Maternal obesity accelerates rat offspring metabolic aging in a sex dependent manner

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Abbreviations: AI, adiposity index; C, control; DCF, dichlorofluorescein; DHEA, dehydroepiandrosterone; F_0, female founder generation; F_1, offspring; GPx, glutathione peroxidase; H&E, hematoxylin and eosine; HOMA, homeostatic model assessment; MDA, malondialdehyde; MO, maternal obesity; PND, postnatal day; RIA, radioimmunoassay; ROS, reactive oxygen species; SOD, superoxide dismutase.

KEY POINTS

- Maternal obesity predisposes to metabolic dysfunction in male and female offspring
- Maternal high-fat diet consumption prior and throughout pregnancy and lactation accelerates offspring metabolic aging in a sex dependent manner
- This study provides evidence for programming-aging interactions

ABSTRACT

Human epidemiological studies show that maternal obesity (MO) shortens offspring life and health span. Life course cellular mechanisms involved in this developmental programming-aging interaction are poorly understood. In a well-established rat MO model, female Wistar rats ate chow (controls - C) or high energy, obesogenic diet to induce MO from weaning through pregnancy and lactation. Females were bred at postnatal day (PND) 120. Offspring (F_1) of mothers on control diet (CF_1) and MO diet (MOF_1) delivered spontaneously at terms. Both CF_1 and MOF_1 ate C diet from weaning throughout the study. Offspring were euthanized at PND 36, 110, 450 and 650. We determined body and liver weights, liver and serum metabolite concentrations, hormones and oxidative stress biomarkers. Male and female CF_1 body weight, total fat, adiposity index, serum leptin, insulin, insulin resistance, and liver weight, fat, triglycerides, malondialdehyde, reactive oxygen species and nitrotyrosine all rose with differing aging trajectories. Female CF_1 triglycerides were unchanged with age. Age-related increases were greater in MOF_1 than CF_1 in both sexes for all variables except glucose in males and females and cholesterol in

males. Cholesterol fell in CF_1 female but not MOF_1. Serum corticosterore levels were higher in male and female MOF_1 than CF_1 and declined with age. DHEA serum levels were lower in male and female MOF_1 than CF_1. Liver antioxidant enzymes decreased with age (CF_1 and MOF_1). Conclusions: Exposure to the developmental challenge of MO accelerates progeny aging metabolic and endocrine profiles in a sex specific manner providing evidence for programming-aging interactions.

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INTRODUCTION

The ***Developmental Programming*** hypothesis states that challenges in critical developmental time windows alter development with persistent effects on offspring (F_1) phenotype. To date the focus has been on developmental programming of predisposition to noncommunicable diseases such as type 2 diabetes, cardiovascular disease, and obesity (Hanson & Gluckman, 2014; Prescott, 2016). Both programming outcomes (Desai *et al.*, 2015) and aging processes (Chahal & Drake, 2007) have been shown to result in metabolic and endocrine dysfunction and increased oxidative stress. This study addresses the urgent need for studies that determine interactions between programming and aging mechanisms (Rodriguez-Gonzalez *et al.*, 2014; Rodriguez-Gonzalez *et al.*, 2015; Tarry-Adkins *et al.*, 2016; Tarry-Adkins *et al.*, 2018; Vaiserman, 2018). Most studies on aging compare only two categorical life course time points. From this limited life course coverage, it is difficult to evaluate aging trends because changes may not be linear, and the direction of change cannot be accurately predicted either between two widely separated time points or outside the interval covered by the restricted time points chosen. It is necessary to take a full life course approach to study the effects of programming challenges as modifiers of the rate of aging, slowing or acceleration.

Thus, data should first be obtained as early in life as possible relevant to the systems under study. We have found that it is preferable to evaluate subjects over at least four ages, spread over as much of the life course as possible, that occur with normative aging in control offspring (CF_1) of normally fed mothers (Zambrano *et al.*, 2015). The more time points evaluated the stronger the conclusions. Thus, to provide the data needed to detect direction of aging changes in the fall in rat serum glucocorticoids across the life course resulting from programming, we obtained data at four time points to compare offspring of obese mothers (Zambrano *et al.*, 2015). In the present study we evaluated programming aging interactions in key metabolic, endocrine and oxidative stress markers at four time points, postnatal day (PND) 36 – puberty time, 110 – young adult, 450 – mature adult and 650 – old adult, in a colony of rats in which CF_1 start to die spontaneously soon after PND 850. The choice of the oldest age was determined by the fact that MO offspring (MOF_1) in

this colony begin to die soon after 650 days while CF_1 generally live past 850 days. This decreased life span in MOF_1 indicates acceleration of aging processes in MOF_1. We chose puberty as the earliest age in order to start the study after the rapid changes that precede puberty while still beginning early in life. 110 days represents the young adult and was chosen to enable comparisons with our paper on RNASeq in programming, addressing mechanisms of MO programming of the liver at 110d (Lomas-Soria *et al.*, 2018). 450 days represents a mature adult and we have also published programming outcomes at this age (Rodriguez-Gonzalez *et al.*, 2014; Rodriguez-Gonzalez *et al.*, 2015; Santos *et al.*, 2015; Zambrano *et al.*, 2015) which represents approximately 50% of the life course of CF_1. Thus, the period studied represents 75% of the normal life course. In contemporaneous studies we determined the aging profile of the same markers in F_1 of obese rats (MO – MOF_1). At each age we evaluated a wide range of metabolic, hormonal and oxidative stress markers to enable us to follow phenotype aging closely. We hypothesized that age-related changes in metabolic, endocrine and oxidative stress markers would be accelerated in MOF_1.

METHODS

Standardization of females recruited to breed as mothers to produce study offspring.

All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico (CINVA 271 and 1868) and are consistent with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; Schulz *et al.*, 2010; Grundy, 2015; Dickinson *et al.*, 2016; Morrison *et al.*, 2018). Female albino Wistar rats were born and maintained in the INCMNSZ animal facility, which is accredited by and adheres to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Rats were maintained at 22–23◦C under controlled lighting (lights on 07:00 to 19:00 h) and fed normal laboratory chow (Zeigler Rodent RQ22-5, Gardners, PA, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fiber, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g^{-1}. At age 14 – 16 weeks, when they weighed 200 – 240 g, females were bred to randomly assigned, non-litter mate, proven male breeders. At delivery on day 0 litters that provided Founder Generation (F_0)

mothers were culled to 10 pups, each containing at least four females. At weaning (day 21) one female F_0 pup from each litter was randomly assigned to either a maternal control (C) group fed

laboratory chow or to a maternal obesity group (MO) fed a high energy, obesogenic diet containing 22.5% protein, 20.0% animal lard, 5.0% fat, 20.5% polysaccharide, 20.5% simple sugars, 5.0% fiber, 5.0% mineral mix, 1.0% vitamin mix, (w/w), energy 4.9 kcal g^{-1}. The high energy obesogenic diet was produced in the specialized dietary facility of the INCMNSZ (Table 1). Thus, each F_0 group had only one female from any litter and F_0 females in different groups, but not within groups, were sisters, providing homogeneity in F_0 mothers' own developmental programming and genetics. We report here data with the following number of animals at 36, 110, 450 and 650 – CF1: males n= 8, 7, 6, 6; females n= 8, 7, 6, 6 and MOF1: males n= 7, 7, 6, 6; females n= 7, 7, 5, 6.

Production of Study Offspring

Control and MO F_0 female rats were placed with proven male breeders on day 120 and conceived during the next cycle. Lactating mothers were maintained on their pregnancy diet (C or MO). C and MO F_1 litter size and pup weights were recorded at birth. Ano-genital distance was measured to identify males and females (Zambrano *et al.*, 2006). Litters with more than 14 pups were excluded from the study. To ensure study F_1 homogeneity, on postnatal day (PND) 2 all F_0 litters studied were adjusted to 10 pups with equal numbers of males and females wherever possible. Offspring were weaned at PND 21 and fed Chow diet throughout the study. Litters were divided into male and female offspring in different cages. There was no mixing of litters or sexes from different treatment or age groups. Offspring were maintained in this situation until 50 days of life after which no more than 4 rats were placed in one cage. After 110 days the number was reduced to a minimum of two or a maximum of three per cage. Four hours of fasting at PND 36 and 6 hours of fasting at PND 110, 450 and 650, one male and female F_1 per litter/age were euthanized under general anesthesia with isoflourane, followed by decapitation with a rodent guillotine (Thomas Scientific, Swedesboro, NJ, USA) by trained personnel experienced in the procedure. Thus male and female F_1 evaluated at the four ages were siblings. Offspring rats were decapitated

between 12.00 to 14.00 hours. For each age group, trunk blood was collected in tubes cooled on ice and serum was separated and stored at -70°C. Liver was dissected, cleaned and weighed. The right inferior lobe was fixed in 4% paraformaldehyde and treated as described below for histological analysis. The left lobe was stored at -70°C for further oxidative stress analysis. Fat depots were excised and weighed to determine the adiposity index (AI). AI= total fat (g) x body weight g^{-1} .100^{-1}.

Measurement of food intake

CF_1 and MOF_1 food intake was measured for 14 consecutive days between 95 to 110, 435 to 450 and 635 to 650 days of age. A maximum of 3 rats from the same experimental (C or MO), sex and group were housed per cage. Food was provided in the form of large flat biscuits. The amount of food provided each day was weighed as was the amount remaining after 24 h. The amount consumed daily was averaged per rat.

Blood measurements

Glucose, triglycerides, and cholesterol were determined enzymatically in a Synchron CX auto analyzer (Beckman Coulter, Co. Fullerton, CA, USA) (Zambrano *et al.*, 2006). Serum Leptin was determined by radioimmunoassay (RIA) (Linco Research,Inc., St Charles, MO, USA) (Zambrano *et al.*, 2006). Serum insulin was determined using RIA (Millipore, Burlington, MA, USA) (Zambrano *et al.*, 2006). Homeostatic model assessment (HOMA) was calculated from HOMA= glucose (mmol-1) x insulin (µU/mL). 22.5^{-1} (Cacho *et al.*, 2008; Zambrano *et al.*, 2010; Antunes *et al.*, 2016). Corticosterone serum concentrations were determined by RIA using a commercial rat DPC Coat-a-count kit (TKRC1) (Diagnostic Products, Los Angeles, CA, USA) (Rodriguez *et al.*, 2012). Dehydroepiandrosterone (DHEA) serum concentrations were determined by the enzyme-linked immunosorbent assay (ELISA) using a DHEA ELISA (DRG Instruments GmbH, Germany) (Herrera-Perez *et al.*, 2017). At PND 36 there was not enough serum available to measure corticosterone and DHEA concentrations.

Liver total fat, cholesterol and triglycerides content

Liver fat was extracted by a modified Folch technique (Folch *et al.*, 1957). Samples were homogenized with 2 ml of 0.9% NaCl and 5 ml of chloroform:methanol (2:1). Homogenate phases were separated by centrifugation (1,500 g for 15 min at 4°C) and the organic phase was evaporated under a stream of nitrogen and the extracted fat was weighed. The fat was re-suspended in a solution of isopropanol:triton (1:1000) to measure liver triglycerides content by using a RANDOX triglycerides kit, according to the manufacturer's instructions (RANDOX, Crumlin, UK).

Liver morphometric analysis

The right inferior liver lobe was dissected, sectioned longitudinally and immediately immersion fixed in 4% paraformaldehyde in neutral phosphate saline buffer. After 24h of fixation, liver sections were dehydrated with ethanol at increasing concentrations from 75 to 95% and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin (H&E) to determine the extent of steatosis. The percentage area stained for liver fat was evaluated using an Olympus BX51 light microscope (Melville, NY, United States) at 100x magnification in five random fields in each section. Microscope fields were analyzed using the AxioVisio LE software real 4.8 version (Zeiss® copyright 2006–2010 Stuttgart-Germany). Masson´s trichrome in sections was analysed using ImageJ software (Image-Pro Plus Version 3.1; Media Cybernetics, Inc.). All histological measurements were performed by two independent observers without knowledge of the source of the tissues, and the results were averaged (Bautista *et al.*, 2016).

Oxidative stress biomarkers

The left liver lobe was homogenized in saline at 4°C and aliquots obtained and frozen at -70°C for later protein quantification using the Bradford method and for determination of biomarkers of oxidative stress (reactive oxygen species and antioxidant enzymes). Lipid peroxidation was determined at the time of tissue homogenization. All determinations were performed in duplicate and averaged for statistical analysis.

Lipid peroxidation assay

Lipid peroxidation was determined in 100 μl aliquots of serum and liver homogenate by measuring malondialdehyde (MDA) with the thiobarbituric acid-reactive substances assay. All samples were read in a plate at 532 nm in a Perkin-Elmer LS50-B luminescence spectrometer. Results were expressed as nmol MDA.mg protein^{-1} (Rodriguez-Gonzalez *et al.*, 2014; Vega *et al.*, 2015).

Reactive oxygen species (ROS) assay

ROS formation in 5 μl of homogenized liver was estimated using methods previously reported in detail (Perez-Severiano *et al.*, 2004). A standard curve was obtained using increasing concentrations of 2',7'-dichlorofluorescein (DCF) and incubated in parallel with the samples (37°C for 60 min). At the end of the incubation period fluorescent signals at an excitation wavelength of 488 nm and an emission wavelength of 525 nm were recorded in a Perkin-Elmer LS50-B luminescence spectrometer. Results were expressed as nmoles of DCF formed mg protein^{-1} minute^{-1} (Rodriguez-Gonzalez *et al.*, 2014; Vega *et al.*, 2015).

Superoxide dismutase (SOD) activity

SOD activity was determined in 10 μl aliquots of liver homogenate with a RANSOD kit (RANDOX; Crumlin, UK) as previously described (Rodriguez-Gonzalez *et al.*, 2014; Vega *et al.*, 2015). A standard curve was obtained according to the manufacturer's instructions. All samples were read in a plate at 505 nm in a Perkin-Elmer LS50-B luminescence spectrometer at 0, 30 sec and 3 min at 37 °C. Results were expressed as activity units mg protein^{-1} minute^{-1}.

Glutathione peroxidase (GPx) activity

GPx activity was determined in a 10 μl aliquot liver homogenate with the RANSEL kit (RANDOX; Crumlin, UK). All samples were read at 304 nm in a Perkin-Elmer LS50-B

luminescence spectrometer at baseline, 1, 2 and 3 min at 37 °C. Results were expressed as milliunits mg protein^{-1} minute^{-1} (Rodriguez-Gonzalez *et al.*, 2014; Vega *et al.*, 2015).

Nitrotyrosine histology

5 µm paraffin sections of the right liver lobe were immunostained with affinity-purified mouse monoclonal antibody anti-nitrotyrosine MAB5404 (Millipore, Burlington, MA, USA) at 1:200 using an ABC elite kit (Vector Laboratories, Burlingame, CA, USA) and visualized using 2.5% nickel sulfate with 0.02% chromogen in 0.175 M sodium acetate (Vega *et al.*, 2015). From one tissue section, at least 30–40 photographs per animal were taken at × 40 with an Olympus BX51 light microscope (Melville, NY, USA) using image analysis software (Image-Pro Plus Version 3.1, Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

Data are expressed as mean±SEM; group n= 5 to 8 offspring per sex and group from different F_0 litters. Male and female body weight, total fat, AI were analyzed by one-sided t test and were different, therefore sexes were analyzed separately. Differences between groups at the same age and within the same groups but at different ages were analyzed using two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test.

To determine accelerated aging in MOF_1, we first determined the normative life course trajectory in CF_1 in the variable under study. We then determined whether the change in the variable under study was in the same direction in MOF_1 as in CF_1 – an increase or decrease. Accelerated aging was considered to occur if the variable in MOF_1 changed in the direction of the change (either an increase or decrease) in normative CF_1 aging but at a younger age than in CF_1. There are two ways accelerated aging is reported in our data. Firstly, in what we designate as the "same age method" acceleration is represented by the first age at which MOF_1 and CF_1 differ significantly in the direction of normative aging (see Fig 2 Male Insulin 110 d panel B). Secondly, in what we designate as the "consecutive age method", using our current system where a letter change is used to denote a difference from the earlier age, for accelerated aging to occur there has to be an earlier letter change

in MOF_1 than CF_1. (See Fig 4I Female Liver triglycerides (TG) at 110d). Here we describe accelerated aging as having occurred if at least one of these two criteria are met.

RESULTS

Absolute food intake fell similarly with age in CF_1 of both sexes (Table 2). Food intake relative to body weight decreased between PND 450 and 650 in CF_1 males but earlier in CF_1 females, between PND 110 and 450 (Table 3). In both male and female MOF_1 relative food intake decreased between PND 110 and 450 with a further decrease between PND 450 and 650 in MOF_1 males. The only differences between CF_1 and MOF_1 were higher absolute intake in MOF_1 females at PND 650 and decreased relative intake in MOF_1 males at PND 650.

Male and female CF_1 and MOF_1 body weight increased similarly until PND 110. In both sexes, PND 450 was the first age at which body weight was greater in MOF_1 compared with CF_1. At PND 650 body weight was further increased in male but not female MOF_1 compared with PND 450 (Fig 1A, 1G). Total fat in MOF_1 exceeded CF_1 in both male and female at PND 110 (Fig 1B, 1H). Changes in adiposity index showed the same tendency as total fat in female MOF_1 while in male MOF_1 the increase in adiposity index occurred at PND 450 (Fig 1C, 1I). Serum leptin levels in male MOF_1 were greater than CF_1 at PND 36 while in female MOF_1 this difference was first seen at PND 110 (Fig 1D, 1J). Serum triglycerides were higher in male MOF_1 than CF_1 at all ages evaluated (Fig 1E); in females this increase first occurred at PND 110 (Fig 1K). There were no differences with age or maternal diet in male cholesterol serum levels; while in CF_1 female cholesterol fell between PND 36 and 110 and remained similar thereafter (Fig 1F, 1L). Cholesterol remained unchanged across the lifespan in males. We observed accelerated aging in MOF_1 compared to CF_1 in body weight by PND 450, (here and throughout at which acceleration was first noted is presented in a sex specific manner: male age first and female age second), total fat (PND 110 in both sexes), AI (PND 450, 110) leptin (PND 36, 110), and TG (PND 36, 110) (Table 4).

Glucose concentrations were similar in males and females at all ages and in both treatment groups (Fig 2A, 2D). We observed accelerated aging changes in, serum insulin concentration

(PND 110, 36) and HOMA (PND 110, 36). Male MOF1 showed further increase in insulin and HOMA between PND 110 and 450 (Fig 2B, 2C), while in female MOF_1 this further increase in insulin and HOMA occurred between PND 450 and 650 (Fig 2E, 2F and Table 4).

Corticosterone fell between PND 450 and 650 in CF_1 and MOF_1 males and females. Corticosterone concentrations were increased in both male and female MOF_1 when compared to CF1 at all ages (Fig. 3A, 3D). In CF1 but not MOF1, corticosterone was higher in females than males at all ages. There were no age-related trends in DHEA in CF_1 males or females (Fig 3B, 3E). DHEA serum levels were lower in male MOF1 than CF_1 at all ages and in females at PND 450 and 650. In males and females DHEA decreased in MO between PND 110 and 650 (Fig 3B, 3E). As a result of these changes, in both males and females at PND 450 and 650 DHEA/corticosterone ratio was lower in MOF_1 than CF1 (Fig 3C, 3F). In MOF_1 males the ratio was also lower at an earlier age, PND 110. Table 4.

We observed premature liver aging changes in MOF_1 at the following PND: weight (PND 450, in males only), total fat observed by H&E staining (PND 110, 650), percent fat (PND 36 in both sexes) and TG (PND 110, 450) (Fig 4A, 4B, 4D, 4F, 4G, 4I; and Table 3). Histology clearly showed larger fat droplets in MOF_1 (Fig. 4C, 4E) both accelerated aging. Female MOF1 showed a small increase in percent liver fat at the youngest and oldest age evaluated (Fig 4H, 4J).

In MOF_1 we observed accelerated liver aging changes at the following PND (Fig 5A, 5B, 5F, 5G): serum MDA (110, 450), liver MDA (PND 110 in both sexes), ROS (PND 450 in both sexes). Liver SOD in males was similar at all ages, while in females liver SOD in MOF_1 was increased by PND 110. SOD showed a decrease with aging that was accelerated at PND at 650 only in females (Fig 5D, 5I). Liver GPx in male MOF_1 was increased by PND 110. GPx showed a fall with aging which was accelerated in MOF_1 at PND 450, while in females at PND 650 (Fig 5E, 5J) and Table 4.

The nitrotyrosine aging trajectory in CF_1 males and females was similar. At all ages evaluated nitrotyrosine was higher in both sexes of MOF_1 in comparison to CF_1 (Fig 6A-6D). The first increase in MOF_1 from both sexes was observed at PND 36.

DISCUSSION

In recent years the incidence of metabolic disorders has greatly increased (Federico *et al.*, 2017; Heindel *et al.*, 2017; Saklayen, 2018). There is now compelling evidence that environmental challenges, including suboptimal maternal nutrition experienced *in utero* and/or in early neonatal life can increase susceptibility to later life diseases such as type 2 diabetes (Portha *et al.*, 2011; Bouret *et al.*, 2015), hypertension (Ingelfinger & Nuyt, 2012; Skrypnik *et al.*, 2019) and obesity (Plagemann *et al.*, 1992; Sarr *et al.*, 2012; Zambrano & Nathanielsz, 2013). The significance of the studies presented here lies in the exponential increase in the proportion of women of reproductive years who are overweight or obese (Hanson *et al.*, 2015; Howell & Powell, 2017). We were able to show that MO prior and throughout pregnancy and lactation programmed MOF_1 to metabolic and endocrine dysfunction and oxidative stress and predisposed MOF_1 to premature aging and the progression of non-alcoholic fatty acid liver disease in a sex specific manner.

Several studies have shown that MO during gestation is an important determinant of offspring weight (Villamor & Cnattingius, 2006; Samuelsson *et al.*, 2008; Zambrano *et al.*, 2010; Maffeis & Morandi, 2017; Wang *et al.*, 2019). We have previously reported that even when MOF_1 eat a normal control laboratory diet, they exhibit increased body weight, fat, and adiposity index (Zambrano *et al.*, 2010; Vega *et al.*, 2015). In the present study both male and female MOF_1 were heavier than CF_1 and had more fat and a higher adiposity index showing the reproducibility of F_1 outcomes in our model of programming by MO. Food intake is controlled through highly complex processes; modifications of this system during the aging process result in the anorexia of aging (Horwitz *et al.*, 2002; Landi *et al.*, 2016), as we observed in the relative food intake in the MOF_1. Complex mechanisms are involved in the age-related deterioration of food intake such as the decline in saliva secretion as well as the decrease of the sense of smell and taste; changes in gastrointestinal function,

chronic low-grade inflammation and alterations in circulating hormones (Landi *et al.*, 2016; Kummer *et al.*, 2019).

Body fat is primarily stored in adipose tissue, which is an active endocrine organ that secretes numerous hormones including leptin (Sethi & Vidal-Puig, 2007; Kalupahana *et al.*, 2012). Under normal physiological conditions leptin plays a key role in regulating body weight by balancing energy intake and expenditure. However, in obesity serum leptin levels increase in correlation with total body fat (Maffei *et al.*, 1995) as leptin resistance develops (Rehman Khan & Awan, 2016). In the present study both male and female MOF_1 had elevated adipose mass and leptin concentrations. The failure of leptin to normalize body weight in obese individuals indicates the presence of leptin resistance (Crujeiras *et al.*, 2015). Energy balance and intake are also regulated by insulin and glucocorticoids. An example of the synergic effects of insulin and glucocorticoids in increasing weight and obesity can be seen in patients with Cushing's syndrome who have intraabdominal obesity and increased cortisol, insulin, and glucose serum levels (la Fleur *et al.*, 2004). Therefore, the increased insulin and corticosterone serum levels observed here in MOF_1 would lead to hyperlipidemia and obesity. The changes observed in adrenal steroids with age are of great interest as both an increase (as in Cushing's disease) and decrease in glucocorticoids (as in Addison's disease) are associated with frailty and premature aging (Clegg & Hassan-Smith, 2018). The fall in corticosterone between 450 and 650 days confirms our finding reported previously (Zambrano *et al.*, 2015). In male MOF_1 by PND 110 the DHEA:corticosterone ratio was already at its lowest level observed, so we cannot determine the timing of any life course associated fall in the ratio without data prior to 110 days. In female MOF_1 however, the ratio fell between PND 110 and 450. Since DHEA is protective against aging, this later fall in the ratio may represent one of the mechansms responsible for the so called "female aging advantage" (Cheng *et al.*, 2019).

MO and high fat diet consumption during pregnancy and lactation are associated with MOF_1 changes in lipid metabolism, increased cholesterol levels, development of fatty liver, impaired energy homeostasis and food intake, and deterioration of β-cell function (Elahi *et al.*, 2009; McCurdy *et al.*, 2009; Long *et al.*, 2010; Zambrano *et al.*, 2010; Yokomizo *et al.*, 2014; Vega *et al.*, 2015; Zambrano *et al.*, 2016a; Zambrano

et al., 2016b). In our model, the exposure of F_0 to the high fat diet from weaning until the end of lactation establishes a dysfunctional metabolic phenotype (elevated serum triglycerides, leptin and insulin as well as insulin resistance and liver fat accumulation) in MOF_1. Fat tissue dysfunction in obesity is associated with a shortened lifespan (Ahima, 2009; Fasshauer & Bluher, 2015). Reynolds et al. published that the presence of maternal overweight and obesity in human pregnancy is strongly associated with increased risk of premature death and cardiovascular disease in both mothers (Lee *et al.*, 2015) and offspring (Reynolds *et al.*, 2013). In our model, CF_1 survive longer than 850 days while no MOF_1 males are alive at 850 days (Rodriguez-Gonzalez *et al.*, 2014; Rodriguez-Gonzalez *et al.*, 2015).

The liver constitutes a key organ in the control of lipid homeostasis and under normal conditions stores small amounts of fatty acids in the form of triglycerides. However, under certain pathophysiological conditions, hepatic acid metabolism is altered (Alves-Bezerra & Cohen, 2017). Non-alcoholic fatty liver disease is a progressive disease that is characterized by excessive hepatic triglyceride accumulation and is associated with obesity, insulin resistance and dyslipidemia (Bril *et al.*, 2016; Sinton *et al.*, 2019) along with oxidative stress (Perla *et al.*, 2017). For reasons that are not completely clear, non-alcoholic fatty liver disease is more common in men than women. However, the precise incidence is uncertain because of the difficulties of establishing a precise diagnosis (Tsuneto *et al.*, 2010). Our results are in accordance with previously published data, since MOF_1 showed insulin resistance, excessive liver fat, and triglyceride liver accumulation (in sex specific manner) as well as increased oxidative stress which plays a key role in the initiation and progression of non-alcoholic fatty liver disease due to increased ROS formation which leads to lipoperoxidation (Spahis *et al.*, 2017). Oxidative damage can also be caused by an increase in reactive nitrogen species. Nitrotyrosine, a product of tyrosine nitration, is an indicator of oxidative cell damage (Ischiropoulos, 1998). We observed that livers from male and female MOF_1 showed higher levels of nitrotyrosine immunoreactivity. High levels of glucocorticoids and dehydroepiandrosterone may also play a role in the development of non-alcoholic fatty liver disease (Papanastasiou *et al.*, 2017). We have previously reported that MO prior and throughout pregnancy and lactation

programs the offspring to the development of non-alcoholic fatty liver disease in a sex-dependent manner (Lomas-Soria *et al.*, 2018).

Aging is characterized by a progressive decline in cellular function that eventually leads to dysfunction and failure of physiological functions (Stuart *et al.*, 2014). For example, aging is a significant risk factor for the development of obesity and hepatic steatosis (Jin *et al.*, 2013). As we age, fat distribution shifts from subcutaneous to visceral fat, lean mass decrease and triglycerides are ectopically deposited in the liver, muscle, heart, among other tissues (Cartwright *et al.*, 2007; Kuk *et al.*, 2009). Major changes in adipose tissue function occur throught life, such as dysregulation of adipocyte lipid metabolism (Ahima, 2009). The progressive dysfunction of fat metabolism we have observed is an important hallmark of the aging process and contributes to widespread functional metabolic abnormalities. Obesity shares numerous biological similarities with the normal aging; nevertheless, fat tissue dysfunction appears earlier in obesity than in normal aging (Perez *et al.*, 2016). The first step in understanding the role of MO in F_1 metabolic aging is to establish the timing of life-course changes in metabolic parameters with normal aging. Therefore, there is a need for data from many life-course stages as possible, even before a clear aged phenotype emerges. There is also a need to determine any differences between males and females. In our model we found that body weight, fat accumulation, leptin, triglycerides, insulin, insulin resistance index, oxidative stress biomarkers, as well as liver fat accumulation are all increased with aging in both CF_1 and MOF_1 in a sex specific manner. Importantly, the increases occurs from higher early life levels in MOF_1.

It has long been a central principle of the DOHaD hypothesis that developmental programming in response to challenges such as MO predisposes to F_1 non-communicable disease (Roura & Arulkumaran, 2015; Mandy & Nyirenda, 2018). MO has significant effects on aging of systems other than lipid function. Thus we have published data showing that MO during pregnancy and lactation increases MOF_1 testicular and sperm oxidative stress leading to premature aging of reproductive capacity (Rodriguez-Gonzalez *et al.*, 2015; Santos *et al.*, 2015).

In conclusion, MO is a key factor in predisposing offspring to chronic metabolic disease. Our data show clearly that MO leads in a sex-dependent manner to

premature aging potentially as a result of oxidative stress, changes in steroid hormones and other functional changes, providing compelling evidence for programming-aging interactions

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AUTHOR CONTRIBUTIONS

GLRG, LAR, CJB, AAB, CAI, CCV, CLS, CCR, ALEL researched data. GLRG and LAR data analysis. EZ study design. GLRG, PWN and EZ preparation and review of manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

COMPETING INTERESTS

The authors have declared that not competing interesting exists.

FIGURE LEGENDS

Figure 1. Body weight, total body fat, adiposity index and lipid metabolism in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: A, G. body weight (g); B, H. total fat (g); C, I. adiposity index; D, J. leptin (ng/mL); E, K. triglycerides (mg/dL); F, L. cholesterol (mg/dL). Mean ± SEM, CF_1: males n= 8, 7, 6, 6; females n= 8, 7, 6, 6 and MOF_1: males n= 7, 7, 6, 6; females n= 7, 7, 5, 6 rats from different litters at postnatal day 36, 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

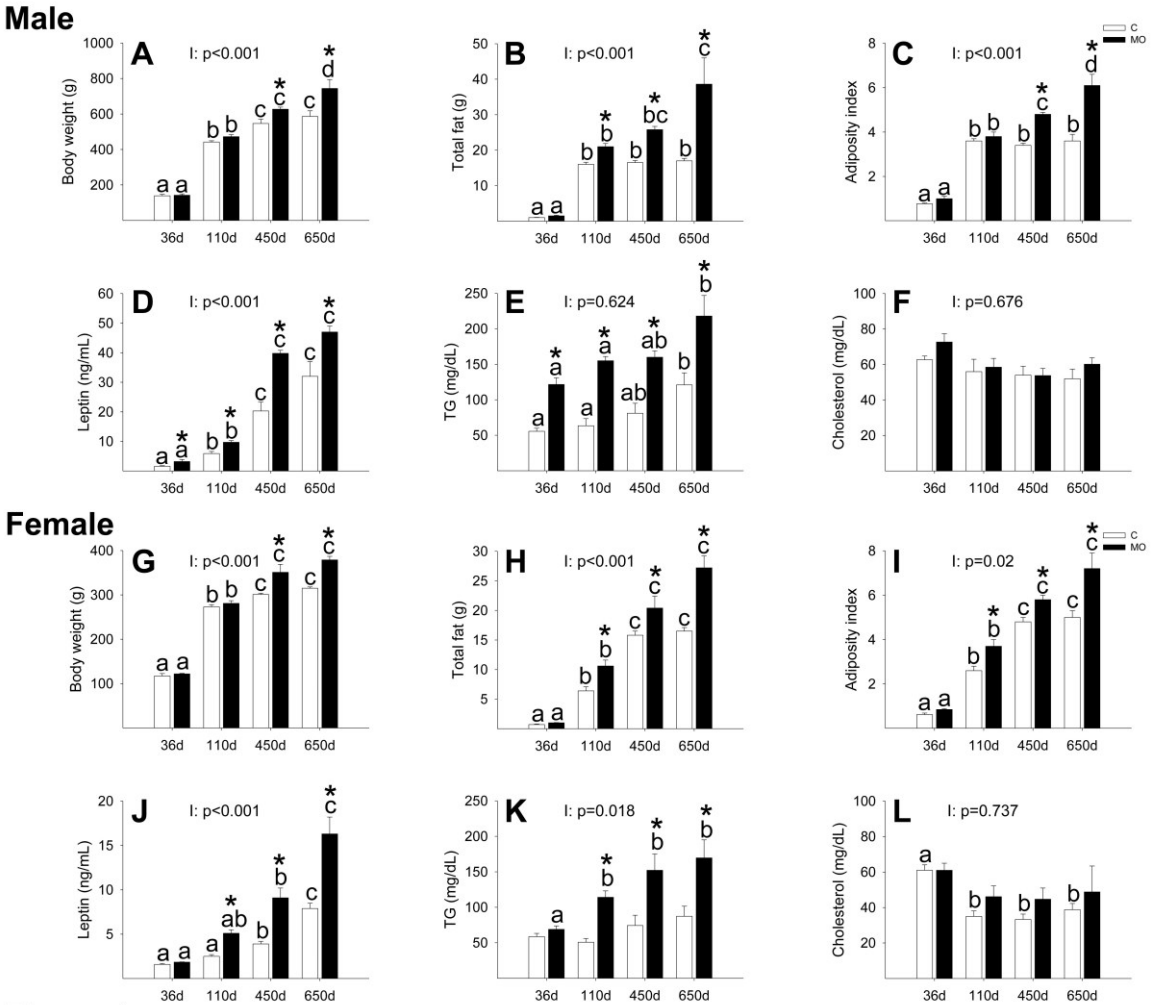

Figure 1.

Figure 2. Carbohydrates metabolism in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: A, D. glucose (mg/dL); B, E. insulin (ng/mL); C, F. insulin resistance index. Mean ± SEM, CF_1: males n= 8, 7, 6, 6; females n= 8, 7, 6, 6 and MOF_1: males n= 7, 7, 6, 6; females n= 7, 7, 5, 6 rats from different litters at postnatal day 36, 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

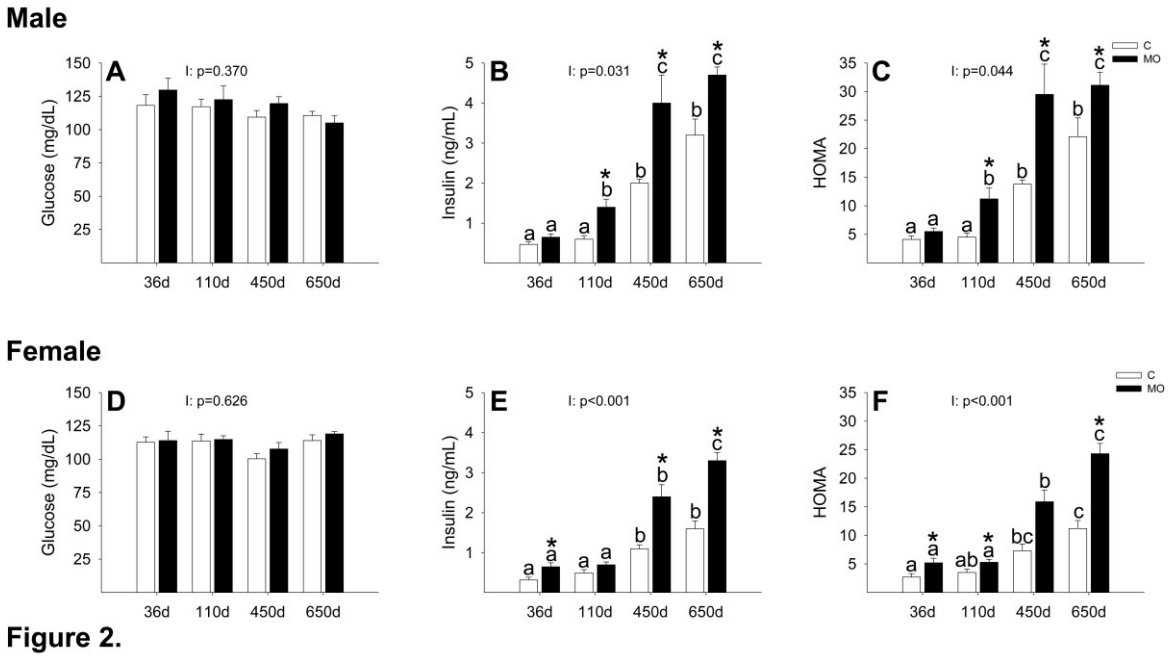

Figure 2.

Figure 3. Adrenal steroid function in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: A, C. corticosterone (ng/mL); B, D. DHEA (ng/mL). Mean ± SEM, CF_1: males n= 7, 6, 6; females n= 7, 6, 6 and MOF_1: males n= 7, 6, 6; females n= 7, 5, 6 rats from different litters at postnatal day 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

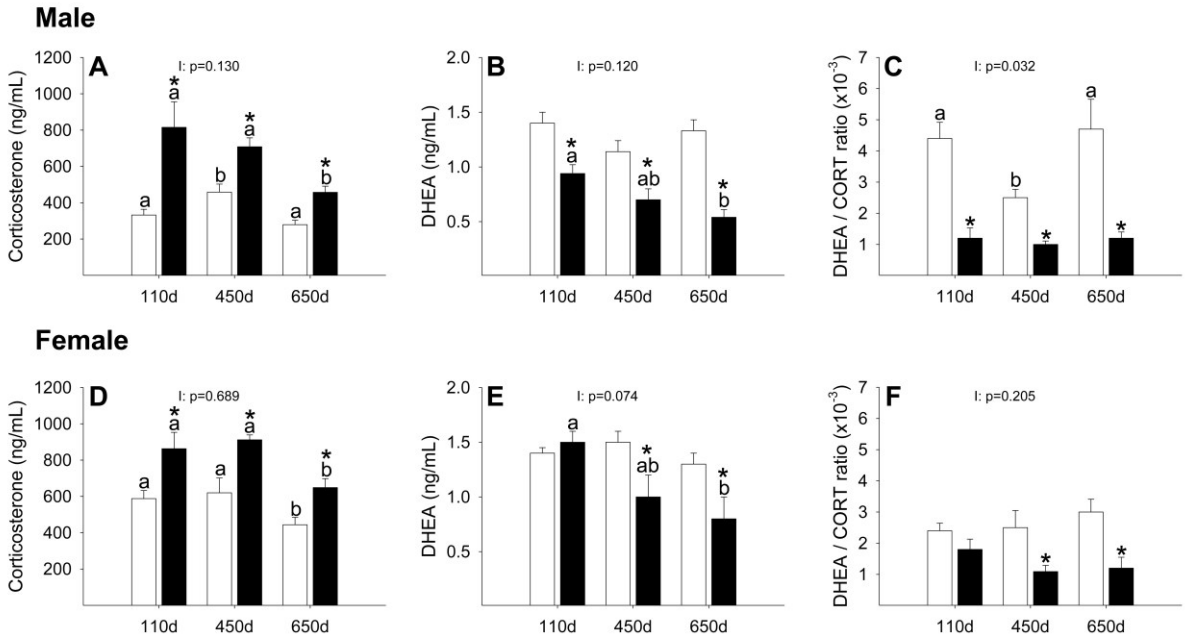

Figure 3.

Figure 4. Liver weight and liver fat accumulation in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: A, F. liver weight (g); B, G. total liver fat (%); C, H. liver fat area (% area); D, I. liver triglycerides (g/g of liver); E, J. representative histology for liver fat. Mean ± SEM, CF_1: males n= 8, 7, 6, 6; females n= 8, 7, 6, 6 and MOF_1: males n= 7, 7, 6, 6; females n= 7, 7, 5, 6 rats from different litters at postnatal day 36, 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

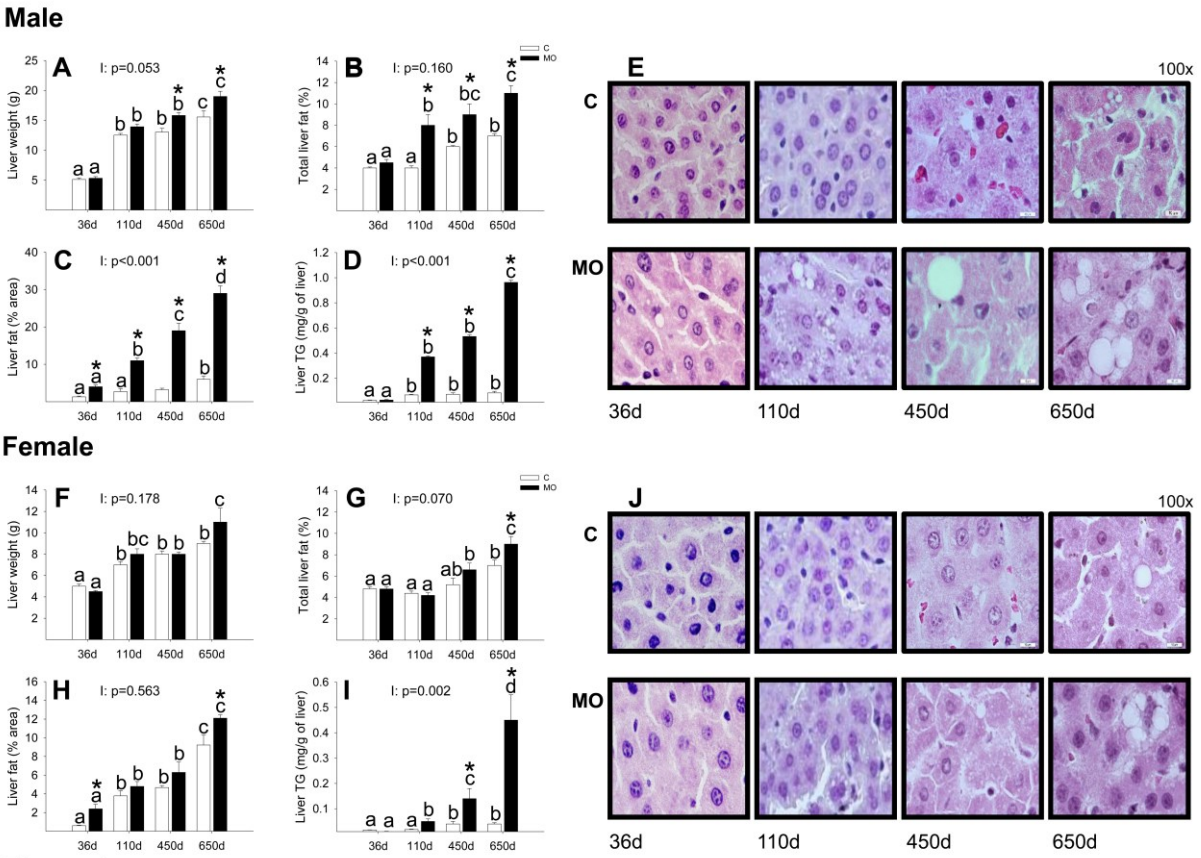

Figure 4.

Figure 5. Serum and hepatic oxidative stress biomarkers in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: Serum- A, F. MDA (nMol/100µL); Liver- B, G. MDA (nMol·mg prot^{-1}); C, H. ROS (nMol DCF·mg prot^{-1}·min^{-1}); D, I. SOD (U SOD·mg prot^{-1}); E. J. GPx (mU GPx·mg prot^{-1}). Mean ± SEM, CF_1: males n= 7, 6, 6; females n= 7, 6, 6 and MOF_1: males n= 7, 6, 6; females n= 7, 5, 6 rats from different litters at postnatal day 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

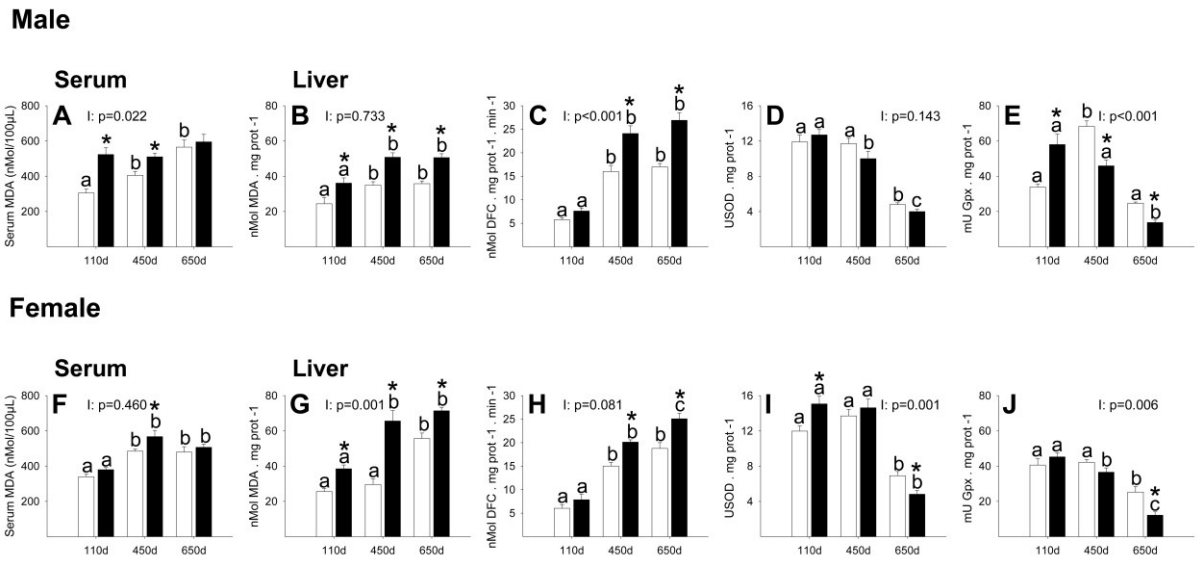

Figure 5.

Figure 6. Hepatic nitrotyrosine fraction stained in F_1 control (C – open bars) and maternal obesity (MO – closed bars) groups. Male and female: A, C. % of area immunostained; B, D. representative immunohistology. Mean ± SEM, CF_1: males n= 8, 7, 6, 6; females n= 8, 7, 6, 6 and MOF_1: males n= 7, 7, 6, 6; females n= 7, 7, 5, 6 rats from different litters at postnatal day 36, 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

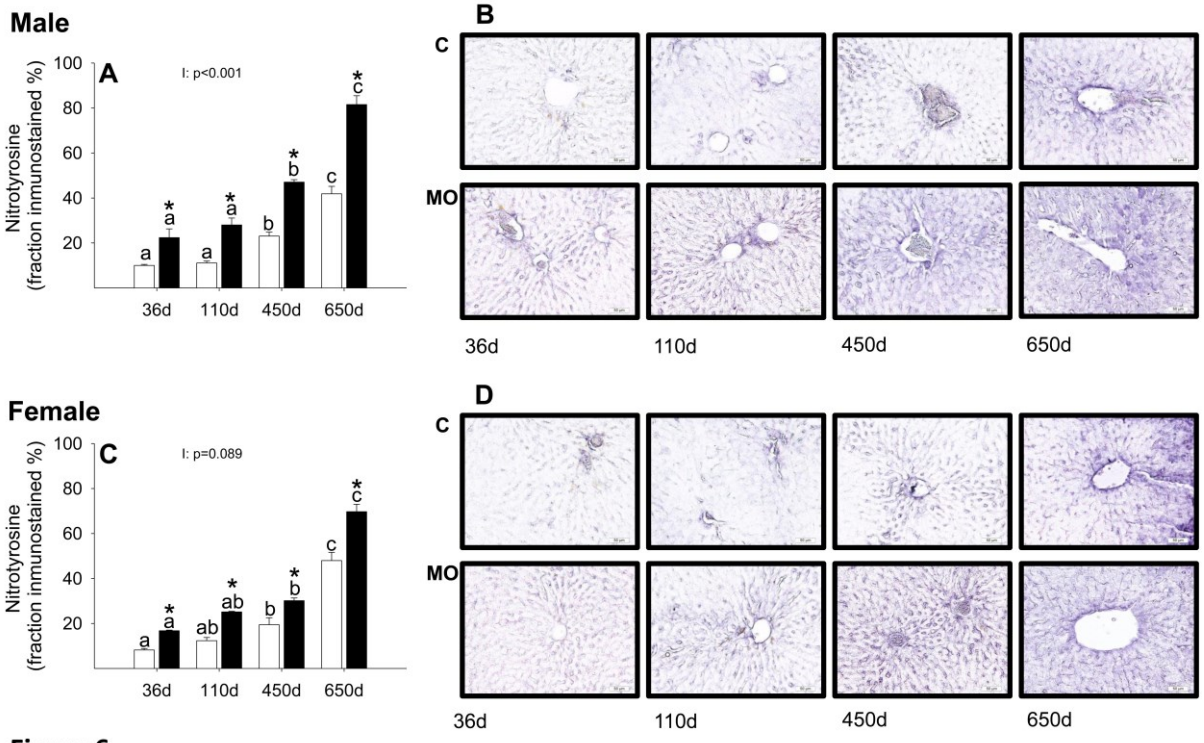

Figure 6.

TABLES

Table 1. High energy obesogenic diet composition

	(%)	**Supplier**
Casein	22.5	Envigo, Huntingdon, UK
L-Cystine dihydrochloride	0.3	Sigma Life Science, St. Louis, MO, USA
Choline dihydrogencitrate	0.17	Sigma Life Science, St. Louis, MO, USA MP Biomedicals LLC, Santa Ana, CA, USA
α-Cellulose	5	
AIN-93VX Vitamin Mix	1	
AIN-93MX Mineral Mix	5	MP Biomedicals LLC, Santa Ana, CA, USA J.C. Fortes, S.A de C.V, Mexico City
Animal lard	20	
Corn oil	5	Gloria, Corfuerte, S.A. de C.V, Mexico City
Carbohydrates		
Corn starch	20.5	Corn Starch Monde México S. de RL de CV
Dextrose	20.5	Droguería Cosmopolita, Mexico City

Energy 4.9 kcal g^{-1}

Table 2. Food intake at postnatal day 110, 450 and 650 in control F_1 and maternal obesity F_1 groups.

		PND 110	PND 450	PND 650	Interactions
Male	C	28.8 ± 8.5 **a**	29.07 ± 1.09 **a**	25.0 ± 1.2 **b**	I: p=0.807
	MO	28.05 ± 0.16 **a**	28.9 ± 2.0 **a**	23.3 ± 1.4 **b**	
Female	C	18.5 ± 0.4 **a**	18.2 ± 0.37 **a**	15.6 ± 0.85 **b**	I: p=0.201
	MO	20.1 ± 0.22 **a**	19.08 ± 0.86 **ab**	18.4 ± 0.19 **b***	

Mean ± SEM, CF_1: males n= 7, 6, 6; females n= 7, 6, 6 and MOF_1: males n= 7, 6, 6; females n= 7, 5, 6 rats from different litters at postnatal day 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

Table 3. Relative food intake to body weight at postnatal day 110, 450 and 650 in control F_1 and maternal obesity F_1 groups.

		PND 110	PND 450	PND 650	Interactions
Male	C	6.2 ± 0.2 **a**	5.4 ± 0.2 **a**	4.3 ± 0.2 **b**	I: p=0.246
	MO	5.9 ± 0.2 **a**	4.6 ± 0.3 **b**	3.1 ± 0.1 **c***	
Female	C	7 ± 0.1 **a**	6 ± 0.2 **b**	4.9 ± 0.2 **c**	I: p=0.288
	MO	7 ± 0.2 **a**	5.5 ± 0.3 **b**	4.8 ± 0.1 **b**	

Mean ± SEM, CF_1: males n= 7, 6, 6; females n= 7, 6, 6 and MOF_1: males n= 7, 6, 6; females n= 7, 5, 6 rats from different litters at postnatal day 110, 450, 650 respectively. Two-way multiple analysis of variance (ANOVA) followed by Holm-Sidak test. $p<0.05$ for data not sharing at least one letter at the same maternal diet, *$p<0.05$ vs C. I = interaction between offspring age and maternal diet.

Table 4. Summary of findings of accelerated aging according to at least on criterion in male and female from in MO F_1 groups.

Serum or liver	Variable	Figure	Accelerated aging in males by day	Accelerated aging in females by day
	Body weight	1A, G	450 ↑	450 ↑
	Total fat	1B, H	110 ↑	110 ↑
	Adiposity index	1C, I	450 ↑	110 ↑
Serum	Leptin	1D, J	36 ↑	110 ↑
	Triglycerides	1E, K	36 ↑	110 ↑
	Cholesterol	1F, L	Not observed	Not observed
Serum	Glucose	2A, D	Not observed	Not observed
	Insulin	2B, E	110 ↑	36 ↑
	HOMA	2C, F	110 ↑	36 ↑
	Corticosterone	3A, D	110 ↑	110 ↑
	DHEA	3B, E	110 ↓	450 ↓
Liver	Weight	4A, F	450 ↑	Not observed
	Total liver fat	4B, G	110 ↑	650 ↑
	Percent liver fat	4C, H	36 ↑	36 ↑
	Liver TG	4D, I	110 ↑	450 ↑
Serum	MDA	5A, F	110 ↑	450 ↑
Liver	MDA	5B, G	110 ↑	110 ↑
	ROS	5C, H	450 ↑	450 ↑
	SOD	5D, I	Not observed	110 ↑
	GPx	5E, J	110 ↑	650 ↓
Liver	Nitrotyrosine	6A, C	36 ↑	36 ↑
	Food intake	Table 1	Not observed	650 ↑

↑ increase and ↓ decrease

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