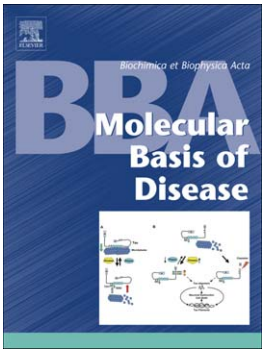

Peri-conceptional obesogenic exposure induces sex-specific programming of disease susceptibilities in adult mouse offspring

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Abbreviations: *Acaca*, Acetyl-coenzyme A carboxylase 1; *Acly*, ATP citrate lyase; *Actb*, Beta-actin; ANOVA, Analysis of variance; AUC, Area under the curve; *Bax*, Bcl2-associated X protein; *Bcl2*, B cell leukemia/lymphoma 2; *Bscl2*, Berardinelli-Seip congenital lipodystrophy 2 (also known as *seipin*); BW, Body weight; CD, Control diet; *Cd36*, Cd36 antigen; CET, Central European Time; *Cidea*, Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A; *Cpt1*, Carnitine palmitoyltransferase 1; CT, Computed tomography; Dpc, Day post coitum; EEC, European Economic Commission; FA, Fatty acid; *Fabp4*, Fatty acid binding protein 4; *Fasn*, Fatty acid synthase; GR, Glucocorticoid receptor; GTT, Glucose tolerance test; H&E, Hematoxylin-eosin; *Hes1*, Hairy and enhancer of split 1; HFD, High-fat, high-calorie diet; HMW, High-molecular-weight; HOMA-IR, Homeostatic model assessment of insulin resistance; HP, Heat production; *Lep*, Leptin; mat-CD, Exposure to maternal control diet; mat-HFD, Exposure to maternal high-fat, high-calorie diet; MD, Maintenance diet; MDA, Malondialdehyde; *Me1*, Malic enzyme 1; *Mest*, Mesoderm-specific transcript/imprinted paternally expressed gene 1 (also known as *Peg1*); MRI, Magnetic resonance imaging; N, Nitrogen; NAFLD, Non-alcoholic fatty liver disease; NMRI, Naval Medical Research Institute; NEFA, Non-esterified fatty acid; *Nr3c1*, Nuclear receptor subfamily 3, group C, member 1 (also known as *Gr*, glucocorticoid receptor); *Nr1h3*, Nuclear receptor subfamily 1, group H, member 3 (also known as *Lxra*, liver X receptor alpha); NRL, Nose-rump-length; PFA, Paraformaldehyde; *Plin2*, Perilipin 2; *Pnpla2*, Patatin-like phospholipase domain-containing protein 2 (also known as *Atgl,* adipose triglyceride lipase); *Ppara*, Peroxisome proliferator activated receptor alpha; *Pparg*, Peroxisome proliferator activated receptor gamma; *Ppia*, Peptidylprolyl isomerase A; RER, Respiratory exchange ratio; ROI, Region of interest; *Scd2*, Stearoyl-coenzyme A desaturase 2; S.e.m., Standard error of the mean; *Sfrp5*, Secreted frizzled-related sequence protein 5; *Srebf1*, Sterol regulatory element binding transcription factor 1; TBARS, Thiobarbituric acid-reactive substances; *Ube2d2*, Ubiquitin-conjugating enzyme E2D 2; VCO_2, carbon dioxide production; VO_2, oxygen consumption

Abstract:

Vulnerability of the fetus upon maternal obesity can potentially occur during all developmental phases. We aimed at elaborating longer-term health outcomes of fetal overnutrition during the earliest stages of development. We utilized NMRI mice to induce pre-conceptional and gestational obesity and followed offspring outcomes in the absence of any postnatal obesogenic influences. Male adult offspring developed overweight, insulin resistance, hyperleptinemia, hyperuricemia and hepatic steatosis; all these features not being observed in females. Instead, those showed impaired fasting glucose and a reduced fat mass and adipocyte size. Influences of the interaction of maternal diet*sex concerned offspring genes involved in fatty liver disease, lipid droplet size regulation and fat mass expansion. These data suggest that a peri-conceptional obesogenic exposure is sufficient to shape offspring gene expression patterns and health outcomes in a sex- and organ-specific manner, indicating varying developmental vulnerabilities between sexes towards metabolic disease in response to maternal overnutrition.

Keywords:

Obesity, Peri-conceptional, Pregnancy, Offspring, Programming, Sex-specificity

1. Introduction

Human epidemiological studies show that the offspring's risk of developing obesity in later life is strongly associated with maternal obesity [1]. Among the various perinatal risk factors for the development of childhood obesity, pre-conceptional maternal obesity confers the strongest risk [2, 3]. This finding is supported by evidence of a decreased offspring obesity risk subsequent to maternal weight-loss surgery [4].

It seems likely that more than one critical time window during development plays a role in programming offspring disease risks [5]. To address this hypothesis, animal studies are needed in order to separate the wide range of confounding exposures and factors during the developmental phases that can influence offspring health outcome. Various animal models have already been used to address the trans-generational impact of diet-induced obesity by fetal exposures throughout pregnancy and also the lactation period [5], which represents a critical time window for programming in rodents [6, 7]. In humans, this period of developmental plasticity most likely corresponds to the third trimester of pregnancy and may expand into postnatal life [8]. However, only very few rodent studies have specifically investigated the transgenerational effects of maternal pre-gravid obesity and during the gestational period [9, 10], although an impact on oocyte and early embryo development has been suggested [11, 12].

Whereas rodent offspring outcomes have so far mostly been determined in males [13], few studies analyzed the impact of an adipogenic exposure during the pregnancy and lactation period by comparing both sexes separately, as reported from rat [14-16] and mouse models [17-19]. In humans, associations between parental body mass index and children's weight and body fat are reported to be sex-dependent, an observation not being explained as yet [20, 21]. Developmental components to sex-specific differences in human disease risk have also been reported for several common disorders [22], such as in diabetes [23].

Most rodent studies utilized inbred mouse strains such as C57BL/6J that upon obesogenic feeding develop pronounced obesity already being complicated by hyperinsulinemia, hyperleptinemia, and dyslipidemia [17, 24, 25]. However, in the human situation, a

substantial proportion of fertile obese women still have largely compensated metabolic homeostasis [26, 27]. We hypothesized that exposure during the earliest developmental stages of life even to milder forms of maternal obesity is already sufficient to induce programming of long-term health risks in offspring of each sex. Thus, we aimed at utilizing a mouse model showing less pronounced maternal adiposity during early developmental stages. For this purpose, we took advantage of the outbred Naval Medical Research Institute (NMRI) mouse stock that is less vulnerable to the consequences of high-fat feeding than the C57BL/6J strain [28].

In the offspring of NMRI dams, we assessed long-term phenotypic outcomes, metabolic features, and underlying gene expression profiles in each sex separately and at different life times. Offspring were transferred to foster dams to specifically preclude programming influences during lactation and were subsequently grown up in the absence of any additional postnatal high-fat diet exposure. Nevertheless, in later life of offspring we demonstrated signs of metabolic disease associated with differential gene expression patterns that strikingly differ between sexes and that are evoked by the obesogenic environment of dams in the pre-conceptional period and early phase of development.

2. Materials and methods

2.1. Experimental design

Male and female NMRI mice (RjHan:NMRI; originally Swiss mice transferred to the US Naval Medical Research Institute, then to the Central Institute for Laboratory Animal Breeding, Hannover, Germany in 1958) were purchased from Janvier (Le Genest ST Isle, France). This outbred stock is characterized by an albino appearance, a rapid growth rate, a high success of reproduction, and large litter sizes [29]. Mice were maintained under specific pathogen-free conditions in the closed barrier facility of the Gene Center Munich at 23 °C, 40% humidity and with a 12 hour light/dark cycle (lights on at 7 AM). All animals had free access to their specific rodent diet and water *ad libitum*. All experiments were approved by the Committee on Animal Health and Care of the local governmental body of the state of Bavaria and performed in strict compliance with the European Economic Commission (EEC) recommendations for the care and use of laboratory animals (European Communities Council Directive of 24th November 1986 [86/609/EEC]).

28 female NMRI mice at 3 weeks of age were randomly distributed into a total of 3 groups. Two groups (7 mice each) received the D12492 high-fat, high-calorie diet (HFD) (E15741-34, Ssniff, Soest, Germany) or the control diet to D12492 (CD) (Ssniff) (Table 1, Supplemental Fig. A.1). We used the term “HFD” being aware that apart from a high-saturated fat content, the carbohydrate composition was characterized by a ratio of 10:1 of sugar to starch. The third group (foster mothers, 14 mice) received a standard maintenance rodent diet (MD) (V1536, Ssniff; Table 1) after arrival to the animal facility (Supplemental Fig. A.1). Body weight was monitored every three days and body composition was measured weekly. At the age of 12 weeks, mice were mated and screened for vaginal plugs every morning and evening. Females of the experimental groups remained on their specific diets and those of the foster group were on CD during pregnancy and lactation to ensure that any postnatal dietary exposure of offspring was only to CD.

The term “peri-conceptional” was used to refer to maternal obesity prior to conception and during the earliest developmental phases including early gestational periods equivalent to the

first and second trimester in humans. Pregnant females were weighed and their body composition was analyzed every three days. All animals were allowed to give birth naturally. Within 12 hours after birth, each litter of the two experimental groups (HFD, CD) was adjusted to a size of 8 animals by culling surplus pups, and directly transferred to one of the dams of the foster group, which gave birth the same day and whose pups were removed (Supplemental Fig. A.1). All litters in each maternal group were greater than 8 pups and none of the dams was excluded due to large variations in litter size. To avoid any selection influence of the experimenter, offspring were randomly chosen regardless of sex, size or other features.

Offspring were weighed every three days. At day 21, all offspring were weaned onto CD. Thereafter, body composition of offspring was monitored weekly and from week eight onwards every two weeks up to 5 months of life, when mice became too large for analysis of body composition. At age 9 months, offspring were sacrificed. In a separate experimental cohort, offspring were sacrificed at age 3 weeks and 5 months, respectively. After fasting for 12 hours, animals were bled from the retro-orbital plexus under short-term (< 30 sec) ether anesthesia and killed by cervical dislocation. Ether anesthesia can induce systemic metabolic and hormonal alterations in rodents and humans [30] through enhanced lipid peroxidation following deep anesthesia of at least several minutes [31, 32]. Therefore, we applied only short-time ether exposition for taking blood samples, along with dietary supplementation of high antioxidant concentrations (150 mg vitamin E per kg diet) [33].

Organs were dissected, blotted dry, and weighed to the nearest mg. Visceral adipose tissue was removed from the entire visceral cavity and comprised perirenal, omental, and periovarian fat depots. Tissue samples were immediately processed and either frozen at -80°C, fixed in 4% paraformaldehyde (PFA) (pH 7.4), or fixed in RNALater (Qiagen, Hilden, Germany) and frozen at -20°C.

2.2. Body composition analysis and metabolic phenotyping

Body composition (% fat and lean mass) was assessed using a magnetic resonance imaging (MRI) analyzer (Minispec LF50; Bruker, Karlsruhe, Germany) without anesthesia.

2.2.1. Offspring calorimetric analysis, movement activity, body temperature and food intake

Offspring prenatally exposed to maternal HFD were named “mat-HFD” and offspring of lean mothers “mat-CD”. Metabolic phenotyping of mat-CD and mat-HFD offspring was performed in the Energy Metabolism Screen of the German Mouse Clinic, Helmholtz Zentrum München, at an age of 5 months (n=7 per maternal diet and sex) [34]. Mice were caged in groups of 3 to 4 animals in individually ventilated Makrolon type II cages. Room temperature was set to 23°C; the light/dark cycle in the room was 12:12 hours (lights on 06:30 Central European Time [CET], lights off 18:30 CET). Mice were weighed every week. About three weeks after entering the Mouse Clinic, energy expenditure was monitored by indirect calorimetry (SM-MARS-8A, Sable Systems, Las Vegas, USA). High precision CO_2 and O_2 sensors measured the difference in CO_2 and O_2 concentrations in air volumes flowing through control or animal cages. O_2 consumption (VO_2, ml $h^{-1}animal^{-1}$) and CO_2 production (VCO_2, ml $h^{-1}animal^{-1}$) were calculated from gas concentrations and the air flow through the cage measured in parallel. Respiratory exchange ratio (RER) as an indicator for metabolic fuel utilization was calculated as the ratio VCO_2/VO_2. Heat production (HP) was calculated from VO_2 and RER using the formula: HP [mW] = (4.44 + 1.43 x RER) x VO_2 [ml h^{-1}]. Each mouse was individually placed in the chamber for a period of about 21 hours (from 14:00 CET to 11:00 CET next day) with free access to food and water. Air from each individual mouse channel was sub-sampled every 10 minutes and used for gas analysis.

Locomotor activity was monitored during this time period, as cages were set up on motion sensitive detector plates that registered fine movements induced by the mouse. These data were used as quantitative estimate for ambulatory behavior. Metabolic chambers were set up in a ventilated cabinet continuously supplied with an overflow of fresh air from outside. In addition, body mass was analyzed before and after the gas exchange measurements. Before

returning the mice to their home cage, rectal body temperature was also determined. Food intake during the trial was monitored by weighing and re-weighing the feeder before and after indirect calorimetry.

2.2.2. Determination of food assimilation coefficient

In a separate experiment, three groups of female NMRI mice were fed either MD (n=6), CD (n=7) or HFD (n=7) starting at the age of three weeks. At the age of 12 weeks, mice were separated in single regular home cages with grid panels on the ground to allow the collection of feces and accurate monitoring of food intake. Mice were kept on their respective diets throughout the experiment. Body mass, food consumption, daily feces production, and the energy content of the feces were measured, whereas energy intake, assimilated energy and the food assimilation coefficient were calculated from raw data. Samples of experimental diets and feces (~1 g) were dried at 60°C for two days, homogenized and squeezed to a pill for determination of energy content in a bomb calorimeter (IKA Calorimeter C7000). Energy intake is determined as the product of food consumed and the caloric value of the food. To obtain assimilated energy, the energy content of feces was subtracted from energy intake. The food assimilation coefficient was calculated as the percentage of total energy intake that was assimilated [food assimilation coefficient = (assimilated energy x 100) / energy intake].

2.3. Glucose tolerance test

To perform a glucose tolerance test (GTT), 1.5 g glucose per kg body weight was administered intraperitoneally. Blood samples were taken from the punctured tail vein at 0, 20, 40, 60, 80, 100 and 120 min after glucose injection and were immediately measured using a glucometer (Abbott MediSense, Wiesbaden, Germany) [35]. In offspring, a GTT was performed at age 5 months after fasting for 12 hours (n=7 per maternal diet and sex). For a GTT during gestation, a separate cohort of dams was used to avoid possible *in utero* influences of the GTT on the investigated offspring. Two groups (7 mice each) were housed individually, fed either HFD or CD, monitored for weight and body composition, and mated as

described above. Pregnant females were fasted 6 h at day 16.5 post coitum (dpc) prior to the GTT. One day later, cesarean sections were performed and dams were sacrificed. Placenta and fetal weights were measured.

2.4. Computed tomography method for determining liver fat content

A computed tomography (CT) X-ray scanner (La Theta, Aloka Ltd., Japan) was used for the non-invasive determination of liver fat content of male and female offspring at age 5 months. CT values of defined tissues decrease with increasing fat content (typically -300 to -100 Hounsfield units for adipose tissue). We made use of this relation and estimated the degree of liver steatosis by measuring X-ray attenuation in CT images of the liver [36]. The X-ray source was pre-set at a current of 1 mA and a voltage of 50 kV. An animal holder with an outer diameter of 48 mm and inner diameter of 41 mm was applied, resulting in pixel resolutions of 100 µm on 480 x 480 pixel images. Mice were sedated by continuous 2-2.5% isoflurane inhalation. Based on a sagittal pre-scan, fixed anatomical landmarks were used to define a region from the proximal end of the eleventh thoracic vertebra to the distal end of the third lumbar vertebra. The scan speed was set to 4.5 s/image; the slice pitch was 1 mm. Recorded scans were analyzed using the software La Theta 2.10. A polygonal region of interest (ROI) in the ventral part of the liver was defined in three consecutive slices. CT values of these ROIs were averaged to determine liver attenuation as an estimate for fat content.

2.5. Histological analyses and determination of adipocyte size

Liver and adipose tissue specimens were fixed in 40 g/l buffered PFA (Serva Electrophoresis, Heidelberg, Germany) for 24 hours, embedded in paraffin (SAV LP, Flintsbach am Inn, Germany), sectioned at 3 µm (HM 315; Microm, Walldorf, Germany), and stained with hematoxylin-eosin (H&E) for routine histopathological examination. For liver fat staining, 10 µm cryosections (Reichert-Jung 2800 Frigocut; Cambridge Instruments, Nussloch, Germany) were prepared from frozen PFA-fixed liver tissue, mounted on glass

slides, incubated with Fat Red 7B (Sigma-Aldrich Chemie, Taufkirchen, Germany), washed with distilled water, and counterstained with hemalum (AppliChem, Darmstadt, Germany). All specimens were interpreted by a pathologist in a blinded fashion. In addition, the severity of hepatic steatosis in dams was assessed in H&E stained sections of paraffin embedded liver samples, applying a semiquantitative grading system [37]. Liver sections were evaluated in a blinded manner twice over the course of 2 days by the same investigator and were additionally assessed by an independent pathologist.

The histological sections of visceral and subcutaneous adipose tissue of offspring were captured at 20 x magnification on an Olympus Fluoview FV1000 microscope equipped with Cell B image capturing software (Olympus Europe, Hamburg, Germany). The images were converted into binary format using ImageJ (http://rsb.info.nih.gov/ij) and GIMP (www.gimp.org) according to [38]. Binary images were then manually compared to the original images to ensure an accurate conversion. If necessary, minor adjustments were made by separating overlapping areas. Cross-sectional profiles of 50 adipocytes per histological sample of each animal were analyzed in a blinded fashion to obtain areas in μm^2.

2.6. Analyses of serum biochemistry, adipocytokines, endocrine status, and reactive oxygen species

Prior to sacrifice, mice were starved for 12 hours, blood was taken, and serum samples were stored at -80°C. Analyses of clinical chemical parameters were carried out as described previously [39]. Briefly, samples were thawed and analyzed the same day using an AU400 autoanalyzer (Olympus, Hamburg, Germany) and adapted reagent kits from Olympus (now available from Beckman Coulter, Krefeld, Germany) or Wako Chemicals GmbH (Neuss, Germany) for the analysis of non-esterified fatty acids (NEFAs). Serum leptin concentrations were measured using the Quantikine Mouse Leptin Immunoassay (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. Serum samples of the pregnant females were subjected to acid/urea activation and subsequently neutralized prior to performing the ELISA to avoid cross-reactivity with a soluble form of the

leptin receptor which is secreted by the mouse placenta from midgestation onwards [40]. Adiponectin secretion was determined by using the high-molecular-weight (HMW) and total adiponectin (mouse) ELISA kit from ALPCO (Salem, USA). Each sample was assayed in duplicate 1:10 diluted for total adiponectin and 1:5 diluted for HMW adiponectin.

For the measurement of serum insulin concentrations, an Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden) was used according to the manufacturer's protocol. Thiobarbituric acid-reactive substances (TBARS) were measured using a TBARS Assay Kit (Cayman, Tallin, Estonia) to assess the level of lipid peroxidation in undiluted serum samples. Serum H_2O_2 was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions.

2.7. Gene expression analyses

Total RNA was extracted from RNALater-conserved frozen liver and visceral adipose tissue of offspring using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany), and 1 µg of RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), both according to the manufacturer's instructions. Quantitative real-time RT-PCR was carried out on the Step One Plus System (Applied Biosystems, Foster City, USA) using Power SYBR Green (Applied Biosystems). 40 cycles were run for each reaction with 15 sec at 95°C for denaturation, 15 sec at 55°C for annealing and 1 min at 60°C for extension. All measurements were done in triplicates. mRNA expression levels were calculated relative to the mean of the housekeeping genes peptidylprolyl isomerase A (*Ppia*) (liver and visceral fat, age 9 months), ubiquitin-conjugating enzyme E2D 2 (*Ube2d2*) (visceral fat, age 5 months), or beta-actin (*Actb*) (liver, age 5 months) used as reference.

2.8. Statistical analyses

Data for body weight and body composition as well as glucose tolerance were statistically analyzed using PROC MIXED (linear mixed models; SAS release 8.2, SAS Institute, Cary, NC, USA) taking into account the fixed effects of diet (CD, HFD), age/time, the interaction of

diet*age/time and the random effect of animal [41]. For the analysis of offspring data (body weight, body composition), mother within diet [mother(diet)] was included in the model as an additional fixed effect to account for the fact that offspring of the same litter developed in the same maternal environment.

For subgroup selections, offspring were ranked according to body fat (%) and selected across the range. Square root transformed gene expression data and serum parameter concentrations were analyzed using PROC GLM (general linear models; SAS release 8.2) taking into account the fixed effects of diet, sex, and the interaction of diet*sex. Significance of individual differences was tested by Tukey's post hoc test. Linear regression models were applied for analysis of metabolic phenotyping data [42]. Two-way analysis of variance (ANOVA) of square root transformed data followed by Holm-Sidak post hoc test was used to compare differences in liver CT Hounsfield units. Kruskal-Wallis test followed by Dunn's test was used to detect differences in adipocyte size.

The remainder of the statistics was performed in GraphPad Prism 4.0 (GraphPad Software, San Diego, USA) using the nonparametric Mann-Whitney *U*-test (two-tailed) to assess differences in organ weights, fetal and placenta weights, birth weights, litter size, area under the glucose curve of the GTT, liver steatosis score, food and energy intake, serum parameters, and transcript levels between two groups of animals each. Data are presented as mean ± standard error of the mean [s.e.m.]; $p < 0.05$ was considered significant.

3. Results

3.1. Composition and digestibility of maternal experimental diets

To ensure comparable digestibility of CD and HFD, which is enriched in long-chain saturated fatty acids, content and source of protein and fiber were kept equal (Table 1). Similarly high digestibility of both diets was confirmed in a separate experiment by analyzing food assimilation efficiencies following HFD or CD intake in NMRI mice from 3 to 12 weeks of age (mean ± s.e.m.; HFD: 93.20 ± 0.39%, CD: 91.53 ± 0.22%). Mineral, trace element and vitamin content of the diets were also matched. Both diets differed in total fat content with 60% of energy derived from fat in the HFD compared to 13% in the CD and, in carbohydrate composition, with a ratio of 10:1 of sugar to starch in the HFD compared to a ratio of 1:1 in the CD. The HFD exceeded the CD in total energy content. Although food intake was lower in the HFD fed dams (mean ± s.e.m.; HFD: 3.1 ± 0.1, CD: 3.5 ± 0.1 g/mouse*day, p=0.014) most likely as a regulatory response [43], the resulting total energy intake was significantly higher in the HFD group (mean ± s.e.m; HFD: 67.3 ± 2.1, CD: 53.3 ± 1.3 kJ/mouse*day $p < 0.0001$). Consequently, protein intake (mean ± s.e.m.; HFD: 0.8 ± 0.02, CD: 0.9 ± 0.02 g/mouse*day, p=0.014) and energy intake resulting from protein (mean ± s.e.m.; HFD: 12.8 ± 0.4, CD: 14.4 ± 0.4 kJ/mouse*day, p=0.014) were slightly lower in the HFD fed dams.

3.2. Influence of obesogenic nutrition before and during pregnancy on NMRI dams

From the age of 8 to 9 weeks onwards, pre-conceptional weight gain and body fat mass of the HFD-fed NMRI dams were significantly increased compared with CD-fed animals (Fig. 1A, B). Reproductive performance appeared to be only slightly influenced by the diet: the fertility rate was 100% (7/7) for each dietary group of mothers, which provided the adult offspring, whereas in the cohort sacrificed at 17.5 dpc, it was 86% (6/7) for the HFD-fed dams versus 100% (7/7) for the CD-fed dams. At the end of gestation, animals fed with HFD showed a moderately impaired glucose tolerance, whereas fasting glucose concentrations and values of the homeostatic model assessment of insulin resistance (HOMA-IR) were unchanged compared to controls (Fig. 1C, Supplemental Table A.1). NEFA levels were

increased in the HFD group (mean ± s.e.m.; HFD: 0.95 ± 0.11 mmol/l, CD: 0.48 ± 0.06 mmol/l, $p < 0.01$); however, there were no other signs of dyslipidemia. Urea values were significantly lower in the HFD-fed group (mean ± s.e.m.; HFD: 5.39 ± 0.47 mmol/l, CD: 7.74 ± 0.32 mmol/l, $p < 0.01$) possibly pointing to a lower protein catabolism than in the CD-fed groups.

Liver histology suggested a tendency, although not statistically significant, towards a higher microvesicular fat deposition in HFD-fed dams compared to the control group, as evidenced by steatosis grading (mean ± s.e.m.; HFD: 2.1 ± 0.2, CD: 1.5 ± 0.1, p=0.057) (Fig. 1D). However, there was no histological and clinical chemical evidence of liver injury and inflammation (Supplemental Table A.1). Serum H_2O_2 was significantly elevated in HFD-fed dams (mean ± s.e.m.; HFD: 114.81 ± 17.02 nmol/ml, CD: 29.33 ± 6.46 nmol/ml, $p < 0.01$); however, there were no significant differences in the levels of serum adipocytokines (leptin, adiponectin) or the inflammatory marker C-reactive protein between both groups.

While the litter size was not influenced by the maternal diet (cohort of 9-month-old offspring: mean ± s.e.m.; HFD: 14.7 ± 0.7, n= 103; CD: 13.6 ± 0.8, n= 95, p=0.26; cohort of dams sacrificed at 17.5 dpc: HFD: 11.7 ± 1.0, n= 72; CD: 12.2 ± 1.3, n= 81, p=0.73), both mean placenta and corresponding fetal weights at 17.5 dpc were significantly increased in the HFD group (Fig. 1E, F).

3.3. Sex-specific effects on body composition and metabolic outcome in adult offspring

Only a few days after birth, the initial differences in birth weight between mat-HFD and mat-CD offspring groups of both sexes disappeared, while all offspring were suckling on lean dams (Fig. 2A, B, inserts). In the absence of any postnatal HFD challenge throughout lifetime (Supplemental Fig. A.1), male mat-HFD offspring developed a significant increase in body weight and fat mass compared to mat-CD males from 10 and 6 weeks of age onwards, respectively (Fig. 2C, E). Unexpectedly, mat-HFD female offspring showed an entirely different response and, although body weight was unaffected, they presented with a

significantly lower body fat content than controls, from age 16 weeks onwards (Fig. 2D, F). These alterations in mat-HFD offspring could not be attributed to changes in food intake, physical activity, body temperature, or energy expenditure (Table 2).

Differences in body weight and body composition were maintained and confirmed at the time of dissection at 5 and 9 months of life, respectively (Table 3). Dissection data at 9 months confirmed an increased subcutaneous fat weight in the mat-HFD males and a significant reduction in visceral fat weight in the mat-HFD females compared to their mat-CD controls. However, liver and carcass weights relative to body weight were significantly increased in the mat-HFD females, accounting for similar body weights in the two female subgroups. Notably, as compared to their controls, visceral fat was not yet detectable at early times of 3 weeks of age in the mat-HFD females and a significant reduction persisted throughout life. In addition, their subcutaneous fat weight was also reduced at 5 months of age, a difference that vanished at the later age of 9 months.

Fasting glucose levels and the results of a GTT performed at the age of 5 months were not different between mat-HFD and mat-CD offspring, both in males and females (data not shown). At 9 months of age, prior to sacrifice, fasting glucose levels were still similar between mat-HFD and mat-CD males but hyperinsulinemia and an increase in HOMA-IR values were evident in the male mat-HFD subgroup (Table 4). In contrast, the mat-HFD females displayed significantly elevated fasting glucose levels at the same age of 9 months, whereas there was no hyperinsulinemia or increased HOMA-IR as in males. Furthermore, male mat-HFD offspring showed hyperleptinemia and hyperuricemia but no significant changes in serum lipid concentrations (Table 4). Female offspring had none of those metabolic features. As in the HFD-fed dams, but unlike in mat-HFD male offspring, urea levels were decreased in the mat-HFD female offspring.

When sex-related differences of metabolic serum parameters were analyzed by ANOVA, we found that fasting glucose, insulin, HOMA-IR, triglycerides, cholesterol, LDL cholesterol, HDL cholesterol, HMW adiponectin, uric acid, urea and TBARS were significantly influenced by sex (Table 4). Among those, insulin, HOMA-IR and urea, as well as leptin were affected by

the interaction diet*sex, pointing to an influence of the peri-conceptional dietary exposure on the sex-related differences.

3.4. Sex-specific effects on liver metabolism in adult offspring

Fat content of the offspring livers was quantitated at the age of 5 months by measuring the X-ray attenuation in liver CT images. Lower Hounsfield units in the mat-HFD males indicated increased liver fat accumulation compared to mat-HFD females (Fig. 3A). In contrast, there was no difference between sexes after *in utero* exposure to a maternal CD environment.

In affected male mat-HFD livers, expression of genes involved in the development of liver steatotic changes was altered compared to mat-CD males at the ages of 5 and 9 months (Fig. 3B, Supplemental Fig. A.2A). Alterations referred to lipogenic genes including fatty acid synthase (*Fasn)* as well as sterol regulatory element binding transcription factor 1 (*Srebf1*) and peroxisome proliferator activated receptor gamma (*Pparg*) 2, two nuclear receptors mediating nutrient effects and previously found to be elevated in steatotic livers [44, 45]. We also found the mRNA levels of the steatosis regulators liver X receptor alpha (*Nr1h3*, also known as *Lxra*) and glucocorticoid receptor (*Nr3c1,* also known as *Gr*) being significantly upregulated in 9-month-old mat-HFD versus mat-CD males (Fig. 3B). The GR target gene hairy and enhancer of split 1 (*Hes1*) was downregulated, consistent with the transcriptionally repressive effects of GR on *Hes1* [46] (Fig. 3B). In contrast to males, there were no significant alterations in gene expression between the mat-CD and mat-HFD females at that age, except for a difference in hepatic *Pparg1* transcript (age 9 months: mean ± s.e.m.; CD: 1.06 +/- 0.11; HFD: 0.68 +/- 0.16; p=0.017) and, at age 5 months, in acetyl-coenzyme A carboxylase 1 (*Acaca*) (mean ± s.e.m.; CD: 1.09 +/- 0.15; HFD: 1.72 +/- 0.13; p=0.008).

Next, liver transcripts of males were compared with females for sex-related differences in response to the peri-conceptional dietary exposure. When offspring were exposed to maternal CD *in utero,* there were no sex-related differences in liver gene expression (Fig. 3C, Supplemental Fig. A.2B). However, maternal HFD exposure led to increased mRNA expression of *Srebf1, Fasn, Pparg* 1 and 2 in male compared to female livers at age 9

months (Fig. 3D). *Pparg* 1 and 2 were significantly influenced by sex and the interaction diet*sex, i.e. maternal diet had an impact on their sex-specific expression (Table 5). Expression of the PPARG target genes Cd36 antigen (*Cd36*), perilipin 2 (*Plin2*) and fatty acid binding protein 4 (*Fabp4*), involved in fatty acid uptake and storage, was not significantly different in the comparisons of groups (Fig. 3B, 3D). Furthermore, transcripts of β-oxidation regulators (peroxisome proliferator activated receptor alpha*, Ppara,* and *Cpt1*) also did not show significant alterations, whereas genes required for *de novo* lipogenesis were upregulated in male offspring exposed to maternal HFD (Fig. 3B, D). These findings point to an imbalance between *de novo* lipogenesis and β-oxidation which could contribute to the excess fat accumulation in male liver. Similar sex-specific relationships were obtained for offspring at an earlier adult age of 5 months (Supplemental Fig. A.2C).

3.5. Sex-specific effects on adipose tissue expansion in adult offspring

As compared to mat-CD males, there was a considerably increased percentage of body fat (Fig. 2 E, F, p=0.002) and visceral fat mass in adult mat-CD females through life (Table 3, $p < 0.001$: ages 5 and 9 months), which consisted of adipocytes of larger size than in males (Fig. 4A). Similar results were obtained for subcutaneous fat mass (Table 3, $p < 0.01$: age 5 months, $p < 0.001$: age 9 months) and adipocyte size (Supplemental Fig. A.3). These findings represent a constitutive sex-specific difference being evident in the absence of any HFD exposure.

Upon maternal HFD exposure, the mean adipocyte size in visceral and subcutaneous adipose tissue of adult mat-HFD female offspring was significantly decreased compared to mat-CD females, even to lower levels than that of male adipocytes (Fig. 4A, Supplemental Fig. A.3A). In males, there was no such effect of maternal HFD on adipocytes, neither on mean size nor fat cell size distribution (Fig. 4A, B, Supplemental Fig. A.3A, B), whereas the adipocyte size distribution of the mat-HFD females showed a leftward shift confirming a smaller cell size in this group (Fig. 4C, Supplemental Fig. A.3C).

Less stored lipids, more apoptosis, or the emergence of smaller brown adipocytes [47] might explain these findings in adult mat-HFD females. We therefore measured transcripts indicative of induced cell death, brown adipose tissue, adipose mass and lipid droplet size regulation in visceral fat pads and found a significantly upregulated expression of genes relevant to lipid biosynthetic capacity by generating NADPH (malic enzyme 1, *Me1*) and acetyl-CoA (ATP citrate lyase, *Acly)* in mat-HFD females compared to their controls (Fig. 4D).

Next, we compared both mat-HFD offspring sexes and analyzed the results relative to the respective mat-CD controls (Fig. 4D, Supplemental Fig. A.4). Striking sex-specific expression differences between male and female mat-HFD offspring at age 9 months were identified for two key genes involved in lipid droplet size regulation: patatin-like phospholipase domain-containing protein 2 (*Pnpla2,* also known as *Atgl*), the rate-limiting enzyme of adipose tissue lipolysis and Berardinelli-Seip congenital lipodystrophy 2 (*Bscl2*, also known as s*eipin*), a regulator of lipid droplet formation [48] (Fig. 4D, Supplemental Fig. A.4). Higher mRNA expression levels in mat-HFD females than males were also measured for Bcl2-associated X protein (*Bax*), which codes for a protein involved in apoptotic signaling. Furthermore, analysis of adipocyte transcripts relevant to *de novo* lipogenesis revealed significant increases in *Fasn*, *Acaca* and *Me1* expression in mat-HFD females compared to mat-HFD males. Remarkably, none of them was differentially expressed between sexes in mat-CD offspring (Supplemental Fig. A.4A). ANOVA revealed that among those genes, expression of *Fasn, Me1, Bscl2* and *Pnpla2* was significantly affected by both sex and the interaction diet*sex, indicating an additional influence of the maternal HFD (Table 5). For *Bax*, a sex-specific expression pattern was only evident through the effects of the interaction diet*sex.

The sex-specificity of this gene expression pattern was even more pronounced in mat-HFD offspring at an earlier adult age of 5 months, and several additional genes were shown to be expressed in a sex-specific manner (Supplemental Fig. A.5): stearoyl-Coenzyme A desaturase 2 (*Scd2), Acly*, leptin (*Lep*), cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea)* and the mesoderm-specific transcript/imprinted paternally

expressed gene 1 (*Mest,* also known as *Peg1*). Among the analyzed genes, only *Acly* showed a differential expression between mat-HFD and mat-CD females, which persisted over time (Supplemental Fig. A.5; Fig. 4D).

4. Discussion

Our data provide evidence that obesogenic stress during the earliest developmental phases can be sufficient to induce a sex-specificity in health outcomes of offspring mice. We found the adverse influence on offspring outcome to be organ-specific, with manifestations at different times during life. In adulthood of male offspring, overweight, insulin resistance, hyperleptinemia, hyperuricemia and hepatic steatosis were observed; all these features not being noted in female offspring. Instead, those showed impaired fasting glucose suggesting a prediabetic condition and a reduction in fat mass with adipocytes of smaller size than in males. Females did not show any visceral fat as early as at 3 weeks of life, corroborating the hypothesis that this phenotype may be due to an early "primary" insult. These phenotypes were accompanied by an underlying HFD-induced sex-specific regulation of genes involved in processes determining fatty liver disease, lipid droplet size and fat mass expansion.

The exposure to the fetus was defined as an adverse maternal diet consisting of a higher saturated fat content, sugar-to-starch ratio, and total energy content than the control diet, which was associated with only a slightly lowered maternal food and protein consumption, whereas diet digestibility was kept similarly high. Based on emerging evidence on programming effects through peri-conceptional and early gestational nutritional influences [2, 3, 49, 50], we limited HFD exposure to this developmental period by fostering all pups to lean dams. Thereby, influences of the lactation phase, a period of developmental plasticity critical to programming of adiposity, energy balance and hypothalamic development [6, 7] were excluded. Furthermore, any subsequent postnatal obesogenic challenges were avoided during the offspring's lifetime. We are aware, however, that suckling of pups from obese dams on lean fosters and subsequent feeding with control diet may cause a disadvantageous mismatch between the pre- and postnatal environment that might influence offspring outcome via epigenetic mechanisms [51]. The process of fostering itself has recently been reported to lead to sex-specific cardiovascular and metabolic alterations and increased white adipose tissue accumulation specifically in males [52]. Thus, as we carried out fostering of all offspring of both the HFD-fed and CD-fed experimental group of dams, we

cannot completely exclude that an interaction between the prenatal maternal HFD and the "fostered" phenotype might have contributed to adult adiposity and metabolic phenotypes observed in our study.

NMRI outbred dams were chosen because we aimed at generating a model of mild diet-induced maternal adiposity that has less severe metabolic consequences than standard inbred strains to HFD [28] and thus shows closer correspondence to the considerable share of overweight and obese women without metabolic complications during pregnancy [53]. Although glucose tolerance of the HFD-fed dams was impaired in end-term pregnancy, fasting serum glucose and insulin levels were similar to controls and there was neither a difference in the insulin resistance index HOMA-IR nor in adipocytokine levels between the groups. HFD-fed dams had elevated serum NEFA levels and reactive oxygen species (H_2O_2) but a normal serum lipid profile and no signs of low-grade inflammation. They showed a microvesicular hepatic fat deposition, which only tended to be higher than in controls, in the absence of any signs of liver injury and inflammation. Pregnancy phenotypes of both NMRI and C57BL/6J dams showed similarities as to normoglycemia and unaffected lipid levels [25]. The NMRI phenotype, however, contrasts to diet-induced obese C57BL/6J mice in that these developed hyperinsulinemia and hyperleptinemia in late pregnancy. Despite the only few changes in the maternal metabolism of obese NMRI dams, those could be potentially relevant as mechanistic factors for adverse offspring outcome. Increased systemic NEFA levels, presumably arising from adipose tissue lipolysis, are transported through the placenta and might have an impact on fetal lipid exposure and regulation of gene expression [54]. Similarly, increased reactive oxygen species produced in the large adipose tissue mass of dams have been implicated in the development of offspring adiposity, possibly by enhancing adipogenesis [55].

Consistent with observations in humans [56], both male and female offspring of obese dams were found heavier at birth than offspring of lean dams. Increased expression of placental nutrient transporters and increased nutrient availability, as observed in HFD-fed pregnant C57BL/6 mice [24], might contribute to the higher placenta and fetal weights in the offspring

of HFD-fed NMRI dams. However, growth trajectories of the differently exposed offspring groups became aligned within a few days of life, excluding alterations in early postnatal growth velocity as a critical determinant of adult disease risk [57]. Notably, this also shows the absence of an immediate short-term effect of fostering on each of the offspring sexes.

Male mat-HFD offspring mice showed an adult onset of adiposity, whereas adult female mat-HFD offspring displayed a reduced fat mass already as early as at 3 weeks after birth and onwards. These findings were attributed to an increased deposition of subcutaneous fat in male and a diminished visceral fat depot in female offspring. Imbalances in energy regulation at the early onset of obesity are generally small requiring the precise measurement of energy flux in early life time [42]. We could not identify imbalances in energy regulation by monitoring energy expenditure and energy intake resulting in increased or decreased body fat. In late adult age, male offspring also showed insulin resistance, hyperinsulinemia, hyperleptinemia, and hyperuricemia. Comparable effects on male offspring have previously been shown only upon more pronounced obesogenic alterations of the peri-conceptional environment in rat models of maternal obesity [9, 10] and were most evident when male offspring were additionally overfed postnatally.

Distinct from our offspring outcomes, C57BL/6J mouse studies that prolonged the obesogenic exposure until the end of the lactation phase showed a broader spectrum of offspring features, including adiposity, hyperlipidemia, hyperinsulinemia, hyperleptinemia and glucose intolerance manifesting at an early age of 3 months [25, 58]. Studies in Wistar rats applying the same obesogenic exposure followed by weaning onto standard chow and investigating both offspring sexes separately revealed insulin resistance only in males of the same early age [16]. Comparison of offspring outcomes of rodents being lard-fed during pregnancy and lactation indicated that more adverse effects were confined to females than males, such as the development of a raised blood pressure in Sprague-Dawley rats [15] and adiposity in C57BL/6J mice [17]. Similar early and detrimental metabolic effects on female C57BL/6J mouse offspring were seen when offspring were exposed to a sucrose-rich maternal diet until weaning [59].

Notably, NMRI mat-HFD males did not show fasting hyperglycemia suggesting that the hyperinsulinemia observed was still sufficient to maintain normoglycemia. In contrast, mat-HFD females showed fasting hyperglycemia at the same late adult age of 9 months, which points to a more pronounced impairment of pancreatic islet function and to a prediabetic metabolic status at this advanced age. Thus, subsequent to a peri-conceptional obesogenic exposure, adult male NMRI mice appear to have greater compensatory capacities regarding glucose homeostasis than females, also reflected by the ANOVA results indicating an impact of the maternal adipogenic diet on the sex-specific differences in insulin and HOMA-IR. This finding is concordant with a significantly higher insulin secretion observed in human male adult offspring of obese mothers than in female offspring [23] and, as noted in adult mouse offspring of obese dams, an impaired beta-cell function only in females [19].

Furthermore, in male but not in female offspring, hepatic derangement was demonstrated morphologically by CT and accompanied by a mat-HFD induced upregulation of genes involved in the progression from steatosis to steatohepatitis and non-alcoholic fatty liver disease (NAFLD) development. This pattern of sex-linked liver gene regulation persisted throughout adulthood. Among those sex-specific genes induced in male offspring were nutrient-sensing mediators including *Pparg* (both isoforms) and *Srebf1* shown to be increased in human and mouse fatty livers [44, 45]. Such induction of nutrient-sensing mediators may facilitate triglyceride deposition in the liver, thereby serving an anti-lipotoxic function as a buffer for toxic lipid species, such as suggested for PPARG2 [60]. Additionally, the imbalanced gene expression we observed between upregulated *de novo* lipogenesis and unaltered fatty acid β-oxidation might have contributed to the steatotic hepatic changes identified only in male but not in female offspring. This sex difference also reflects epidemiological data in humans of male gender being a risk factor for NAFLD [61].

In addition to the differences in fat masses between sexes, we also observed a sex-specific constitutive variation in visceral and subcutaneous adipocyte size revealing larger cells in mat-CD females than in mat-CD males. This finding corroborates prior knowledge on known sex differences in the development of body composition and adipocyte size in mice and

humans [62, 63]. However, in view of the fat mass expansion we found in mat-HFD males, the contrary reduction in weight and adipocyte size of subcutaneous and visceral fat depots of mat-HFD female offspring mice was surprising. Those findings were accompanied by hyperleptinemia in mat-HFD males and, despite the very low fat mass, relatively high leptin levels in mat-HFD females, which might suggest an increased susceptibility towards leptin secretion subsequent to the peri-conceptional HFD exposure. This female adipocyte phenotype, already being evident in visceral fat at 3 weeks of life, is pointing to an early developmental origin and may suggest a retardation of fat mass expansion in female offspring. Several mechanisms and genes contributing to an abnormal adipocyte expandability have been proposed to induce the metabolic dysregulations associated with reduced fat depots [64]. Among these genes known to be involved in pathways influencing adipocyte size, we identified genes that showed a general (inherent) sex-specific regulation, which was additionally influenced by the adipogenic exposure, i.e. *Bscl2, Pnpla2, Fasn* and *Me1*.

The significant upregulation of *Bscl2* in mat-HFD female compared to male adipocytes is particularly interesting in the light of the recent finding that overexpression of the *Bscl2* gene in mice markedly reduced the mass of white adipose tissue and the size of adipocytes and lipid droplets [65]. In conjunction with the upregulation of *Pnpla2* coding for the adipose tissue triglyceride lipase PNPLA2, the key enzyme of lipolysis initiation [66], such differential expression might provide an additional mechanistic clue to explain the mat-HFD female adipocyte phenotype. While we found a significantly upregulated expression of *de novo* lipogenesis-related genes (*Fasn, Me1)*, also in comparison of mat-HFD and mat-CD females (*Acly, Me1)*, this does not necessarily reflect corresponding changes in enzymatic activity or metabolic flux. Upregulated gene expression might equally be an adaptive response to reduced *de novo* lipogenesis activity [67] as our offspring mice did not get any postnatal HFD challenge. Small adipocytes apparently have high lipogenic potential during the early stages of differentiation to initiate the process of lipid accumulation, whereas *de novo* lipogenesis is subsequently downregulated as adipocytes expand [68, 69].

A specific HFD-induced sexual dimorphic regulation was additionally identified for the pro-apoptotic signaling target BAX, a key regulator of mitochondrial outer membrane permeabilization and mediator of sensitivity to necrosis [70]. This suggests that apoptotic signals might additionally have contributed to the visceral adipose tissue phenotype of mat-HFD female offspring.

Taken together, these data are consistent with the hypothesis of an early obesogenic nutritional “hit” and developmental origin of sex-dependent susceptibilities towards adipose tissue formation. The placenta might play a crucial role in mediating such effects to the fetus and offspring. Previous studies report that feeding C57BL/6J mice a HFD during pregnancy leads to modified expression of imprinted genes and local or genome-wide DNA methylation in placenta and that these effects are sex-specific [18, 71]. One may speculate that the sex-dimorphic differences we observed in offspring adult outcome may represent the consequence and transgenerational fixation of detrimental dietary exposures on the placenta during the early prenatal period. Consistent with such a hypothesis is the important finding of this study that sex-specific adverse offspring outcomes occurred even in the absence of any postnatal HFD challenges.

The peri-conceptional obesogenic exposure in the NMRI dams resulted in rather mild metabolic complications of maternal adiposity. In humans, this would correspond to the commonly encountered gestational conditions of overweight or milder forms of obesity. Due to the lack of evidence on long-term health outcomes those conditions have not yet been included in recent clinical guidelines for preventive action to ameliorate obesity in adults or specifically in women of fertile age [72]. Nonetheless, at least in our rodent model, such an obesogenic environment during the earliest phases of development was sufficient to induce long-term adverse effects in offspring phenotypic outcomes and sex-dimorphic gene expression patterns. This indicates varying developmental vulnerabilities between sexes towards overnutrition.

Figure captions

Fig. 1: Maternal obesogenic environment before and during pregnancy. (A) Pre-pregnancy body weight and **(B)** body fat of the control diet (CD) group and high-fat, high-calorie diet (HFD) group of dams (n=7/group). ANOVA revealed significant effects of age ($p < 0.0001$) and the interaction diet*age ($p < 0.0001$) on body weight and significant effects of diet ($p < 0.05$), age ($p < 0.0001$) and diet*age ($p < 0.001$) on body fat. Differences between groups were analyzed by Tukey's post hoc test. **(C)** Intraperitoneal glucose tolerance test performed on gestational day 16.5 (n=5/group). ANOVA revealed significant effects of the diet ($p < 0.01$), time ($p < 0.0001$) and diet*time interaction ($p < 0.001$). Differences between groups were analyzed by Tukey's post hoc test. AUC = area under the curve of the HFD group versus CD group. **(D)** Maternal liver histology of animals of the HFD and CD group. Paraffin sections, H&E stain (left panel), cryosections, fat-red stain (right panel). Scale bars represent 100 µm. **(E)** Placenta weights and **(F)** body weight of fetuses from CD- and HFD-fed mice on gestational day 17.5 (E17.5) (n=81 CD /72 HFD). Mean ± s.e.m., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig. 2: Male and female offspring body composition. (A) Body weight in early postnatal life, **(C)** body weight until adulthood and **(E)** relative body fat of male offspring following peri-conceptional exposure to maternal control diet (mat-CD) or maternal high-fat, high-calorie diet (mat-HFD) (n=22 mat-CD/30 mat-HFD). ANOVA revealed a significant effect of diet for **(C)** ($p < 0.05$) and **(E)** ($p < 0.01$) and a significant effect of age ($p < 0.0001$) for **(A, C, E)**. A significant effect of the interaction diet*age was detected for **(A)** ($p < 0.01$) and **(C, E)** ($p < 0.0001$) and effect of mother for **(A, C)** ($p < 0.0001$) and **(E)** ($p < 0.01$). Significance of individual differences was tested by Tukey's post hoc test. **(B)** Body weight in early postnatal life, **(D)** body weight until adulthood and **(F)** relative body fat of female offspring of the mat-CD group and mat-HFD group (n=27 mat-CD/18 mat-HFD). ANOVA revealed a significant difference in the effect of age and mother ($p < 0.001$) for **(B, D, F)** and diet ($p < 0.01$) for **(F)** accompanied by a trend towards an effect of diet*age interaction ($p=0.069$) for **(F)**.

Differences between groups were tested by Tukey's post hoc test. Data are presented for a period up to 5 months of life, when mice became too large for analysis of body composition by the MRI analyzer (Minispec LF50; Bruker). The inserts **(A, B)** show significant differences in birth weight between mat-CD and mat-HFD male offspring (p=0.036) and female offspring (p=0.006), which vanished while they were foster-nursed by lean dams. Mean ± s.e.m., *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 3: Liver steatotic alterations in adulthood of male but not female offspring exposed to a peri-conceptional obesogenic milieu. (A) Liver computed tomography (CT) of male and female offspring following peri-conceptional exposure to maternal control diet (mat-CD; n=6 male/7 female) or maternal high-fat, high-calorie diet (mat-HFD; n=8 male/6 female) at the age of 5 months. Statistically significant differences in Hounsfield units among offspring groups are shown by different letters (p < 0.05); mean ± s.e.m. **(B, C, D)** mRNA expression of hepatic genes involved in *de novo* lipogenesis, fatty acid (FA) storage and transport, mitochondrial fatty acid β-oxidation, and steatosis regulation by glucocorticoid receptor (GR) signaling relative to the housekeeping gene *Ppia* of **(B)** male offspring (n= 11 mat-CD/13 mat-HFD), **(C)** mat-CD offspring (n=11 male/12 female) and **(D)** mat-HFD offspring (n=13 male/9 female) at the age of 9 months. Mean ± s.e.m., *p < 0.05, **p < 0.01. *Nr1h3* = also known as *Lxra; Nr3c1* = also known as *Gr*.

Fig. 4: Visceral fat cell size reduction in adult female but not male offspring following peri-conceptional obesogenic exposure. (A) Adipocyte size of visceral adipose tissue of male and female offspring following peri-conceptional exposure to maternal control diet (mat-CD) or maternal high-fat, high-calorie diet (mat-HFD) at the age of 9 months (n=5/group). Statistically significant differences among offspring groups are shown by different letters (p < 0.05); mean ± s.e.m. **(B, C)** Fat cell size distribution of visceral fat pads expressed as percentage of cells of male **(B)** and female **(C)** offspring at the age of 9 months (n=5/group, 50 adipocytes per animal). **(D)** mRNA expression of genes involved in lipid droplet size

regulation relative to the housekeeping gene *Ppia* in visceral adipose tissue of male (n=13) and female (n=9) mat-HFD offspring at the age of 9 months. Results are presented relative to the mean of the groups of male (n=11) and female (n=12) mat-CD offspring, respectively. Mean ± s.e.m.; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (statistically significant differences between male versus female mat-HFD offspring in relation to mat-CD offspring); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (statistically significant differences between mat-HFD versus mat-CD male and female offspring, respectively). *Mest* = also known as *Peg1*; *Bscl2* = also known as *seipin*; *Pnpla2* = also known as *Atgl*. Gene names not mentioned in the text: *Bcl2*, B cell leukemia/lymphoma 2; *Sfrp5*, secreted frizzled-related sequence protein 5.

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Table 1: Diet compositions.

	High fat diet (HFD)	Control diet (CD)	Maintenance diet (MD)
Crude nutrients (g/100g)			
Crude protein	24.1	24.1	19.3
Crude fat	34.0	5.1	3.4
Crude fiber	6.0	6.4	5.0
Crude ash	6.1	6.2	6.5
N free extracts	27.0	54.7	55.3
Myristic acid (C14:0)	1.03	0.06	0.01
Palmitic acid (C16:0)	8.06	0.70	0.47
Stearic acid (C18:0)	5.61	0.37	0.08
Oleic acid (C18:1)	12.13	1.44	0.62
Starch	2.2	26.0	37.5
Sugar / Dextrines	22.4	26.1	4.7
Energy (%) derived from			
Protein	19.0	27.0	33.0
Carbohydrates	21.0	60.0	58.0
Fat	60.0	13.0	9.0
Total energy (MJ/kg)	21.4	15.1	13.0

Single diet components are denoted in g per 100 g diet.
CD, control diet; HFD, high-fat, high-calorie diet; MD, maintenance diet; N, nitrogen

Table 2: Metabolic phenotyping. Food intake, movement activity, indirect calorimetry and rectal body temperature of mat-CD and mat-HFD offspring at the age of 5 months.

	Male		Female	
Parameter	**mat-CD** (n=7)	**mat-HFD** (n=7)	**mat-CD** (n=7)	**mat-HFD** (n=7)
Food intake (g)/mouse/day	6.2 ± 0.5	6.3 ± 0.3	4.6 ± 0.5	5.2 ± 0.3
Activity (counts x 100)	7.37 ± 3.0	8.79 ± 3.8	5.88 ± 3.3	8.41 ± 2.9
Respiratory Quotient (VCO_2/VO_2)	0.92 ± 0.01	0.90 ± 0.01	0.87 ± 0.01	0.87 ± 0.01
Metabolic rate (mW)	761.7 ± 25.9	850.0 ± 20.9	680.6 ± 18.6	756.2 ± 21.5
Body temperature (°C)	36.0 ± 0.2	35.9 ± 0.1	36.6 ± 0.2	36.5 ± 0.0

mat-CD, exposure to maternal control diet; mat-HFD, exposure to maternal high-fat, high-calorie diet; VCO_2, carbon dioxide production; VO_2, oxygen consumption. Data are presented as mean ± s.e.m.

Table 3: Absolute and relative organ weights of mat-CD and mat-HFD offspring at the ages of 3 weeks, 5 months and 9 months.

Parameter	3 weeks		5 months		9 months	
Male	mat-CD (n=5)	mat-HFD (n=5)	mat-CD (n=8)	mat-HFD (n=10)	mat-CD (n=21)	mat-HFD (n=29)
Body weight (g)	11.06 ± 0.49	10.92 ± 1.16	37.61 ± 0.94	39.24 ± 0.70	**52.79 ± 1.35**	**56.45 ± 1.08***
NRL (cm)	-	-	11.85 ± 0.13	11.56 ± 0.08	11.99 ± 0.08	12.08 ± 0.07
Liver (g)	0.44 ± 0.04	0.45 ± 0.60	1.50 ± 0.08	1.63 ± 0.03	2.12 ± 0.10	2.36 ± 0.09
Liver (g/g BW)	3.95 ± 0.25	4.10 ± 0.27	4.00 ± 0.18	4.16 ± 0.06	4.03 ± 0.12	4.17 ± 0.12
Visceral fat (g)	0.00 ± 0.00[#]	0.00 ± 0.00[#]	0.18 ± 0.04	0.27 ± 0.02	1.87 ± 0.14	1.98 ± 0.09
Visceral fat (g/g BW)	0.00 ± 0.00[#]	0.00 ± 0.00[#]	0.48 ± 0.09	0.68 ± 0.06	3.48 ± 0.21	3.49 ± 0.13
Subcutaneous fat (g)	0.11 ± 0.02	0.10 ± 0.04	0.49 ± 0.06	0.53 ± 0.02	**1.72 ± 0.13**	**2.99 ± 0.13*****
Subcutaneous fat (g/g BW)	0.96 ± 0.18	0.89 ± 0.29	1.30 ± 0.14	1.34 ± 0.06	**3.19 ± 0.19**	**5.26 ± 0.17*****
Scrotal fat (g)	0.03 ± 0.003	0.003 ± 0.008	0.07 ± 0.01	0.08 ± 0.01	2.26 ± 0.14	2.06 ± 0.10
Scrotal fat (g/g BW)	0.23 ± 0.03	0.23 ± 0.06	1.52 ± 0.22	1.84 ± 0.15	4.27 ± 0.25	3.69 ± 0.20
M. quadrizeps (mg)	0.13 ± 0.02	0.14 ± 0.01	0.54 ± 0.02	0.53 ± 0.02	248.12 ± 6.42	256.01 ± 6.96
M. quadrizeps (g/g BW)	1.14 ± 0.17	1.27 ± 0.06	1.44 ± 0.05	1.35 ± 0.04	0.47 ± 0.01	0.45 ± 0.01
Carcass (g)	4.42 ± 0.24	4.35 ± 0.44	15.82 ± 0.30	15.70 ± 0.34	**18.48 ± 0.33**	**20.06 ± 0.31****
Carcass (g/g BW)	40.0 ± 2.37	39.89 ± 0.53	**42.11 ± 0.43**	**40.00** ± **0.39****	35.22 ± 0.58	35.66 ± 0.38
Female	mat-CD (n=5)	mat-HFD (n=5)	mat-CD (n=9)	mat-HFD (n=10)	mat-CD (n=26)	mat-HFD (n=18)
Body weight (g)	10.86 ± 0.78	10.76 ± 0.70	**33.42 ± 1.00**	**30.26 ± 0.70***	53.20 ± 1.70	51.56 ± 2.38
NRL (cm)	-	-	11.17 ± 0.08	11.07 ± 0.11	11.87 ± 0.10	11.99 ± 0.10
Liver (g)	0.43 ± 0.06	0.46 ± 0.04	1.44 ± 0.05	1.31 ± 0.06	2.05 ± 0.10	2.21 ± 0.16
Liver (g/g BW)	3.98 ± 0.40	4.31 ± 0.30	4.30 ± 0.12	4.31 ± 0.14	**3.83 ± 0.11**	**4.25 ± 0.18***
Visceral fat (g)	**0.015 ± 0.008**	**0.00 ± 0.00[#]**	**1.38 ± 0.24**	**0.76 ± 0.09***	**9.14 ± 0.64**	**7.11 ± 0.60***
Visceral fat (g/g BW)	**0.137 ± 0.08**	**0.00 ± 0.00[#]**	4.06 ± 0.63	2.49 ± 0.28	**16.59 ± 0.80**	**13.18 ± 0.69****
Subcutaneous fat (g)	0.10 ± 0.03	0.08 ± 0.02	**1.33 ± 0.26**	**0.71 ± 0.07***	3.99 ± 0.24	3.51 ± 0.36
Subcutaneous fat (g/g BW)	0.97 ± 0.29	0.77 ± 0.18	**3.86 ± 0.68**	**2.33 ± 0.21***	7.35 ± 0.33	6.51 ± 0.50
M. quadrizeps (mg)	0.13 ± 0.01	0.14 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	211.53 ± 5.90	216.47 ± 7.70
M. quadrizeps (g/g BW)	1.22 ± 0.08	1.30 ± 0.06	1.23 ± 0.05	1.34 ± 0.05	0.40 ± 0.01	0.43 ± 0.02
Carcass (g)	4.42 ± 0.18	4.43 ± 0.44	13.31 ± 0.38	12.48 ± 0.30	16.78 ± 1.47	17.65 ± 2.11
Carcass (g/g BW)	40.78 ±	41.20 ±	39.88 ±	41.23 ±	**32.04 ±**	**34.84 ±**

	1.44	1.61	0.73	0.32	**0.70**	**0.89***

BW, body weight; mat-CD, exposure to maternal control diet; mat-HFD, exposure to maternal high-fat, high-calorie diet; NRL, nose-rump-length. Data are presented as mean ± s.e.m.; *p<0.05, **p<0.01, ***p<0.001. # Visceral fat was not detectable.

Table 4: Fasting serum analyses of mat-CD and mat-HFD offspring at the age of 9 months. The influence of the maternal diet, sex and the interaction of maternal diet*sex on serum parameters was analyzed, and *p*-values obtained using ANOVA are shown in the right part of the table. $p < 0.05$ was considered significant (designated in boldface type).

	Male		Female				
Parameter	mat-CD (n=10)	mat-HFD (n=13)	mat-CD (n=12)	mat-HFD (n=8)	Maternal diet	Sex	Maternal diet*sex
Glucose (mmol/l)	7.87 ± 0.75	8.78 ± 0.70	**5.40 ± 0.33**	**6.96 ± 0.32****	**0.0240**	**0.0006**	0.4360
Insulin (pmol/l)	**119.01 ± 16.99**	**276.62 ± 35.41****	144.92 ± 23.93	97.60 ± 18.12	0.1175	**0.0075**	**0.0006**
HOMA-IR[a]	**7.20 ± 1.24**	**17.58 ± 2.35****	5.89 ± 1.07	4.90 ± 0.90	**0.0203**	**0.0001**	**0.0026**
NEFA (mmol/l)	0.99 ± 0.05	1.05 ± 0.07	0.98 ± 0.07	1.02 ± 0.09	0.5416	0.7714	0.9061
Triglycerides (mmol/l)	1.41 ± 0.15	1.20 ± 0.11	1.80 ± 0.21	1.74 ± 0.18	0.4756	**0.0105**	0.5912
Cholesterol (mmol/l)	4.21 ± 0.35	4.61 ± 0.27	2.59 ± 0.31	3.01 ± 0.13	0.1251	**<0.0001**	0.7259
LDL cholesterol (mmol/l)	0.43 ± 0.03	0.44 ± 0.03	0.32 ± 0.04	0.37 ± 0.01	0.2686	**0.0144**	0.4293
HDL cholesterol (mmol/l)	3.42 ± 0.26	3.79 ± 0.22	1.92 ± 0.22	2.32 ± 0.11	0.0637	**<0.0001**	0.6799
LDL/HDL ratio	0.13 ± 0.01	0.12 ± 0.005	0.17 ± 0.01	0.16 ± 0.01	0.2679	**<0.0001**	0.6166
Leptin (ng/ml)	**14.27 ± 2.99**	**22.19 ± 1.56***	24.81 ± 4.13	19.47 ± 4.85	0.6014	0.5041	**0.0394**
HMW adiponectin (µg/ml)	10.26 ± 1.78	7.65 ± 0.84	20.87 ± 1.93	19.89 ± 2.03	0.2274	**<0.0001**	0.4703
HMW/total adiponectin ratio	0.76 ± 0.26 (n=5)	0.32 ± 0.04 (n=5)	0.48 ± 0.01 (n=5)	0.51 ± 0.04 (n=5)	0.1192	0.9160	0.0809
Uric acid (µmol/l)	**95.91 ± 8.24**	**144.81 ± 18.09****	72.00 ± 4.63	88.21 ± 9.37	**0.0074**	**0.0008**	0.2360
Urea (mmol/l)	8.37 ± 0.26	8.47 ± 0.40	**6.60 ± 0.37**	**5.22 ± 0.41***	0.0555	**<0.0001**	**0.0393**
Creatine kinase (U/l)	615.78 ± 80.16	628.31 ± 119.29	753.67 ± 71.98	719.50 ± 155.95	0.6186	0.3125	0.7633
Alanine transaminase (U/l)	58.80 ± 25.49	59.85 ± 14.04	35.17 ± 2.39	38.50 ± 5.12	0.6278	0.1416	0.8456
Aspartate transaminase (U/l)	148.80 ± 23.16	146.92 ± 21.15	139.50 ± 8.40	166.50 ± 19.71	0.5382	0.5896	0.4646
H_2O_2 (nmol/ml)	64.69 ± 4.18	59.99 ± 3.11	66.71 ± 3.07	63.52 ± 4.26	0.3066	0.4250	0.8620
TBARS (nmol MDA/ml)	3.23 ± 0.12	3.48 ± 0.23	2.54 ± 0.26	2.76 ± 0.29	0.7396	**0.0160**	0.4792
C-reactive protein (mg/l)	24.40 ± 6.50	27.23 ± 6.14	18.67 ± 5.44	13.50 ± 1.35	0.8175	0.0563	0.7770

HMW adiponectin, high-molecular-weight adiponectin; HOMA-IR, homeostatic model assessment of insulin resistance; mat-CD, exposure to maternal control diet; mat-HFD, exposure to maternal high-fat, high-calorie diet; MDA, malondialdehyde; NEFA, non-esterified fatty acids; TBARS, thiobarbituric acid-reactive substances. Data are presented as mean ± s.e.m.; *p<0.05, **p<0.01. [a] HOMA-IR = [fasting insulin (µU/ml) x fasting glucose (mmol/l)]/22.5

Table 5: Influence of the maternal diet, sex and the interaction of maternal diet*sex on offspring gene expression levels in the liver and visceral adipose tissue at the age of 9 months. *p*-values obtained using ANOVA are shown. $p < 0.05$ was considered significant (designated in boldface type).

		Age 9 months		
	Genes	Maternal diet	Sex	Maternal diet*sex
Liver tissue	*Nr1h3*	**0.0025**	0.8542	0.8308
	Srebf1	0.0740	0.0506	0.0980
	Fasn	**0.0082**	0.1259	0.1011
	Acaca	0.0533	0.7464	0.7498
	Pparg1	0.9606	**0.0137**	**0.0205**
	Pparg2	**0.0362**	**0.0051**	**0.0084**
	Cd36	0.4150	0.1749	0.2209
	Fabp4	0.4631	0.3302	0.3037
	Plin2	**0.0235**	0.9699	0.9831
	Ppara	0.9240	0.9608	0.8520
	Cpt1	0.5860	0.7605	0.9821
	Nr3c1	**0.0081**	0.4901	0.4402
	Hes1	0.1906	0.3315	0.1752
Visceral adipose tissue	*Pparg2*	0.6694	0.6460	0.4903
	Fasn	0.5665	**0.0144**	**0.0121**
	Acaca	0.5900	0.1979	0.1426
	Scd2	0.1404	0.3466	0.6589
	Me1	0.9944	**0.0355**	**0.0109**
	Acly	**0.0367**	0.3609	0.4089
	Bax	0.8084	0.0722	**0.0372**
	Bcl2	0.6038	0.2920	0.1990
	Mest	0.2245	0.0837	0.0977
	Sfrp5	0.1925	0.2237	0.4403
	Bscl2	0.9581	**0.0342**	**0.0208**
	Pnpla2	0.0868	**0.0017**	**0.0010**
	Cidea	0.8331	0.9598	0.3733
	Lep	0.5093	0.0708	0.4108

Acaca, acetyl-Coenzyme A carboxylase 1; *Acly*, ATP citrate lyase; *Bax*, Bcl2-associated X protein; *Bcl2*, B cell leukemia/lymphoma 2; *Bscl2,* Berardinelli-Seip congenital lipodystrophy 2 (also known as *seipin)*; *Cd36*, Cd36 antigen; *Cidea*, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A; *Cpt1*, carnitine palmitoyltransferase 1; *Fabp4*, fatty acid binding protein 4; *Fasn*, fatty acid synthase; *Hes1*, hairy and enhancer of split 1; *Lep,* leptin; *Me1,* malic enzyme 1; *Mest,* mesoderm-specific transcript/imprinted paternally expressed gene 1 (also known as *Peg1*); *Nr3c1*, nuclear receptor subfamily 3, group C, member 1 (also known as *Gr,* glucocorticoid receptor*)*; *Nr1h3*, nuclear receptor subfamily 1, group H, member 3 (also known as *Lxra,* liver X receptor alpha); *Plin2*, perilipin 2; *Pnpla2*, patatin-like phospholipase domain-containing protein 2 (also known as *Atgl*); *Ppara*, peroxisome proliferator activated receptor alpha; *Pparg*, peroxisome proliferator activated receptor gamma; *Scd2*, stearoyl-Coenzyme A desaturase 2; *Sfrp5*, secreted frizzled-related sequence protein 5; *Srebf1*, sterol regulatory element binding transcription factor 1.

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Highlights

- Peri-conceptional obesity induces programming of mouse offspring health risks.
- Adult offspring disease occurs despite excluding lactational and postnatal exposure.
- Offspring outcomes including adipose tissue expansion are strikingly sex-specific.
- Early developmental exposure is sufficient to induce sex-specific gene regulation.

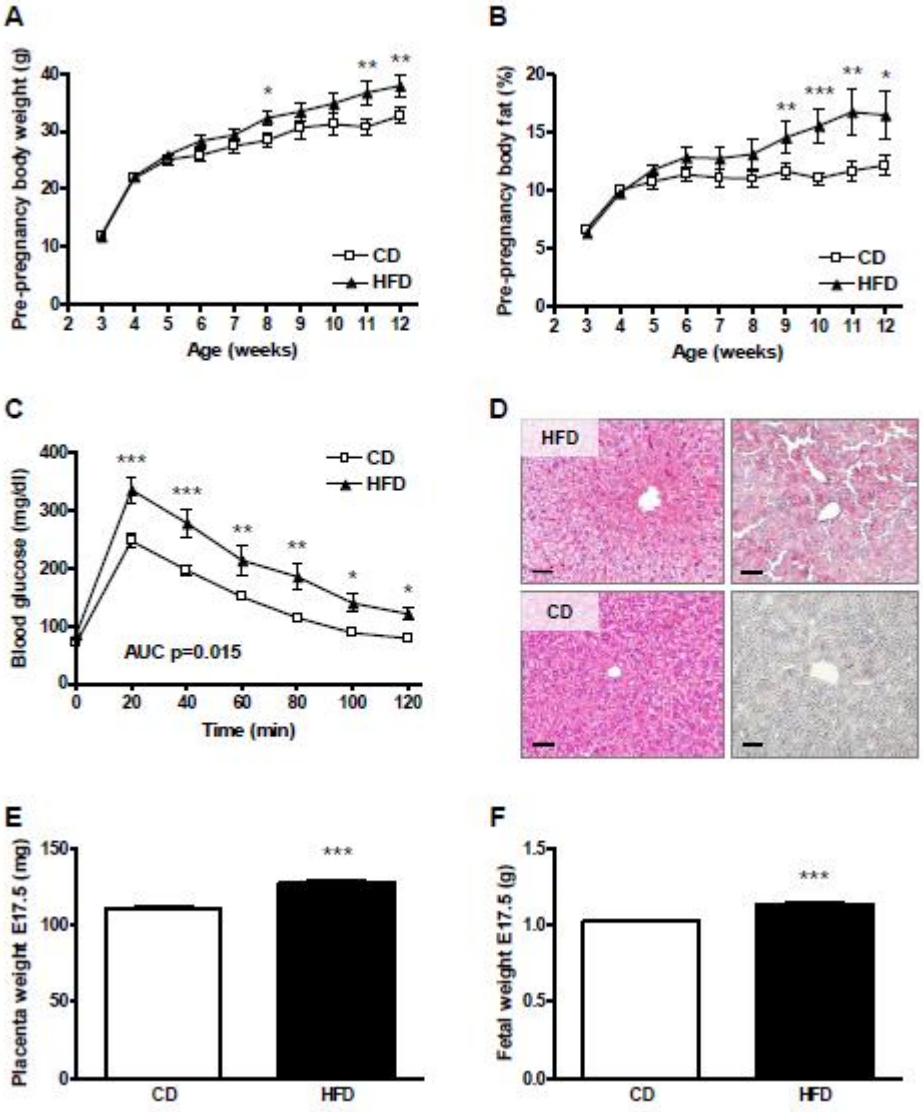

Figure 1

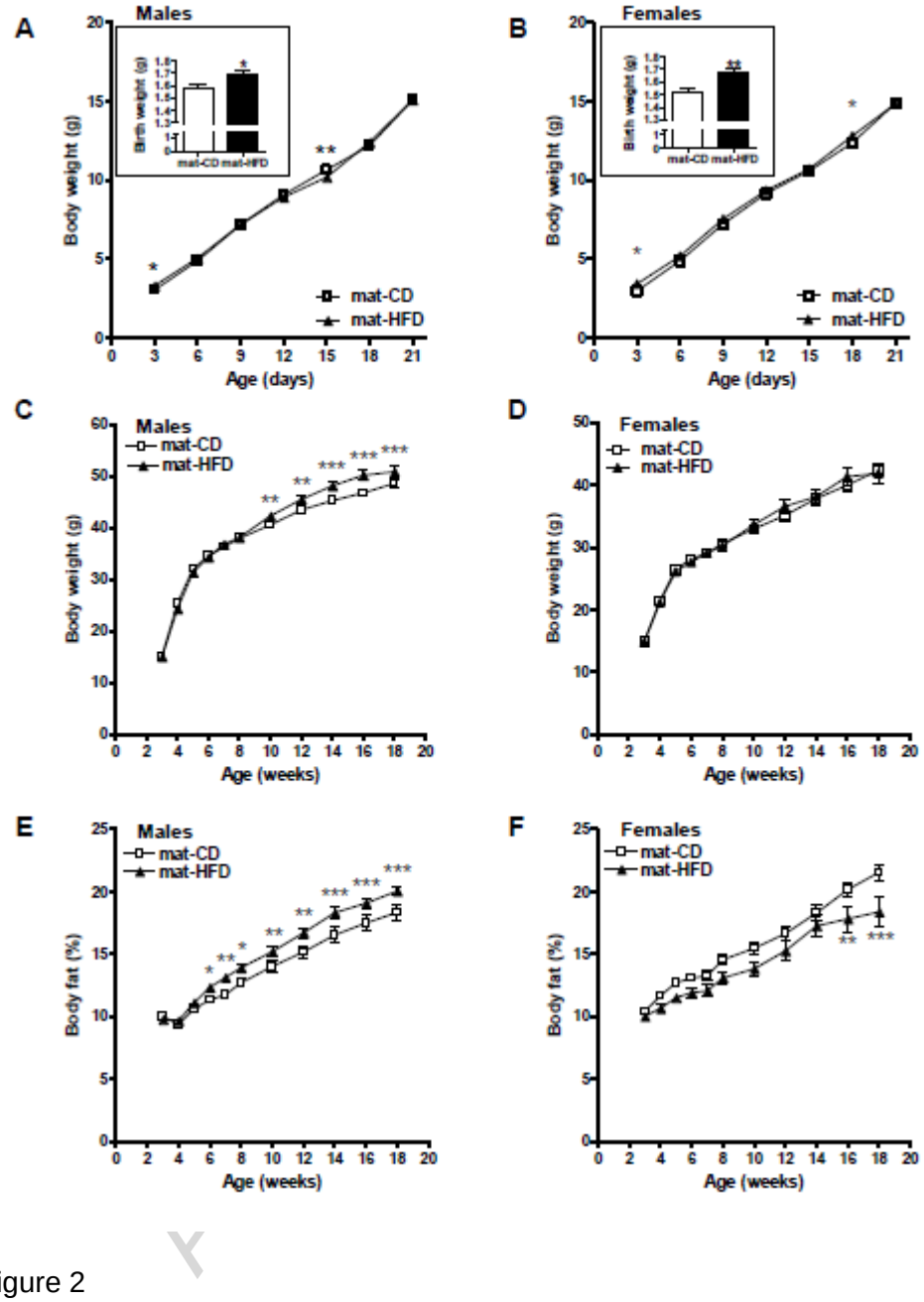

Figure 2

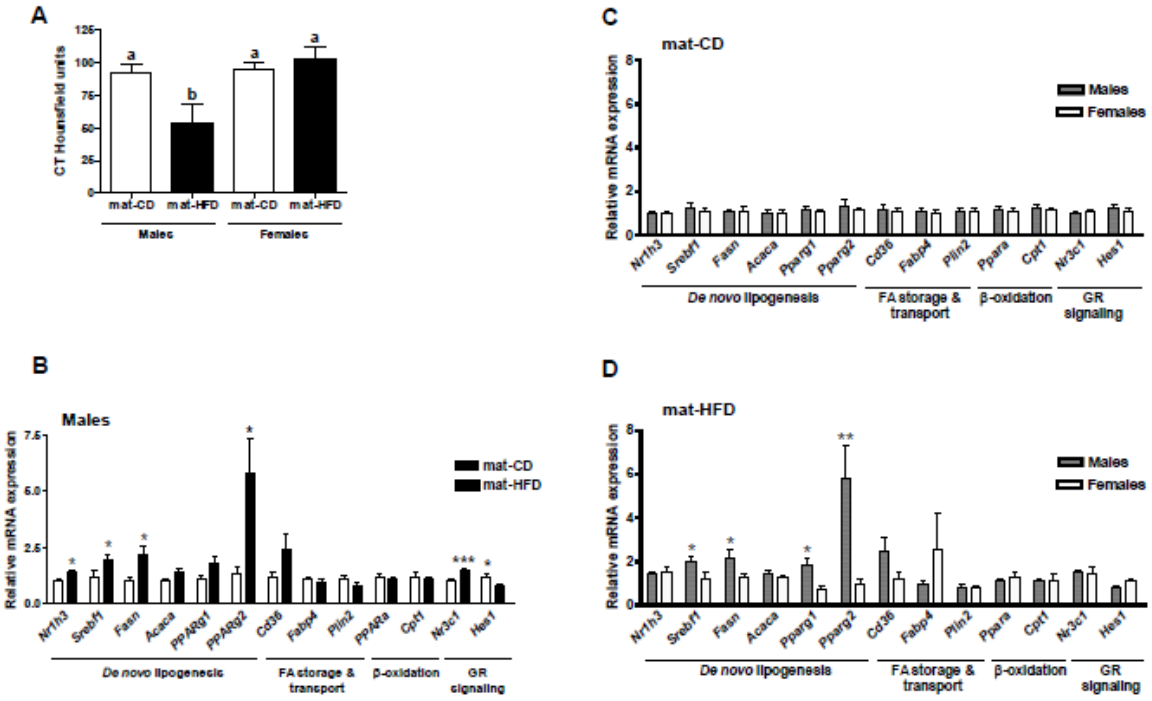

Figure 3

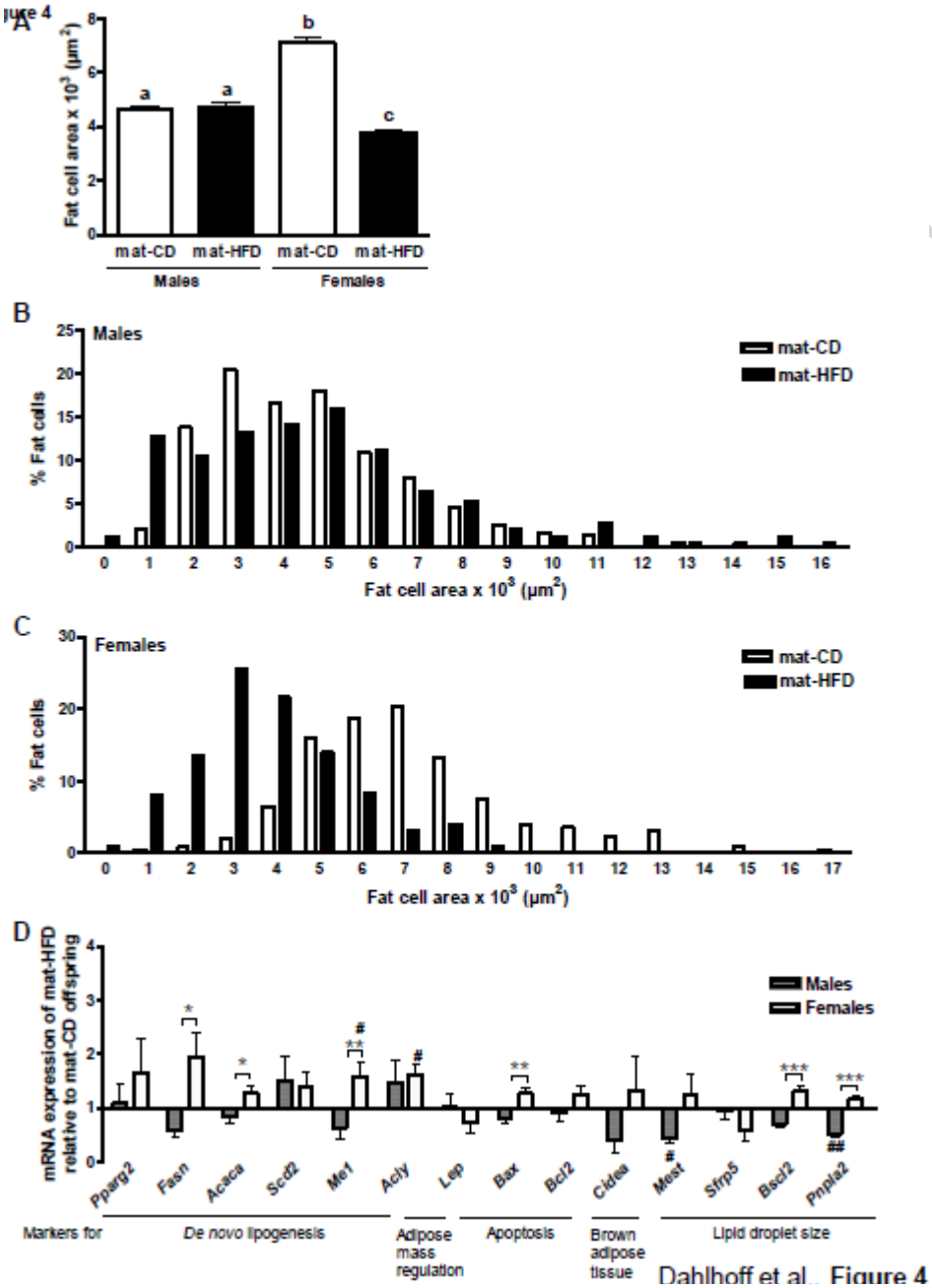

Figure 4