumbar adipose tissue mass, which is a visceral depot (6) and under certain conditions in rats, its changes have been shown to parallel the proportion of fat in carcass (22). Its decreased value in males of the FO group was found here even when corrected by body weight. These findings agree with the evidence that the fatty acids in fish oil decrease preadipocyte proliferation and reduce adiposity in rodents, although these effects may be sex and strain specific (4, 5, 11, 33). Furthermore, it has been proposed that the age-related reductions in insulin sensitivity are due to an age-related increase in adiposity rather than a consequence of advanced chronological age per se (17), and the central role of adipose tissue in the insulin resistance of aging has been shown (31), so the decreased fat mass may contribute to the higher insulin sensitivity seen here in females than in males as well as the higher insulin sensitivity found in adult male offspring of dams fed the FO diet during early pregnancy.

In conclusion, the current study shows that the intake of high amounts of n–3 fatty acids during early pregnancy reduces both fat accretion and age-related decline in insulin sensitivity in male offspring but not in females. It is proposed that the smaller age-related adiposity caused by the increased availability of n–3 fatty acids during intrauterine life is responsible for the lower age-related insulin resistance in male offspring. Further studies are necessary to elucidate the epigenetic mechanism involved and to compare these findings to others using different doses and timings of treatment.

**Perspectives and Significance**

Human epidemiological data and animal studies have shown that interventions in maternal diet during pregnancy or lactation have implications for the long-term health outcomes. The

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00392.2012 • www.ajpregu.org
present study shows for the first time in rats the association between exposure to a moderate amount of dietary n–3 LCPUFA during early pregnancy and the age-dependent reduction of both insulin resistance and increases in adipose tissue mass in male but not in female offspring. These findings raise a number of questions that deserve attention. The first one concerns the timing of the intervention, which was confined to early pregnancy, indicating that the effects of increased n–3 LCPUFA status can occur during just the embryonic stage of intrauterine development. The molecular mechanism, through which the effect is brought about, deserves further investigation.

The second point concerns the sex dependency of the nutritional programming. Our findings extend previous reports indicating a sex differentiation in the response to dietary interventions during fetal or early postnatal life. Thus, by assuming that the mark caused by the presence of an increased proportion of dietary n–3 LCPUFA may occur at a stage of embryonic development before the sexual differentiation of the gonads, it appears that the differential hormonal environment in both adult sexes is responsible for their differential response to the age-dependent insulin resistance.

The third point concerns the potential relationship between changes in body fat mass and insulin sensitivity. The present study does not permit us to establish which occurs first in response to the n–3 LCPUFA intervention, either a decline in fat depot or a delay in the age-dependent loss of insulin sensitivity in males. An investigation into the mechanism of the relationship is warranted and would open new perspectives.

ACKNOWLEDGMENTS

The authors acknowledge Milagros Morante for excellent technical assistance.

GRANTS

This study was carried out with the financial support of the Spanish Ministry of Science and Innovation (SAF2008-04518), Universidad San Pablo CEU (USP09-12), a grant from Fundación Ramón Areces (Spain) to E. Herrera (CIVP16A1835, 2012), and the Cooperation Program between Brazil and Universidad San Pablo CEU. Data were generated in the Faculty of Pharmacy, University of San Pablo-CEU. The authors thank Fundación de Amparo a Pesquisa do Rio de Janeiro (FAPERJ) Tecnológico (CNPq) for the scholarship awarded to F. S. Fernandes, a postgraduate student at the Universidade Federal do Rio de Janeiro (UFRJ).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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