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Short running title : maternal vitamin D programs offspring metabolism

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List of nonstandard abbreviations :

25(OH)D: 25-hydroxyvitamin D

AGRP: Agouti related-peptide

AUC: Area under the curve

CTRL: Control

EE: Energy expenditure

GOX: Carbohydrate oxidation

HF: High fat

HOMA-IR: Homeostasic model assessment of insulin resistance

IPA: Ingenuity pathway analysis

ITT: Insulin tolerance test

LF: Low fat

LOX: Lipid oxidation

MC4R: Melanocortin receptor type 4

NPY: Neuropeptide Y

RQ: Respiratory quotient

VDD: Vitamin D deficiency

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 17β-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 processes[1], including control of adipose tissue biology[2–9]. Consequently, vitamin D insufficiency is considered as a risk factor in several pathologies, including auto-immune diseases, musculoskeletal defects[10], 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/L[11]. 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 new-borns present a 25(OH)D status < 50nmol/L, also 18% of pregnant women and 29% of new-borns 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 outcomes[15] such as increased risk factor for preeclampsia[16], gestational diabetes mellitus[17], higher risk of small-for-gestational-age, reduced term birth weight, and lower head circumference[18], even if these results are sometimes controverted [19].

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) offspring[20]. 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 years[21]. 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 offspring[22]. Nevertheless, in a long term follow up (20 years), no association between maternal vitamin D status and cardio-metabolic risk factors was highlighted[23]. 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 peri-conceptional 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 offspring[26].

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/6JRJ 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 17β-estradiol were measured in plasma using an enzyme-linked immuno-sorbent assay ELISA (Insulin ALPCO Diagnostics, New Hamshire, 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

Visceralal adipose tissue samples were fixed in 10% buffered formalin, embedded in paraffin and sliced to prepare 5 µm 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 (μm^2) 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 described[6]. The calorimetric appliance was composed of a gas analyzer (to measure O_2 consumption and CO_2 production as VO_2 and VCO_2) 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 cm^3/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 O_2 consumed (VO_2) and CO_2 produced (VCO_2) 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 = $(1.69\times VO_2 - 1.69\times VCO_2)\times(9.46\times 4.186/60)$ (watt) and GOX = $(4.57\times VCO_2 - 3.23\times VO_2)\times(3.74\times 4.186/60)$ (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 synthetize 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 described[28]. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method[29].

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-firstrand, 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.

Ingenuity Pathway Analysis (IPA)

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

Statistical analysis

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

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 (108.6nmol/L ± 2.852) compared with the CTRL (128.9nmol/L ± 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 intake[30]. 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 CRTL 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 CRTL 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 mice[24] or at weaning[31]. Other studies reported that VDD male offspring displayed higher body weight[32] 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 mice[36], and it has been reported that in human, VDD during pregnancy is strongly correlated with the risk of attention deficit and hyperactivity disorder in children[37]. Nevertheless, in juvenile offspring of VDD rats[38], no modification of locomotor activity was observed. The origin of this phenotype is presently not well understood. Neurobehavioral development could explain this phenotype[39], 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

reports[35]. 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 rats[32]. 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 reports[31], 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 development[40]. 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 weeks[33], and in VDD male mice to an

increase of insulinemia[34]. Such differences could be related to the model used (mice vs rats[33]) or duration of the protocol (14 weeks vs 6 months[34]). 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 adiposity[41], 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 be 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 WAT[42–44]. 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 status[45]. Indeed, it is well-established that estrogens promotes subcutaneous fat accumulation[46] and improve glucose homeostasis[47]. 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

481 women[45]. Thus, we focussed on 17β-estradiol plasma level to explain observed phenotypes.
482 Interestingly, we noticed that in males, no significant differences of plasma concentrations
483 were observed, suggesting that estradiol by itself is not a driver element. It is important to
484 keep in mind that estradiol plasma level is not the sole element that impact adipose tissue
485 biology, and estrogen receptors are also important[45] and would deserve attention in further
486 experiments. Nevertheless, in females, when comparing CTRL-LF to CTRL-HF, 17β-
487 estradiol plasma level tended to increase but did not reach statistical significance, whereas in
488 VDD mice (VDD-LF vs VDD-HF), the plasma level of 17β-estradiol was strongly and
489 significantly induced. This is an important point since 17β-estradiol is well-known to induce
490 metabolic catabolism, leading to weight gain limitation and glucose homeostasis
491 improvement[45,47],and such increase of 17β-estradiol could explain by itself the metabolic
492 improvement observed in VDD-HF female mice. The origin of the induction of 17β-estradiol
493 is presently unknown, even if epigenetic mechanism are suspected to be involved. Further
494 investigations will be mandatory to explain this observation.
495 To conclude, our study brings new informations on the impact of VDD in metabolic
496 disruption in offspring and notably its predisposition to long term metabolic health
497 complications. Importantly it sheds light on the sex-specific adipose tissue / adiposity
498 response, that need to be taking into account in terms of public health.
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Author Contributions: JF Landrier and E Seipelt designed research; JF Landrier, E Seipelt, L Mounien, B Loriod, M Puceat analysed data; E Seipelt, F Tourniaire, C Couturier, J Astier, H Vachon performed research; JF Landrier, E Seipelt, L Mounien wrote the paper.

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

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