



Prenatal maternal vitamin D deficiency sex-dependently programs adipose tissue metabolism and energy homeostasis in offspring

Eva Seipelt, Franck Tourniaire, Charlène Couturier, Julien Astier, Béatrice B. Loriod, Hortense Vachon, Michel Puceat, Lourdes Mounien, Jean-Francois Landrier

► To cite this version:

Eva Seipelt, Franck Tourniaire, Charlène Couturier, Julien Astier, Béatrice B. Loriod, et al.. Prenatal maternal vitamin D deficiency sex-dependently programs adipose tissue metabolism and energy homeostasis in offspring. *FASEB Journal*, 2020, 34 (11), pp.14905-14919. 10.1096/fj.201902924RR . hal-03156949

HAL Id: hal-03156949

<https://hal.inrae.fr/hal-03156949>

Submitted on 20 May 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Prenatal maternal vitamin D deficiency sex-dependently programs adipose tissue metabolism and energy homeostasis in offspring

Eva M. Seipelt^{1,4}, Franck Tourniaire^{1,2}, Charlène Couturier¹, Julien Astier¹, Béatrice Lloriod³, Hortense Vachon³, Michel Pucéat^{4*}, Lourdes Mounien^{1*}, Jean-François Landrier^{1,2*}

1 Aix-Marseille Université, C2VN, INRAE, INSERM, 13000, Marseille, France

2 CriBioM, Criblage Biologique Marseille, Faculté de médecine de la Timone, Marseille, France.

3 Aix-Marseille Université, TGML, TAGC, INSERM, 13000 Marseille, France

4 Aix-Marseille Université, MMG, INSERM U1251, Marseille, France.

* joint last authors.

Short running title : maternal vitamin D programs offspring metabolism

Corresponding author and person to whom reprint requests should be addressed:

Jean-François Landrier, C2VN ; UMR 1260 INRAE/1263 INSERM/Université d'Aix-Marseille, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France. Phone: +33 4 91 29 42 75; E-mail: jean-francois.landrier@univ-amu.fr

25 **List of nonstandard abbreviations :**

26

27 25(OH)D: 25-hydroxyvitamin D

28 AGRP: Agouti related-peptide

29 AUC: Area under the curve

30 CTRL: Control

31 EE: Energy expenditure

32 GOX: Carbohydrate oxidation

33 HF: High fat

34 HOMA-IR: Homeostatic model assessment of insulin resistance

35 IPA: Ingenuity pathway analysis

36 ITT: Insulin tolerance test

37 LF: Low fat

38 LOX: Lipid oxidation

39 MC4R: Melanocortin receptor type 4

40 NPY: Neuropeptide Y

41 RQ: Respiratory quotient

42 VDD: Vitamin D deficiency

43

Abstract

In utero environment is crucial to ensure normal development of the foetus and to program metabolic health throughout the life. Beside macronutrients, the role of micronutrients, including vitamin D, begins to be explore. The aim of this study was to decipher the impact of maternal vitamin D deficiency (VDD), in normal and high fat (HF) diet context, on adipose tissue metabolism and energy homeostasis in offspring, considering sex-specific responses. Body weight, energy expenditure and spontaneous activity was differential impacted in juvenile male and female offspring born from VDD mice. In adulthood, a HF diet combined with maternal VDD disrupted glucose homeostasis and adiposity in male offspring but not in females. Such phenotypes were associated to different transcriptomic profiles in adipose tissue, that could be related to differential modulation of plasma 17 β -estradiol concentrations. Thus, maternal VDD sex-dependently modulated metabolic fate of the offspring, especially when associated with HF diet in adulthood.

Keywords: Vitamin D, Maternal vitamin D deficiency, offspring, adipose tissue, metabolism.

Introduction

Vitamin D is an essential micronutrient that is suspected to display an important role on normal foetal development, and throughout the life of the child and future adult. Its role in calcium and phosphate metabolism is well-established and it also appears to be involved in many other physiological processes¹, including control of adipose tissue biology²⁻⁹. Consequently, vitamin D insufficiency is considered as a risk factor in several pathologies, including auto-immune diseases, musculoskeletal defects¹⁰, cardiovascular and metabolic diseases. Such insufficiency is characterised by plasma concentration in 25-hydroxyvitamin D (25(OH)D) below the cut-off value of 50nmol/L¹¹. In fact, vitamin D insufficiency has become a large public health issue^{12,13} and is common for women in child bearing age, pregnant and breastfeeding women. Currently, 54% of pregnant women and 75% of newborns present a 25(OH)D status < 50nmol/L, also 18% of pregnant women and 29% of newborns present a severe vitamin D deficiency (25(OH)D < 25nmol/L)^{14,15}. It is noteworthy that maternal vitamin D deficiency (VDD) was associated with pregnancy, foetal and neonatal outcomes¹⁵ such as increased risk factor for preeclampsia¹⁶, gestational diabetes mellitus¹⁷, higher risk of small-for-gestational-age, reduced term birth weight, and lower head circumference¹⁸, even if these results are sometimes controverted¹⁹.

Interestingly, recent studies linked maternal vitamin D insufficiency to overweight and increased fat mass in offspring. Indeed, in the INMA cohort, including 2358 pregnant women, maternal VDD was associated with increased risk of overweight in foetal and early postnatal (1-year-old) offspring²⁰. Similarly, in the prospective cohort study Southampton Women's survey (977 pregnant women), maternal VDD was associated with lower fat mass at birth and greater fat mass at 4 and 6 years²¹. These results were confirmed in the prospective cohort Rhea (532 pregnant women), where the maternal 25(OH)D concentrations < 37.7 nmol/L were associated with higher body mass index and central adiposity in the 4 and 6-years-old offspring²². Nevertheless, in a long term follow up (20 years), no association between maternal vitamin D status and cardio-metabolic risk factors was highlighted²³. Thus, those associations remain controversial.

To gain further insight on the relationship, several preclinical studies have been implemented. Overall, it has been established that maternal VDD as potential programming long-term effect on metabolic health^{24,25}, nevertheless several important points remain pending. Based on the Developmental origins of Health and disease (DOHaD concept), we hypothesized that the response to prenatal maternal VDD may be sex-specific and influenced by the nutritional

environment during adulthood. Indeed, the maternal environment during the pre- and periconceptional period, especially nutrition, can modify epigenetic marks, leading to long term phenotypic consequences that may vary according to the sex and the environment of the offspring²⁶.

Thus, the aim of the present study is to determine the impact of maternal VDD combined with the impact of obesogenic environment, induced by a high-fat diet, on the sex-specific response during adulthood on energy homeostasis and adipose tissue metabolism.

Material and Methods

Animal Experiments

The protocol received the agreement of Aix-Marseille University Ethics Committee and the French Ministry of Research (APAFIS#1300-2015072112279135). Eight-week-old female and male C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), fed ad libitum with control food (chow diet A04 from Safe-diets, Augy France) during the 1-week acclimation period and with full access to drinking water. The animals were maintained at 22°C under a 12-hour light, 12-hour dark cycle and a 20% humidity level. Female mice (15 per group) were randomly assigned into one of the two experimental groups depending on the diet *i.e.* control (AIN-93G with vitamin D3, 1.0 IU/g) or vitamin D-depleted (AIN-93G without vitamin D3, 0.0 IU/g) for eight-weeks (Supplemental Figure 1), and were mated with males. Weight gain was measured once a week and dietary at 3-weeks of pre-mate diet, at 5 days and 15 days of gestational stage (Supplemental Figure 2). After delivery, all females were fed with control diet (AIN-93G) until weaning of the offspring. The litter size was adjusted to 6 pups per females. The body weight of the offspring was evaluated weekly from the weaning until the study end, and not prior weaning to avoid maternal cannibalization and perinatal stress. At six-weeks of age both males and females of the offspring were randomly assigned to receive Low Fat diet (AIN-93M Maintenance Purified Diet) or High Fat diet (DIO Rodent Purified Diet w/45% Energy from Fat) for eight weeks. At the end of the protocol, mice were subjected to food restriction overnight and blood was collected by cardiac puncture anesthesia, serum was isolated by centrifugation at 3000 rpm for 15 min at 4°C and was stored at -80°C. Animals were euthanized by cervical dislocation and various tissue (liver, spleen, hypothalamus and various white adipose tissue deposits) were collected, weighted and stored at -80°C. Eight groups of offspring mice (males and females) were designed to study the impact of maternal diet (CTRL vs VDD) and adult diet (LF vs HF).

Biochemical analysis

To confirm the maternal and offspring vitamin D status, 25(OH)D serum concentration were measured using an in vitro diagnostic enzyme immunoassay kit 25-OH Vitamin D (direct) ELISA kit (PromoKine). Insulin, leptin, testosterone and 17 β -estradiol were measured in plasma using an enzyme-linked immuno-sorbent assay ELISA (Insulin ALPCO Diagnostics, New Hampshire, United States; DuoSet mouse leptin, R&D systems, Minneapolis, United States, Testosterone Demeditec Diagnostics GmbH, Germany; ab108667, Abcam, Cambridge,

England, respectively). The manufacturer's protocols were followed. For glucose concentration, mice were subjected to food restriction for 5h and glycemia was measured from tail blood (Accu-Check glucometer, Roche). The HOMA-IR index was calculated according to the following formula: fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5.

Histological analysis

Visceral adipose tissue samples were fixed in 10% buffered formalin, embedded in paraffin and sliced to prepare 5 μ m tissue sections whose were stained with hematoxylin and eosin (H&E). The images were captured by a light microscope (EZAD, Leica, Germany; 10X magnification). The adipocyte area (μ m²) were determined using (Image J) software.

Indirect calorimetry

At 5 and 12 weeks old the offspring was acclimated and kept for 24h in an indirect calorimetric cage (Physiocage, Bioseb, Vitrolles, France), as previously described⁶. The calorimetric appliance was composed of a gas analyzer (to measure O₂ consumption and CO₂ production as VO₂ and VCO₂) and an activity recorder (locomotion and rearing). The temperature of the calorimetric room was set to 22°C. Four were connected to each gas analyzer, but each cage had specific inlets and outlets. A constant inlet flow (5 cm³/min) was maintained throughout the experiment. Gases were continuously analyzed with the following sequence: 3 min from cage 1, 3 min from cage 2, 3 minutes from cage 3, 3 minutes from cage 4 and then 3 min from room air and thus the volume (mL/min) of O₂ consumed (VO₂) and CO₂ produced (VCO₂) were measured for each mouse. Energy expenditure (EE) was calculated as following (EE= (16.3× VO₂ + 4.57× VCO₂)/60 (watt)). Lipid (LOX) and carbohydrate (GOX) oxidation were calculated according to the following equations: LOX = (1.69×VO₂ - 1.69×VCO₂)×(9.46×4.186/60) (watt) and GOX = (4.57×VCO₂ - 3.23×VO₂)×(3.74×4.186/60) (watt)²⁷. Total activity was evaluated by summing spontaneous activity and rearing activity, measured in indirect calorimetric cages (Physiocage, Bioseb, Vitrolles, France), and was normalized to the control value.

Insulin Tolerance Test (ITT)

One week before the end of the protocol, mice were subjected to ITT. Mice were subjected to food restriction for 5h and stuffed with an insulin solution (0.05 UI/mL) prepared in saline

(0.5 UI/ kg body weight). Glycemia was measured from tail blood at 0, 15, 30, 60, 90, 120 minutes after injection (Accu-Check glucometer, Roche Diagnostic, Meylan, France).

RNA extraction real time PCR and RNA sequencing

Total RNA was extracted from retroperitoneal adipose tissue or hypothalamus using TRIzol reagent (Thermo Fischer Scientific, Les Ulis, France). For the real time PCR, one µg of total RNA from hypothalamus was used to synthesize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Thermo Fischer Scientific, Les Ulis, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, USA) as previously described²⁸. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method²⁹.

The sequences of the primers used in this study are reported in supplemental data (Supplemental Table 1).

For the RNA sequencing, total RNA was isolated from 3 mice per group and was used for the RNA-seq library preparation, using the kit TruSeq Stranded mRNA by Illumina.

Libraries were paired-end sequenced on the Illumina NextSeq 500 sequencer. Reads with a phred score lower than 20 and shorter than 25 bp were removed using Sickle (v1.33). Quality of trim reads were checked using multiQC (v1.0). Trim reads were aligned using STAR aligner (v2.7.0d) with arguments “outFilterMismatchNoverLmax” and “outFilterMultimapNmax” set to 0.08 and 1, respectively.

Transcripts discovery was performed using Cufflinks (v2.2.1) with the “library-type” argument set to fr-firststrand, and a GTF file obtained from GENCODE (“Comprehensive gene annotation”, vM1) provided as the genomic annotation. The GTF files produced for each sample by Cufflinks were combined using Cuffmerge. The “class code” assigned to each transcript by Cuffmerge was used to defined unknown transcripts (class code “u”). Only de novo transcripts with counts greater than 0 in at least one RNA-seq sample were kept for subsequent analyses. These de novo transcripts were combined with the GENCODE GTF file to produce the final genomic annotation that was provided to FeatureCounts (v1.6.1) for quantification.

Differential gene expression was performed using DESEQ2 between conditions. To create bigwig files, reads from Watson and Crick strands were selected using SAMtools (v1.9) and provided to the bam2wig.py script from the RseQC program suite (v2.6.4). RNA-seq profiles were visualized using the IGV genome browser.

204

205 ***Ingenuity Pathway Analysis (IPA)***

206 Differential gene expression (with p-value adjusted < 0.05), obtained from the RNA seq
207 analysis between our conditions, were used in the IPA software to identify the canonical
208 pathways differentially impacted by the maternal diet.

209

210 ***Statistical analysis***

211 Data are expressed as mean \pm SEM. Significant differences were determined by unpaired
212 Student's *t* test or by ANOVA followed by the Fisher's LSD post hoc test using GraphPad
213 Prism. $p < 0.05$ was considered to be statistically significant.

214

215

Results

Maternal vitamin D deficiency affects post-weaning energy metabolism of offspring.

Maternal VDD diet consumption prior and during gestation led to significant smaller post-weaning (5 weeks old) body weight in male mice offspring (Fig.1A), that was non-significant in female offspring (Fig.1H). The food consumption remained the same between CTRL and VDD, either for males or females (Fig. 1B, 1I). In VDD males, spontaneous activity, respiratory quotient (RQ) and energy expenditure (EE) were increased (Fig. 1C, D, E) compared with the control group ($p < 0.05$). No difference of lipid oxidation (LOX) was noticed in male (Fig. 1F), but carbohydrate oxidation (GOX) was increased in VDD males (Fig. 1G). For female, despite the increased activity and RQ (Fig. 1J, K) of the VDD group, there was no statistical difference for the EE (Fig. 1L). This was associated with decreased LOX and increased GOX (Fig. 1M, N).

Maternal vitamin D deficiency combined with HF diet affects energy metabolism in adult offspring.

To explore the combined effect of maternal VDD and post-weaning nutrition, mice were submitted to low fat (LF) or high fat (HF) diet, leading thus to 8 groups (CTRL-LF, CTRL-HF, VDD-LF and VDD-HF, for both male and female). Firstly, plasma 25(OH)D concentration was measured in 6 weeks old offspring. Only VDD females presented decreased 25(OH)D values ($108.6\text{nmol/L} \pm 2.852$) compared with the CTRL ($128.9\text{nmol/L} \pm 6.186$; $p < 0.01$) (Fig. 2A, B). During the diet period (from week 6 to 12), no statistical difference between CTRL-LF and VDD-LF males body weight was observed (except at 9 weeks old, $p < 0.05$; Fig. 2C). Under HF diet, the body weight of CTRL males was gradually higher than VDD males (significant from week 9 to 12; Fig. 2C). Concerning females, there was non-significant differences between CTRL-LF and VDD-LF (from week 6 to 12). Under HF diet, the CTRL females showed increased body weight compared with the VDD (significant from week 9 to 12; Fig. 2D).

The energy metabolism was explored by indirect calorimetry (performed at 12-weeks of age). No modification of the energy metabolism of VDD offspring (*i.e.* body weight, activity, RQ, EE, LOX and GOX) was observed compared with the CTRL offspring when exposed to LF diet in adulthood, either for male and female (Supplemental Figure 3). Under HF diet, energy metabolism of VDD offspring was disturbed when compared with CTRL (Fig. 2). Males exposed to VDD associated-HF diet displayed a smaller body weight than CTRL-HF ($p <$

0.05) and increased food consumption, EE and GOX ($p < 0.05$) (Fig. 2E, F, I, J). No modification of activity, RQ and LOX was observed in males. Female exposed to VDD-HF diets also exhibited smaller body weight than CTRL-HF (Fig. 2L). The food intake tended to be increased (non-significant) in the VDD-HF compared with the CTRL-HF, whereas the activity decreased and EE and GOX increased ($p < 0.05$) (Fig. 2M, N, P, R). No modification of RQ and LOX was observed in females.

In order to explain the disruption of feeding behaviour in VDD group, we investigated the expression of the genes of hypothalamic melanocortin pathway known to be involved in the regulation of food intake³⁰. For males, exposed to LF diet in adulthood, agouti related-peptide (AGRP) and melanocortin receptor type 4 (MC4R) were under-expressed in the VDD compare to the CTRL (Supplemental Figure 4A). No statistical differences were observed in HF condition (Supplemental Figure 4B). For females in VDD and CTRL-LF there was not statistical difference (Supplemental Figure 4C). Under HF diet, Neuropeptide Y (NPY) and AGRP were overexpressed ($p < 0.05$) and MC4R under-expressed in VDD-HF compare to CTRL HF (Supplemental Figure 4D). There is no difference in POMC expression between the different groups (Supplemental Figure 4A-D).

In order to explain energy metabolism sex discrepancies, the 17β -estradiol and testosterone plasma concentrations were evaluated in all groups (Table 1). No significant modification was observed in male groups (CTRL-LF, CTRL-HF, VDD-LF, VDD-HF) for 17β -estradiol and testosterone. In females, no difference was observed in CTRL condition, whereas in VDD condition, 17β -estradiol was strongly increased under HF diet (Table 1). No modification of testosterone was observed.

Maternal VDD and HF diet-associated affect glucose homeostasis sex-specifically

Biochemical analysis of male offspring highlighted that the glycemia tended to be higher in both CTRL-HF and VDD-HF groups when compared with LF groups (performed at 13 weeks; Fig. 3A). Similar pattern was observed for plasma insulin, with an increased concentration for the VDD-HF compared with the CTRL-HF ($p < 0.05$) (Fig. 3B). Thus, the VDD-HF males presented the higher values of plasma insulin, glycemia and HOMA-IR when compared with CTRL-HF, and to CTRL-LF and VDD-LF males (Fig. 3C, D).

When challenged with an HF diet, the glycemia of the VDD female tended to be decreased (nonsignificant) compared with the CTRL-HF group to reach the same values as LF groups (Fig. 3G), and plasma insulin of VDD female (VDD-HF) decreased compared with the control group (CTRL-HF) ($p < 0.05$) to reach the same values as LF groups (both CTRL-LF

and VDD-HF) (Fig. 3H). The CTRL-HF females displayed higher values than the other three groups regarding of combined plasma insulin, glycemia and HOMA-IR (Fig. 3I, J).

Insulin tolerance tests were undertaken in CTRL and VDD offspring (performed at 13 weeks). No statistically differences were observed between LF groups, for both male and female (Fig. 3E, 3K). In obesogenic condition, the area under the curve (AUC) of VDD-HF was increased compared with the CTRL HF group of males ($p < 0.01$) (Fig. 3F) and the AUC of VDD-HF was decreased compared with the CTRL of females ($p < 0.05$) (Fig. 3L).

Maternal VDD and HF diet-associated modify morphological parameters and adipose tissue cellularity in sex-specific manner.

After euthanasia, CTRL-LF and VDD-LF males showed similar body weight and weight gain; similarly, to CTRL-HF and VDD-HF (Table 1). Significant differences were observed between LF (both CTRL and VDD) and HF groups (both CTRL and VDD). Compare to the three other groups, VDD-HF males presented the highest fat pad weight (*i.e.* perigonadal, retroperitoneal, inguinal fat pad; Table 1), leading to increased adiposity index and leptin plasma level (Fig. 4A, Table 1). HF diet in CTRL females increased body weight, weight gain (between weaning and the end of the protocol), fat pad weight, adiposity index and leptin plasma level, whereas VDD-HF displayed similar parameters than LF groups (both CTRL-LF and VDD-LF). HF diet (both CTRL and VDD) increased liver weight in male compare to the LF (both CTRL and VDD). For females, only CTRL-HF increased liver weight compared with other groups. No statistical difference of spleen's weight of males, but the females exposed to HF diet showed increased spleen weight compare to the LF (Table 1).

The mean adipocyte area of CTRL and VDD males under LF diet were reduced compared with the adipocytes area of males under HF diet (both CTRL and VDD; Fig. 4A, C and E). The mean adipocyte area increased in CTRL-HF females, but was not different in VDD-HF females, compared with LF groups (both CTRL-LF and VDD-LF; Fig. 4B, D and F).

Maternal and adult offspring diet modulate the expression of mRNA and associated canonical pathways in adipose tissue.

To identify the impacts of maternal (CTRL, VDD) and adult diets (LF, HF) on the offspring transcriptome, we performed RNA sequencing on visceral adipose tissue. Two set of data were used to characterize the impact of the maternal diet and the adult diet. The first one to study the impact of the HF diet on the same condition of maternal diet (*i.e.* CTRL-LF vs CTRL-HF and VDD-LF vs VDD-HF). The second one to study maternal VDD on the same

condition of adult diet (*i.e.* CTRL-LF vs VDD-LF and CTRL-HF vs VDD-HF). We highlighted the differential expression of transcripts between our conditions as established in the Supplemental Table 2.

Using Ingenuity pathway analysis, we put forward canonical pathways with determinant z-score and non-similar between conditions. We highlighted 16 differentially expressed canonical pathways between CTRL-LF and CTRL-HF males. Interestingly, the triacylglycerol biosynthesis, mitochondrial L-carnitine Shuttle Pathway, and fatty acid beta oxidation were differentially decreased in CTRL-HF compared to CTRL-LF. 19 pathways were differentially regulated between the VDD-LF and the VDD-HF males; including the oxidative phosphorylation which was down-regulated in VDD-HF compared to VDD-LF. Also, 6 pathways seemed to be common between the offspring exposed to maternal CTRL diet or VDD diet (Fig. 5).

When comparing CTRL-LF and VDD-LF males, 1 canonical pathway was expressed, while 3 pathways were expressed between CTRL-HF and VDD-HF males.

For the female born from CTRL mice, 31 canonical pathways were differentially expressed between LF and HF, including the fatty acid beta oxidation, the oxidative phosphorylation and triacylglycerol biosynthesis which decreased in CTRL-HF compared to CTRL-LF (Fig. 6). No pathway was regulated when comparing VDD-LF to VDD-HF. Thus, when studying the impact of LF diet between CTRL and VDD females, 3 canonical pathways were differentially expressed, while 27 were differentially expressed for females on HF diet, including oxidative phosphorylation, fatty acid beta oxidation, mitochondrial L-carnitine Shuttle pathway that were induced in VDD-HF compared to CTRL-HF. Interestingly, estrogen biosynthesis pathway appeared as induced in VDD-HF compared to CTRL-HF. One pathway was common between LF and HF diets.

Discussion

The maternal diet is now well-established as a key player in the foetal development and long-term effects programming in the offspring. In this study, we highlighted the impacts of maternal VDD on offspring metabolism in normal nutritional condition and under HF challenge. We also explored the sexual dimorphism of the metabolic response.

In agreement with previously published data, we reported here that maternal VDD diet differentially affected the energy homeostasis and the body weight of the juvenile offspring (5 weeks old). Indeed, the maternal VDD was associated with reduction of body weight of the male mice offspring in our experiment, similarly to previous reports in 15 days old mice²⁴ or at weaning³¹. Other studies reported that VDD male offspring displayed higher body weight³² or not differences^{33–35}, but it is noteworthy that body weight evaluation have been evaluated in adult rats^{32,33} or mice^{34,35}, not in juvenile. Interestingly, in our experiment, no discrepancy in body weight was observed in female, demonstrating thus a strong metabolic sex-specific response which has never been highlighted so far.

Such body weight modifications are strongly related to energy metabolism as highlight by indirect calorimetry. Indeed, the increased spontaneous activity and energy expenditure (through the carbohydrate oxidation) of the VDD males, together with the absence food intake difference, could explain the smaller body weight of juvenile VDD males. The lack of body weight impact in female may result from the absence of food intake modification in VDD female. The observed increase in VDD female spontaneous activity could explain the increase in carbohydrate oxidation, which was associated to a decrease in lipid oxidation, resulting in overall no modification of energy expenditure. Interestingly, we observed that both male and female VDD of the offspring were characterized by an increase in spontaneous activity. Similar observations have already been reported in males VDD mice³⁶, and it has been reported that in human, VDD during pregnancy is strongly correlated with the risk of attention deficit and hyperactivity disorder in children³⁷. Nevertheless, in juvenile offspring of VDD rats³⁸, no modification of locomotor activity was observed. The origin of this phenotype is presently not well understood. Neurobehavioral development could explain this phenotype³⁹, but this assumption will require further investigations.

In adulthood, morphological parameters (body weight, fat pad weight, liver weight, adipocyte area) and energy balance were not modified in male and female offspring of VDD or CTRL mice, when exposed to a control diet (VDD-LF and CTRL-LF), in agreement with previous

reports³⁵. All altered parameters in juvenile mice were normalized in adulthood, including reduced body weight and energy expenditure, and increased spontaneous activity, for both CTRL and VDD offspring. In offspring of VDD rats, similar results were reported in adulthood, *i.e.* no effect on food intake, spontaneous activity in 14 weeks old male rats³². Nevertheless, this study also reported differences on body weight and others biological parameters (total cholesterol, triglycerides, HDL, blood glucose, that were higher in VDD compared with CTRL offspring)³². The origin of these contradictory observations is not clear but could be due to different compositions of control diets used, that could be sufficient to unveil the metabolic phenotype associated to maternal VDD in offspring. In agreement with this assumption, we highlighted that challenged offspring with an HF diets (45% of energy from lipids from 9 to 12 weeks of age) led to a smaller body weight of VDD offspring compared with the CTRL offspring for both males and females. Such observation is not fully consistent with previous reports³¹, but could be related to the genetic background of the mice which differ between studies. Nevertheless, our results in males are consistent with the fact that the increased energy expenditure (mainly due to the carbohydrate oxidation), could exceed the increased food intake, leading to smaller body weight of the VDD-HF males compared with CTRL-HF males.

Female from VDD-HF diets also presented smaller body weight under HF, compared with LF diet, with a tend to food intake increase. This limitation of body weight might be related to the increase of energy expenditure (through the carbohydrate oxidation). An important issue that remain presently unsolved is the drastic decrease of spontaneous activity in VDD-HF female compared with CTRL-HF.

An interesting observation was that in VDD group the level of expression of the hypothalamic melanocortin pathway genes, *i.e.* NPY, AgRP and MC4R, is different of the CTRL mice level, and could explain at least in part food intake modulations. This suggest that VD is important for the accurate development of the melanocortin pathway. In accordance with this hypothesis, VD is known to be important for the brain development⁴⁰. However, further investigations are needed to understand the impact of VD on the development of neural pathways involved in feeding behaviour.

Beside body weight and energy metabolism, the glucose homeostasis was also investigated in offspring adulthood. In LF condition, both in males and females, no modification of glucose homeostasis was observed, especially glycemia, consistently with other studies^{24,32-34}. Nevertheless, several studies reported that VDD in male rats led to an increase of fasting insulin, HOMA-IR levels and insulin tolerance at 16 weeks³³, and in VDD male mice to an

increase of insulinemia³⁴. Such differences could be related to the model used (mice vs rats³³) or duration of the protocol (14 weeks vs 6 months³⁴). Interestingly, in males submitted to HF diet, we observed higher insulin plasma level, glycemia, HOMA IR and insulin resistance, as previously reported in control conditions^{24,32–34}. Surprisingly, in females, no perturbation of the glucose homeostasis was observed. At the opposite, VDD-HF females displayed similar values as CTRL-LF or VDD-LF for several parameters, including insulinemia, glycemia and HOMA-IR. Such observation has never been reported yet, since most of the studies included only males.

Since insulin resistance is strongly linked to adiposity⁴¹, we investigated adiposity index of animals, leptin plasma level and adipose tissue cellularity. Interestingly, adiposity index and leptin plasma concentration were not impacted in males by the VDD in LF condition but was strongly induced by the combination of VDD and HF, similarly to Belenchia et al.³¹ and appeared to be strongly correlated with insulin resistance. No additive effect was reported for adipose cellularity. In females, under LF diet, no modification was observed for adiposity index, leptin plasma level and adipocytes area. As expected, the adiposity index, plasma leptin and cellularity were induced by HF diet in offspring of CTRL mice (CTRL-HF), but surprisingly, VDD-HF group displayed similar adiposity index, plasma leptin and adipocyte area compared with LF groups (both CTRL-LF and VDD-LF). Such observation is fully consistent with the improvement of insulin sensitivity observed in the VDD-HF group. Nevertheless, such effect has never been reported yet and could correspond to an adaptative mechanism based on an adequate diet supply of macronutrients and micronutrients, that appears to be highly sex-specific.

To investigate the origin of such adiposity discrepancies, RNA-seq experiments were undertaken on retroperitoneal adipose tissue. We hypothesized that differential transcriptomes could explain the variation of adiposity between group. In agreement, when comparing male offspring from control mice exposed to LF or HF diet in adulthood (*i.e.* CTRL-LF vs CTRL-HF), we observed that the “mitochondrial L-carnitine shuttle pathway” and the “fatty acid beta oxidation” were down-regulated in the CTRL-HF, which was associated to increase of adiposity. In males from VDD mice submitted to LF or HF (*i.e.* VDD-LF vs VDD-HF), those canonical pathways related to lipid catabolism were not regulated, but we observed a strong repression of oxidative phosphorylation which correlated with fat pad accretion, weight gain and a higher adiposity index. Thus, the discrepancy between offspring of CTRL or VDD mice could be related to the ability to induce lipid oxidation/oxidative phosphorylation in adipose tissue. In addition, the transcriptomic response to maternal diet combined to

adulthood diet (*i.e.* CTRL-LF vs VDD-LF and CTRL-HF vs VDD-HF) did not results in major differences in term of lipid metabolism pathways, nor major difference in adiposity, which reinforces the putative role of adipose tissue lipid metabolism modulations to drive adiposity phenotype. It is noteworthy that the contribution of white adipose tissue to whole body energy expenditure is considered relatively small. Nevertheless, there are examples of nutritional and pharmacological interventions in animals resulting in obesity resistance associated with increased oxidative capacity in WAT⁴²⁻⁴⁴. In agreement with the modest contribution of white adipose tissue on the whole energy expenditure , we did not lipid oxidation induction by indirect calorimetry, suggesting that such local induction of lipid oxidation may have beneficial effect in terms of adiposity, but not impact the overall lipid oxidation which involve many other organs.

Concerning females, CTRL-HF compared with CTRL-LF repressed several canonical pathways linked to lipid metabolism (*i.e.* fatty acid, beta oxidation, oxidative phosphorylation, triacyl glycerol biosynthesis, mitochondrial l-carnitine shuttle pathway). Similarly to males, such pattern corresponded to a signature of body weight gain and adiposity. Importantly, when comparing VDD-HF to VDD-LF, we did not observe any modification of metabolic canonical pathways, nor modification of adiposity. In addition, the transcriptomic response to maternal diet combined to adulthood diet (*i.e.* CTRL-LF vs VDD-LF and CTRL-HF vs VDD-HF) results in the induction of canonical pathways implicated in lipid metabolism (*i.e.* beta oxidation, oxidative phosphorylation, triacyl glycerol biosynthesis, mitochondrial l-carnitine shuttle pathway) in VDD-HF compared to CTRL-HF. This was in agreement with the decreased of adiposity index, and body weight observed in VDD-HF compared to CTRL-HF. Altogether these observations suggested that adiposity is strongly associated to lipid metabolism pathway that may influence adipose tissue accretion. The origin of such gene expression profiles modifications is presently not established but we speculate that epigenetic mechanisms could link maternal VDD to long-term transcriptional modifications in offspring's adipose tissue. Such assumption will require further investigations.

It is obvious that the adiposity and gene expression profile are strongly sex-specific, and notably the effect of VDD maternal under HF diet which leads to highly divergent adiposity in males and females. Sex-specific metabolic discrepancies and notably adiposity are strongly related to estradiol status⁴⁵. Indeed, it is well-established that estrogens promotes subcutaneous fat accumulation⁴⁶ and improve glucose homeostasis⁴⁷. Consequently, the decrease of estrogens associated to menopause is linked to an increase in visceral fat and greater risk for the metabolic syndrome in postmenopausal compared with premenopausal

women⁴⁵. Thus, we focussed on 17 β -estradiol plasma level to explain observed phenotypes. Interestingly, we noticed that in males, no significant differences of plasma concentrations were observed, suggesting that estradiol by itself is not a driver element. It is important to keep in mind that estradiol plasma level is not the sole element that impact adipose tissue biology, and estrogen receptors are also important⁴⁵ and would deserve attention in further experiments. Nevertheless, in females, when comparing CTRL-LF to CTRL-HF, 17 β -estradiol plasma level tended to increase but did not reach statistical significance, whereas in VDD mice (VDD-LF vs VDD-HF), the plasma level of 17 β -estradiol was strongly and significantly induced. This is an important point since 17 β -estradiol is well-known to induce metabolic catabolism, leading to weight gain limitation and glucose homeostasis improvement^{45,47}, and such increase of 17 β -estradiol could explain by itself the metabolic improvement observed in VDD-HF female mice. The origin of the induction of 17 β -estradiol is presently unknown, even if epigenetic mechanism are suspected to be involved. Further investigations will be mandatory to explain this observation.

To conclude, our study brings new informations on the impact of VDD in metabolic disruption in offspring and notably its predisposition to long term metabolic health complications. Importantly it sheds light on the sex-specific adipose tissue / adiposity response, that need to be taking into account in terms of public health.

502

503 **Funding.** The work has been funded by grants from INRA, INSERM, AMU and the
504 Fondation de France. High throughput sequencing was performed at the TGML Platform,
505 supported by grants from Inserm, GIS IBiSA, Aix-Marseille Université, and ANR-10-INBS-
506 0009-10

507

508

509 **Conflict of interest.** No conflict of interest as to be disclose.

510

511 **Author Contributions:** JF Landrier and E Seipelt designed research; JF Landrier, E Seipelt,
512 L Mounien, B Lioriod, M Puceat analysed data; E Seipelt, F Tourniaire, C Couturier, J Astier,
513 H Vachon performed research; JF Landrier, E Seipelt, L Mounien wrote the paper.

514

Figures legends

Fig 1. Indirect calorimetry on juvenile offspring during 24hours to compare the energetic metabolism of the VDD and the CTRL. Parameters measured during 24 h for males and females were Body weight (A, H), Food intake (B, I), spontaneous activity (C, J), Respiratory quotient (RQ, D, K), Energy expenditure (EE, E, L), Lipid oxidation (LOX, F, M) and carbohydrate oxidation (GOX, G, N). Values are presented as mean \pm SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

Fig 2. Morpho-metabolic follow-up of juvenile and 12 weeks-old offspring. (A, B) 25OH(D) concentration of 5-weeks-old offspring male (A) and female (B) before the adult diet induced (Low fat LF or HF, High fat diet). Growing curves of males (C) and females (D) from the beginning of hf diet to indirect calorimetry at 12-weeks-old. Indirect calorimetry of adults (E-R) offspring during 24hours to compare the energetic metabolism of the VDD and the CTRL on HF diet. Parameters measured during 24 h for males and females were Body weight (E, L), Food intake (F, M), activity (G, N), Respiratory quotient (RQ, H, O), Energy expenditure (EE, I, P), Lipid oxidation (LOX, J, Q) and carbohydrate oxidation (GOX, K, R). Values are presented as mean \pm SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

Fig 3. Glucose homeostasis of 13-weeks old offspring. Measured of glucose from tail blood of males (A) and females (G). Insulinemia of male (B) and females (H), Ratio glucose/insulin for males (C) and females (F), HOMA-IR for males (D) and females (J). Insulin tolerance test for males on LF diet (E) and HF diet (F), also for females on LF diet (K), and HF diet (L). Values are presented as mean \pm SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

Fig 4. Adiposity index and adipose tissue histology of offspring. Adiposity index of the offspring has been established for males (A) and females (B). Representative histological images of visceral adipose tissue of males (E) and females (F) adult offspring after eosin-hematoxylin coloration (10X magnification). Mean adipocyte area determined using Image J software for males (C) and females (D). Values are presented as mean \pm SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

Fig 5. Overview of canonical pathway differentially expressed on visceral adipose tissue of the male offspring.

Fig 6. Overview of canonical pathway differentially expressed on visceral adipose tissue of the female offspring.

Table 1. General morphological parameters and 17 β -estradiol plasma concentrations obtain from the offspring at the protocol end. Values are presented as mean \pm SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

References

1. Bendik I, Friedel A, Roos FF, Weber P, Eggersdorfer M. Vitamin D: A critical and essential micronutrient for human health. *Front Physiol.* 2014;5 JUL(July):1-14. doi:10.3389/fphys.2014.00248
2. Bonnet L, Karkeni E, Couturier C, et al. Gene Expression Pattern in Response to Cholecalciferol Supplementation Highlights Cubilin as a Major Protein of 25(OH)D Uptake in Adipocytes and Male Mice White Adipose Tissue. *Endocrinology.* 2018;159(2):957-966. doi:10.1210/en.2017-00650
3. Karkeni E, Bonnet L, Marcotorchino J, et al. Vitamin D limits inflammation-linked microRNA expression in adipocytes in vitro and in vivo: A new mechanism for the regulation of inflammation by vitamin D. *Epigenetics.* 2018;13(2):156-162. doi:10.1080/15592294.2016.1276681
4. Karkeni E, Marcotorchino J, Tourniaire F, et al. Vitamin D Limits Chemokine Expression in Adipocytes and Macrophage Migration In Vitro and in Male Mice. *Endocrinology.* 2015;156(5):1782-1793. doi:10.1210/en.2014-1647
5. Marcotorchino J, Gouranton E, Romier B, et al. Vitamin D reduces the inflammatory response and restores glucose uptake in adipocytes. *Mol Nutr Food Res.* 2012;56(12):1771-1782. doi:10.1002/mnfr.201200383
6. Marcotorchino J, Tourniaire F, Astier J, et al. Vitamin D protects against diet-induced obesity by enhancing fatty acid oxidation. *J Nutr Biochem.* 2014;25(10):1077-1083. doi:10.1016/j.jnutbio.2014.05.010
7. Landrier J-F, Karkeni E, Marcotorchino J, Bonnet L, Tourniaire F. Vitamin D modulates adipose tissue biology: possible consequences for obesity? *Proc Nutr Soc.* 2015;25(March):1-9. doi:10.1017/S0029665115004164
8. Bonnet L, Hachemi MA, Karkeni E, et al. Diet induced obesity modifies vitamin D metabolism and adipose tissue storage in mice. *J Steroid Biochem Mol Biol.* 2019;185(May 2018):39-46. doi:10.1016/j.jsbmb.2018.07.006
9. Landrier J, Mounien L, Tourniaire F. Obesity and Vitamin D Metabolism Modifications. *J Bone Miner Res.* 2019;34(7):1383-1383. doi:10.1002/jbmr.3739
10. Borg SA, Buckley H, Owen R, et al. Early life Vitamin D depletion alters the postnatal response to skeletal loading in growing and mature bone. *PLoS One.* 2018;13(1):1-17. doi:10.1371/journal.pone.0190675
11. Turck D, Bresson J, Burlingame B, et al. Dietary reference values for vitamin D. *EFSA*

J. 2016;14(10):e04547. doi:10.2903/j.efsa.2016.4547

12. Palacios C, Gonzalez L. Is vitamin D deficiency a major global public health problem? *J Steroid Biochem Mol Biol.* 2014;144 Pt A(787):138-145. doi:10.1016/j.jsbmb.2013.11.003
13. Cashman KD, Dowling KG, Gonzalez-Gross M, et al. Vitamin D deficiency in Europe: pandemic? *Am J Clin Nutr.* 2016;(C):1-12. doi:10.3945/ajcn.115.120873.
14. Saraf R, Morton SMB, Camargo CA, Grant CC. Global summary of maternal and newborn vitamin D status - a systematic review. *Matern Child Nutr.* 2016;12(4):647-668. doi:10.1111/mcn.12210
15. Miliku K, Blanken LME, Gaillard R, et al. Maternal Vitamin D concentrations during pregnancy, fetal growth patterns, and risks of adverse birth outcomes. *Am J Clin Nutr.* 2016;103(6):1514-1522. doi:10.3945/ajcn.115.123752.1514
16. Achkar M. Vitamin D status in early pregnancy and risk of preeclampsia. *Am J Obstet Gynecol.* 2016;212(4):1-14. doi:10.1016/j.ajog.2014.11.009. Vitamin
17. Amraei M, Mohamadpour S, Sayehmiri K, Mousavi SF, Shirzadpour E, Moayeri A. Effects of vitamin D deficiency on incidence risk of gestational diabetes mellitus: A systematic review and meta-analysis. *Front Endocrinol (Lausanne).* 2018;9(FEB):1-11. doi:10.3389/fendo.2018.00007
18. Gernand AD, Simhan HN, Klebanoff MA, Bodnar LM. Maternal serum 25-hydroxyvitamin D and measures of newborn and placental weight in a U.S. multicenter cohort study. *J Clin Endocrinol Metab.* 2013;98(1):398-404. doi:10.1210/jc.2012-3275
19. Wang H, Xiao Y, Zhang L, Gao Q. Journal of Steroid Biochemistry and Molecular Biology Maternal early pregnancy vitamin D status in relation to low birth weight and small-for-gestational-age offspring. *J Steroid Biochem Mol Biol.* 2017;(157):0-1. doi:10.1016/j.jsbmb.2017.09.010
20. Morales E, Rodriguez A, Valvi D, et al. Deficit of vitamin D in pregnancy and growth and overweight in the offspring. *Int J Obes.* 2015;39(1):61-68. doi:10.1038/ijo.2014.165
21. Crozier SR, Harvey NC, Inskip HM, Godfrey KM. Maternal vitamin D status in pregnancy is associated with adiposity in the offspring : prospective observational study. *Am J Clin Nutr.* 2012;96(1):57-63. doi:10.3945/ajcn.112.037473. Maternal
22. Daraki V, Roumeliotaki T, Chalkiadaki G, et al. Low maternal vitamin D status in pregnancy increases the risk of childhood obesity. *Pediatr Obes.* 2018;13(8):467-475. doi:10.1111/ijpo.12267

23. Rytter D, Bech BH, Halldorsson TI, et al. Maternal Vitamin D status at week 30 of gestation and offspring cardio-metabolic health at 20 years: A prospective cohort study over two decades. *PLoS One*. 2016;11(10):1-12. doi:10.1371/journal.pone.0164758
24. Reichetzeder C, Chen H, Föller M, et al. Maternal Vitamin D Deficiency and Fetal Programming - Lessons Learned from Humans and Mice. *Kidney Blood Press Res*. 2014;39(4):315-329. doi:10.1159/000355809
25. Ideraabdullah FY, Belenchia AM, Rosenfeld CS, et al. Maternal vitamin D deficiency and developmental origins of health and disease (DOHaD). *J Endocrinol*. 2019;241(2):R65-R80. doi:10.1530/joe-18-0541
26. Goyal D, Limesand SW, Goyal R. Epigenetic responses and the developmental origins of health and disease. *J Endocrinol*. 2019;242(1):T105-T119. doi:10.1530/JOE-19-0009
27. Even PC, Mokhtarian A, Pele A. Practical aspects of indirect calorimetry in laboratory animals. *Neurosci Biobehav Rev*. 1994;18(3):435-447.
28. Fenni S, Hammou H, Astier J, et al. Lycopene and tomato powder supplementation similarly inhibit high-fat diet induced obesity, inflammatory response, and associated metabolic disorders. *Mol Nutr Food Res*. 2017;61(9):1601083. doi:10.1002/mnfr.201601083
29. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262
30. Derghal A, Djelloul M, Trouslard J, Mounien L. The Role of MicroRNA in the Modulation of the Melanocortinergic System. *Front Neurosci*. 2017;11(APR):1-8. doi:10.3389/fnins.2017.00181
31. Belenchia AM, Johnson SA, Ellersieck MR, Rosenfeld CS, Peterson CA. In utero vitamin D deficiency predisposes offspring to long-term adverse adipose tissue effects. *J Endocrinol*. 2017;234(3):301-313. doi:10.1530/JOE-17-0015
32. Wen J, Hong Q, Wang X, et al. The effect of maternal vitamin D deficiency during pregnancy on body fat and adipogenesis in rat offspring. *Sci Rep*. 2018;8(1):365. doi:10.1038/s41598-017-18770-4
33. Zhang H, Chu X, Huang Y, et al. Maternal vitamin D deficiency during pregnancy results in insulin resistance in rat offspring, which is associated with inflammation and Ikb α methylation. *Diabetologia*. 2014;57(10):2165-2172. doi:10.1007/s00125-014-3316-7

34. Nascimento FAM, Ceciliano TC, Aguila MB, Mandarin-de-lacerda CA. Transgenerational Effects on the Liver and Pancreas Resulting from Maternal Vitamin D Restriction in Mice. *J Nutr Sci Vitaminol (Tokyo)*. 2013;59(5):367-374. <http://jlc.jst.go.jp/DN/JST.JSTAGE/jnsv/59.367?lang=en&from=CrossRef&type=abstract>.
35. Belenchia AM, Jones KL, Will M, et al. Maternal vitamin D deficiency during pregnancy affects expression of adipogenic-regulating genes peroxisome proliferator-activated receptor gamma (PPAR γ) and vitamin D receptor (VDR) in lean male mice offspring. *Eur J Nutr*. 2018;57(2):723-730. doi:10.1007/s00394-016-1359-x
36. Fu L, Chen Y-H, Chen X, Xu S, Yu Z, Xu D-X. Vitamin D deficiency impairs neurobehavioral development in male mice. *Physiol Behav*. 2017;179(July):333-339. doi:10.1016/j.physbeh.2017.07.017
37. Morales E, Julvez J, Torrent M, et al. Vitamin D in Pregnancy and Attention Deficit Hyperactivity Disorder-like Symptoms in Childhood. *Epidemiology*. 2015;26(4):458-465. doi:10.1097/EDE.0000000000000292
38. Pan P, Jin DHS, Chatterjee-Chakraborty M, et al. The effects of vitamin D3 during pregnancy and lactation on offspring physiology and behavior in Sprague-Dawley rats. *Dev Psychobiol*. 2014;56(1):12-22. doi:10.1002/dev.21086
39. Hawes JE, Tesic D, Whitehouse AJ, Zosky GR, Smith JT, Wyrwoll CS. Maternal vitamin D deficiency alters fetal brain development in the BALB/c mouse. *Behav Brain Res*. 2015;286:192-200. doi:10.1016/j.bbr.2015.03.008
40. Eyles D, McGrath J. Vitamin D Brain Development and Function. In: *Vitamin D*. Vol 1. Fourth Edi. Elsevier; 2018:563-581. doi:10.1016/B978-0-12-809965-0.00033-1
41. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*. 2006;444(7121):847-853. doi:10.1038/nature05483
42. Kusminski CM, Scherer PE. Mitochondrial dysfunction in white adipose tissue. *Trends Endocrinol Metab*. 2012;23(9):435-443. doi:10.1016/j.tem.2012.06.004
43. Tourniaire F, Musinovic H, Gouranton E, et al. All- trans retinoic acid induces oxidative phosphorylation and mitochondria biogenesis in adipocytes. *J Lipid Res*. 2015;56(6):1100-1109. doi:10.1194/jlr.M053652
44. Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: A key to lean phenotype. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831(5):986-1003. doi:10.1016/j.bbalip.2013.02.003
45. Brown L, Clegg D. Central effects of estradiol in the regulation of food intake, body

699 weight, and adiposity. *J Steroid Biochem Mol Biol.* 2010;122(1-3):65-73.
700 doi:10.1016/j.jsbmb.2009.12.005

701 46. Krotkiewski M, Bjorntorp P, Sjostrom L, Smith U. Impact of obesity on metabolism in
702 men and women. Importance of regional adipose tissue distribution. *J Clin Invest.*
703 1983;72(3):1150-1162.

704 47. Riant E, Waget A, Cogo H, Arnal J-F, Burcelin R, Gourdy P. Estrogens Protect against
705 High-Fat Diet-Induced Insulin Resistance and Glucose Intolerance in Mice.
706 *Endocrinology.* 2009;150(5):2109-2117. doi:10.1210/en.2008-0971
707
708