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

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

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4

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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

44 **Abstract**

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

57

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

59

## 60 **Introduction**

61

62 Vitamin D is an essential micronutrient that is suspected to display an important role on  
63 normal foetal development, and throughout the life of the child and future adult. Its role in  
64 calcium and phosphate metabolism is well-established and it also appears to be involved in  
65 many other physiological processes<sup>1</sup>, including control of adipose tissue biology<sup>2-9</sup>.  
66 Consequently, vitamin D insufficiency is considered as a risk factor in several pathologies,  
67 including auto-immune diseases, musculoskeletal defects<sup>10</sup>, cardiovascular and metabolic  
68 diseases. Such insufficiency is characterised by plasma concentration in 25-hydroxyvitamin D  
69 (25(OH)D) below the cut-off value of 50nmol/L<sup>11</sup>. In fact, vitamin D insufficiency has  
70 become a large public health issue<sup>12,13</sup> and is common for women in child bearing age,  
71 pregnant and breastfeeding women. Currently, 54% of pregnant women and 75% of new-  
72 borns present a 25(OH)D status < 50nmol/L, also 18% of pregnant women and 29% of new-  
73 borns present a severe vitamin D deficiency (25(OH)D < 25nmol/L)<sup>14,15</sup>. It is noteworthy that  
74 maternal vitamin D deficiency (VDD) was associated with pregnancy, foetal and neonatal  
75 outcomes<sup>15</sup> such as increased risk factor for preeclampsia<sup>16</sup>, gestational diabetes mellitus<sup>17</sup>,  
76 higher risk of small-for-gestational-age, reduced term birth weight, and lower head  
77 circumference<sup>18</sup>, even if these results are sometimes controverted<sup>19</sup>.

78 Interestingly, recent studies linked maternal vitamin D insufficiency to overweight and  
79 increased fat mass in offspring. Indeed, in the INMA cohort, including 2358 pregnant women,  
80 maternal VDD was associated with increased risk of overweight in foetal and early postnatal  
81 (1-year-old) offspring<sup>20</sup>. Similarly, in the prospective cohort study Southampton Women's  
82 survey (977 pregnant women), maternal VDD was associated with lower fat mass at birth and  
83 greater fat mass at 4 and 6 years<sup>21</sup>. These results were confirmed in the prospective cohort  
84 Rhea (532 pregnant women), where the maternal 25(OH)D concentrations < 37.7 nmol/L  
85 were associated with higher body mass index and central adiposity in the 4 and 6-years-old  
86 offspring<sup>22</sup>. Nevertheless, in a long term follow up (20 years), no association between  
87 maternal vitamin D status and cardio-metabolic risk factors was highlighted<sup>23</sup>. Thus, those  
88 associations remain controversial.

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

94 environment during adulthood. Indeed, the maternal environment during the pre- and peri-  
95 conceptional period, especially nutrition, can modify epigenetic marks, leading to long term  
96 phenotypic consequences that may vary according to the sex and the environment of the  
97 offspring<sup>26</sup>.

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

101

102

## 103 **Material and Methods**

104

### 105 ***Animal Experiments***

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

129

### 130 ***Biochemical analysis***

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

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

142

### 143 ***Histological analysis***

144 Visceral adipose tissue samples were fixed in 10% buffered formalin, embedded in paraffin  
145 and sliced to prepare 5  $\mu\text{m}$  tissue sections whose were stained with hematoxylin and eosin  
146 (H&E). The images were captured by a light microscope (EZAD, Leica, Germany; 10X  
147 magnification). The adipocyte area ( $\mu\text{m}^2$ ) were determined using (Image J) software.

148

### 149 ***Indirect calorimetry***

150 At 5 and 12 weeks old the offspring was acclimated and kept for 24h in an indirect  
151 calorimetric cage (Physiocage, Bioseb, Vitrolles, France), as previously described<sup>6</sup>. The  
152 calorimetric appliance was composed of a gas analyzer (to measure  $\text{O}_2$  consumption and  $\text{CO}_2$   
153 production as  $\text{VO}_2$  and  $\text{VCO}_2$ ) and an activity recorder (locomotion and rearing). The  
154 temperature of the calorimetric room was set to 22°C. Four were connected to each gas  
155 analyzer, but each cage had specific inlets and outlets. A constant inlet flow (5  $\text{cm}^3/\text{min}$ ) was  
156 maintained throughout the experiment. Gases were continuously analyzed with the following  
157 sequence: 3 min from cage 1, 3 min from cage 2, 3 minutes from cage 3, 3 minutes from cage  
158 4 and then 3 min from room air and thus the volume (mL/min) of  $\text{O}_2$  consumed ( $\text{VO}_2$ ) and  
159  $\text{CO}_2$  produced ( $\text{VCO}_2$ ) were measured for each mouse. Energy expenditure (EE) was  
160 calculated as following ( $\text{EE} = (16.3 \times \text{VO}_2 + 4.57 \times \text{VCO}_2)/60$  (watt)). Lipid (LOX) and  
161 carbohydrate (GOX) oxidation were calculated according to the following equations:  $\text{LOX} =$   
162  $(1.69 \times \text{VO}_2 - 1.69 \times \text{VCO}_2) \times (9.46 \times 4.186/60)$  (watt) and  $\text{GOX} = (4.57 \times \text{VCO}_2 -$   
163  $3.23 \times \text{VO}_2) \times (3.74 \times 4.186/60)$  (watt)<sup>27</sup>. Total activity was evaluated by summing  
164 spontaneous activity and rearing activity, measured in indirect calorimetric cages  
165 (Physiocage, Bioseb, Vitrolles, France), and was normalized to the control value.

166

### 167 ***Insulin Tolerance Test (ITT)***

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



170 (0.5 UI/ kg body weight). Glycemia was measured from tail blood at 0, 15, 30, 60, 90, 120  
171 minutes after injection (Accu-Check glucometer, Roche Diagnostic, Meylan, France).

172

### 173 *RNA extraction real time PCR and RNA sequencing*

174 Total RNA was extracted from retroperitoneal adipose tissue or hypothalamus using TRIzol  
175 reagent (Thermo Fischer Scientific, Les Ulis, France). For the real time PCR, one µg of total  
176 RNA from hypothalamus was used to synthesize cDNAs using random primers and Moloney  
177 murine leukemia virus reverse transcriptase (Thermo Fischer Scientific, Les Ulis, France).  
178 Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR  
179 System (Stratagene, La Jolla, USA) as previously described<sup>28</sup>. For each condition, expression  
180 was quantified in duplicate, and 18S rRNA was used as the endogenous control in the  
181 comparative cycle threshold (CT) method<sup>29</sup>.

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

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

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

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

200 Differential gene expression was performed using DESEQ2 between conditions. To create  
201 bigwig files, reads from Watson and Crick strands were selected using SAMtools (v1.9) and  
202 provided to the bam2wig.py script from the RseQC program suite (v2.6.4). RNA-seq profiles  
203 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

216 **Results**

217

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

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

229

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

232 To explore the combined effect of maternal VDD and post-weaning nutrition, mice were  
233 submitted to low fat (LF) or high fat (HF) diet, leading thus to 8 groups (CTRL-LF, CTRL-  
234 HF, VDD-LF and VDD-HF, for both male and female). Firstly, plasma 25(OH)D  
235 concentration was measured in 6 weeks old offspring. Only VDD females presented  
236 decreased 25(OH)D values ( $108.6\text{nmol/L} \pm 2.852$ ) compared with the CTRL ( $128.9\text{nmol/L} \pm$   
237  $6.186$ ;  $p < 0.01$ ) (Fig. 2A, B). During the diet period (from week 6 to 12), no statistical  
238 difference between CTRL-LF and VDD-LF males body weight was observed (except at 9  
239 weeks old,  $p < 0.05$ ; Fig. 2C). Under HF diet, the body weight of CTRL males was gradually  
240 higher than VDD males (significant from week 9 to 12; Fig. 2C). Concerning females, there  
241 was non-significant differences between CTRL-LF and VDD-LF (from week 6 to 12). Under  
242 HF diet, the CTRL females showed increased body weight compared with the VDD  
243 (significant from week 9 to 12; Fig. 2D).

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

250 0.05) and increased food consumption, EE and GOX ( $p < 0.05$ ) (Fig. 2E, F, I, J). No  
251 modification of activity, RQ and LOX was observed in males. Female exposed to VDD-HF  
252 diets also exhibited smaller body weight than CTRL-HF (Fig. 2L). The food intake tended to  
253 be increased (non-significant) in the VDD-HF compared with the CTRL-HF, whereas the  
254 activity decreased and EE and GOX increased ( $p < 0.05$ ) (Fig. 2M, N, P, R). No modification  
255 of RQ and LOX was observed in females.

256 In order to explain the disruption of feeding behaviour in VDD group, we investigated the  
257 expression of the genes of hypothalamic melanocortin pathway known to be involved in the  
258 regulation of food intake<sup>30</sup>. For males, exposed to LF diet in adulthood, agouti related-peptide  
259 (AGRP) and melanocortin receptor type 4 (MC4R) were under-expressed in the VDD  
260 compare to the CTRL (Supplemental Figure 4A). No statistical differences were observed in  
261 HF condition (Supplemental Figure 4B). For females in VDD and CTRL-LF there was not  
262 statistical difference (Supplemental Figure 4C). Under HF diet, Neuropeptide Y (NPY) and  
263 AGRP were overexpressed ( $p < 0.05$ ) and MC4R under-expressed in VDD-HF compare to  
264 CTRL HF (Supplemental Figure 4D). There is no difference in POMC expression between  
265 the different groups (Supplemental Figure 4A-D).

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

272

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

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

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

284 and VDD-HF) (Fig. 3H). The CTRL-HF females displayed higher values than the other three  
285 groups regarding of combined plasma insulin, glycemia and HOMA-IR (Fig. 3I, J).  
286 Insulin tolerance tests were undertaken in CTRL and VDD offspring (performed at 13 weeks).  
287 No statistically differences were observed between LF groups, for both male and female (Fig.  
288 3E, 3K). In obesogenic condition, the area under the curve (AUC) of VDD-HF was increased  
289 compared with the CTRL HF group of males ( $p < 0.01$ ) (Fig. 3F) and the AUC of VDD-HF  
290 was decreased compared with the CTRL of females ( $p < 0.05$ ) (Fig. 3L).

291

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

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

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

310

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

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

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

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

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

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

342

343

344

## 345 **Discussion**

346

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

351 In agreement with previously published data, we reported here that maternal VDD diet  
352 differentially affected the energy homeostasis and the body weight of the juvenile offspring (5  
353 weeks old). Indeed, the maternal VDD was associated with reduction of body weight of the  
354 male mice offspring in our experiment, similarly to previous reports in 15 days old mice<sup>24</sup> or  
355 at weaning<sup>31</sup>. Other studies reported that VDD male offspring displayed higher body weight<sup>32</sup>  
356 or not differences<sup>33-35</sup>, but it is noteworthy that body weight evaluation have been evaluated in  
357 adult rats<sup>32,33</sup> or mice<sup>34,35</sup>, not in juvenile. Interestingly, in our experiment, no discrepancy in  
358 body weight was observed in female, demonstrating thus a strong metabolic sex-specific  
359 response which has never been highlighted so far.

360 Such body weight modifications are strongly related to energy metabolism as highlight by  
361 indirect calorimetry. Indeed, the increased spontaneous activity and energy expenditure  
362 (through the carbohydrate oxidation) of the VDD males, together with the absence food intake  
363 difference, could explain the smaller body weight of juvenile VDD males. The lack of body  
364 weight impact in female may result from the absence of food intake modification in VDD  
365 female. The observed increase in VDD female spontaneous activity could explain the increase  
366 in carbohydrate oxidation, which was associated to a decrease in lipid oxidation, resulting in  
367 overall no modification of energy expenditure. Interestingly, we observed that both male and  
368 female VDD of the offspring were characterized by an increase in spontaneous activity.  
369 Similar observations have already been reported in males VDD mice<sup>36</sup>, and it has been  
370 reported that in human, VDD during pregnancy is strongly correlated with the risk of attention  
371 deficit and hyperactivity disorder in children<sup>37</sup>. Nevertheless, in juvenile offspring of VDD  
372 rats<sup>38</sup>, no modification of locomotor activity was observed. The origin of this phenotype is  
373 presently not well understood. Neurobehavioral development could explain this phenotype<sup>39</sup>,  
374 but this assumption will require further investigations.

375

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

379 reports<sup>35</sup>. All altered parameters in juvenile mice were normalized in adulthood, including  
380 reduced body weight and energy expenditure, and increased spontaneous activity, for both  
381 CTRL and VDD offspring. In offspring of VDD rats, similar results were reported in  
382 adulthood, *i.e.* no effect on food intake, spontaneous activity in 14 weeks old male rats<sup>32</sup>.  
383 Nevertheless, this study also reported differences on body weight and others biological  
384 parameters (total cholesterol, triglycerides, HDL, blood glucose, that were higher in VDD  
385 compared with CTRL offspring)<sup>32</sup>. The origin of these contradictory observations is not clear  
386 but could be due to different compositions of control diets used, that could be sufficient to  
387 unveil the metabolic phenotype associated to maternal VDD in offspring. In agreement with  
388 this assumption, we highlighted that challenged offspring with an HF diets (45% of energy  
389 from lipids from 9 to 12 weeks of age) led to a smaller body weight of VDD offspring  
390 compared with the CTRL offspring for both males and females. Such observation is not fully  
391 consistent with previous reports<sup>31</sup>, but could be related to the genetic background of the mice  
392 which differ between studies. Nevertheless, our results in males are consistent with the fact  
393 that the increased energy expenditure (mainly due to the carbohydrate oxidation), could  
394 exceed the increased food intake, leading to smaller body weight of the VDD-HF males  
395 compared with CTRL-HF males.

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

401 An interesting observation was that in VDD group the level of expression of the hypothalamic  
402 melanocortin pathway genes, *i.e.* NPY, AgRP and MC4R, is different of the CTRL mice  
403 level, and could explain at least in part food intake modulations. This suggest that VD is  
404 important for the accurate development of the melanocortin pathway. In accordance with this  
405 hypothesis, VD is known to be important for the brain development<sup>40</sup>. However, further  
406 investigations are needed to understand the impact of VD on the development of neural  
407 pathways involved in feeding behaviour.

408 Beside body weight and energy metabolism, the glucose homeostasis was also investigated in  
409 offspring adulthood. In LF condition, both in males and females, no modification of glucose  
410 homeostasis was observed, especially glycemia, consistently with other studies<sup>24,32-34</sup>.  
411 Nevertheless, several studies reported that VDD in male rats led to an increase of fasting  
412 insulin, HOMA-IR levels and insulin tolerance at 16 weeks<sup>33</sup>, and in VDD male mice to an



413 increase of insulinemia<sup>34</sup>. Such differences could be related to the model used (mice vs rats<sup>33</sup>)  
414 or duration of the protocol (14 weeks vs 6 months<sup>34</sup>). Interestingly, in males submitted to HF  
415 diet, we observed higher insulin plasma level, glycemia, HOMA IR and insulin resistance, as  
416 previously reported in control conditions<sup>24,32-34</sup>. Surprisingly, in females, no perturbation of  
417 the glucose homeostasis was observed. At the opposite, VDD-HF females displayed similar  
418 values as CTRL-LF or VDD-LF for several parameters, including insulinemia, glycemia and  
419 HOMA-IR. Such observation has never been reported yet, since most of the studies included  
420 only males.

421 Since insulin resistance is strongly linked to adiposity<sup>41</sup>, we investigated adiposity index of  
422 animals, leptin plasma level and adipose tissue cellularity. Interestingly, adiposity index and  
423 leptin plasma concentration were not impacted in males by the VDD in LF condition but was  
424 strongly induced by the combination of VDD and HF, similarly to Belenchia et al.<sup>31</sup> and  
425 appeared to be strongly correlated with insulin resistance. No additive effect was reported for  
426 adipose cellularity. In females, under LF diet, no modification was observed for adiposity  
427 index, leptin plasma level and adipocytes area. As expected, the adiposity index, plasma leptin  
428 and cellularity were induced by HF diet in offspring of CTRL mice (CTRL-HF), but  
429 surprisingly, VDD-HF group displayed similar adiposity index, plasma leptin and adipocyte  
430 area compared with LF groups (both CTRL-LF and VDD-LF). Such observation is fully  
431 consistent with the improvement of insulin sensitivity observed in the VDD-HF group.  
432 Nevertheless, such effect has never been reported yet and could correspond to an adaptative  
433 mechanism based on an adequate diet supply of macronutrients and micronutrients, that  
434 appears to be highly sex-specific.

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

447 adulthood diet (*i.e.* CTRL-LF vs VDD-LF and CTRL-HF vs VDD-HF) did not results in  
448 major differences in term of lipid metabolism pathways, nor major difference in adiposity,  
449 which reinforces the putative role of adipose tissue lipid metabolism modulations to drive  
450 adiposity phenotype. It is noteworthy that the contribution of white adipose tissue to whole  
451 body energy expenditure is considered relatively small. Nevertheless, there are examples of  
452 nutritional and pharmacological interventions in animals resulting in obesity resistance  
453 associated with increased oxidative capacity in WAT<sup>42-44</sup>. In agreement with the modest  
454 contribution of white adipose tissue on the whole energy expenditure , we did not lipid  
455 oxidation induction by indirect calorimetry, suggesting that such local induction of lipid  
456 oxidation may have beneficial effect in terms of adiposity, but not impact the overall lipid  
457 oxidation which involve many other organs.

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

474 It is obvious that the adiposity and gene expression profile are strongly sex-specific, and  
475 notably the effect of VDD maternal under HF diet which leads to highly divergent adiposity  
476 in males and females. Sex-specific metabolic discrepancies and notably adiposity are strongly  
477 related to estradiol status<sup>45</sup>. Indeed, it is well-established that estrogens promotes  
478 subcutaneous fat accumulation<sup>46</sup> and improve glucose homeostasis<sup>47</sup>. Consequently, the  
479 decrease of estrogens associated to menopause is linked to an increase in visceral fat and  
480 greater risk for the metabolic syndrome in postmenopausal compared with premenopausal

481 women<sup>45</sup>. Thus, we focussed on 17 $\beta$ -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<sup>45</sup> and would deserve attention in further  
486 experiments. Nevertheless, in females, when comparing CTRL-LF to CTRL-HF, 17 $\beta$ -  
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 17 $\beta$ -estradiol was strongly and  
489 significantly induced. This is an important point since 17 $\beta$ -estradiol is well-known to induce  
490 metabolic catabolism, leading to weight gain limitation and glucose homeostasis  
491 improvement<sup>45,47</sup>, and such increase of 17 $\beta$ -estradiol could explain by itself the metabolic  
492 improvement observed in VDD-HF female mice. The origin of the induction of 17 $\beta$ -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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510

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512 L Mounien, B Loriod, 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

515 **Figures legends**

516

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

523

524 **Fig 2. Morpho-metabolic follow-up of juvenile and 12 weeks-old offspring.** (A, B)  
525 25OH(D) concentration of 5-weeks-old offspring male (A) and female (B) before the adult  
526 diet induced (Low fat LF or HF, High fat diet). Growing curves of males (C) and females (D)  
527 from the beginning of hf diet to indirect calorimetry at 12-weeks-old. Indirect calorimetry of  
528 adults (E-R) offspring during 24hours to compare the energetic metabolism of the VDD and  
529 the CTRL on HF diet. Parameters measured during 24 h for males and females were Body  
530 weight (E, L), Food intake (F, M), activity (G, N), Respiratory quotient (RQ, H, O), Energy  
531 expenditure (EE, LP), Lipid oxidation (LOX, J, Q) and carbohydrate oxidation (GOX, K, R).  
532 Values are presented as mean  $\pm$  SEM. Bars not sharing the same letter were significantly  
533 different in Fisher's LSD post hoc test.  $p < 0.05$

534

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

541

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

548

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

551

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

554

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

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