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1 **Diet induced obesity modifies vitamin D metabolism and adipose tissue storage in mice.**

2

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6

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10

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12

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18

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20 **Abstract**

21 Low circulating levels of total and free 25-hydroxyvitamin D (25(OH)D) indicative of vitamin D
22 status have been associated with obesity in humans. Moreover, obesity is thought to play a causal
23 role in the reduction of 25(OH)D levels, and several theories have been put forward to explain
24 this relationship. Here we tested the hypothesis that obesity disrupts vitamin D homeostasis in
25 key organs of vitamin D metabolism. Male C57BL6 mice were fed for 7 or 11 weeks on either a
26 control diet (control, 10% energy from fat) or a high-fat diet (HF, 60% energy from fat)
27 formulated to provide equivalent vitamin D3 intake in both groups. After 7 weeks, there was a
28 transient increase of total 25(OH)D together with a significant decrease of plasma vitamin D3
29 that could be related to the induction of hepatic genes involved in 25-hydroxylation. After 11
30 weeks, there was no change in total 25(OH)D but a significant decrease of free 25(OH)D and
31 plasma vitamin D3 levels. We also quantified an increase of 25(OH)D in adipose tissue that was
32 inversely correlated to the free 25(OH)D. Interestingly, this accumulation of 25(OH)D in adipose
33 tissue was highly correlated to the induction of Cyp2r1, which could actively participate in
34 vitamin D3 trapping and subsequent conversion to 25(OH)D in adipose tissue. Taken together,
35 our data strongly suggest that the enzymes involved in vitamin D metabolism, notably in adipose
36 tissue, are transcriptionally modified under high-fat diet, thus contributing to the obesity-related
37 reduction of free 25(OH)D.

38

39 **Keywords:** obesity, high fat diet, vitamin D, metabolism, adipose tissue, free 25-hydroxyvitamin

40 D

41 **Introduction**

42 Vitamin D is a secosteroid hormone that plays key roles in phosphocalcium homeostasis and
43 bone metabolism [1] but also has many other biological functions [2]. There are two main
44 sources of vitamin D—one through diet, mainly as vitamin D₃, and the other through
45 endogenous production [1, 3, 4]. To become biologically active, the native vitamin D has to be
46 converted in the liver into 25-hydroxyvitamin D (25(OH)D), in a first hydroxylation step
47 catalyzed by 4 enzymes (CYP2R1, CYP27A1, CYP2J6 and CYP3A11) [5]. A second
48 hydroxylation step catalyzed by CYP2B1 in the kidney then produces 1,25-dihydroxyvitamin D
49 (1,25(OH)₂D), the active form of cholecalciferol, which is a potent activator of the vitamin D
50 receptor (VDR) [6]. 25(OH)D and 1,25(OH)₂D can be catabolized by 24-hydroxylase,
51 CYP24A1, to generate inactive metabolites [7].

52 Vitamin D status is classically reflected by total plasma 25(OH)D concentration, which
53 represents the sum of free 25(OH)D and 25(OH)D bound to vitamin D binding protein (DBP,
54 encoded by the Gc gene) and albumin [8, 9]. Interestingly, vitamin D status is impacted by a
55 number of physio-pathological parameters, including obesity which is classically associated to a
56 decrease of total 25(OH)D [10, 11]. Indeed, plasma 25(OH)D levels are inversely correlated to
57 all parameters of obesity, including BMI, fat mass and waist circumference [12, 13].
58 Furthermore, it was recently shown that the free forms of 25(OH)D and 1,25(OH)₂D were also
59 decreased during obesity [14]. Several hypotheses have been put forward to explain the low total
60 25(OH)D levels observed in obese people: 1) impaired hepatic 25-hydroxylation linked to high
61 levels of 1,25(OH)₂D and parathyroid hormone (PTH) [15]; 2) sequestration of vitamin D in
62 adipose tissue (AT) caused by a passive phenomenon due to the hydrophobic nature of vitamin D
63 [16]; 3) volumetric dilution of 25(OH)D in obese subjects [17]. More recently, Wamberg

64 suggested that obesity alters vitamin D metabolism in AT, as Cyp2j2 expression was modified in
65 biopsies of obese compared to lean patients [18]. In line with idea, Park *et al.* described the effect
66 of a high-fat diet on the expression of vitamin D-metabolizing enzymes in mice [19]. However,
67 the mechanism linking obesity to the decrease in free 25(OH)D remains unknown.

68

69 To go further in determining the impact of obesity on vitamin D status and metabolism in mice,
70 and notably its consequences on free 25(OH)D as an important new parameter, we implemented
71 a longitudinal study of high-fat diet induced-obesity. We tested the hypothesis that obesity
72 disrupts vitamin D homeostasis via gene expression modulations in key organs of vitamin D
73 metabolism (i.e. liver, kidney, and AT). These modulations could participate in the active storage
74 of vitamin D metabolites in AT and could be related to the decrease of free 25(OH)D observed
75 during obesity.

76 **Material and methods**

77 **Reagents** – TRIzol reagent, random primers, and Moloney murine leukemia virus reverse
78 transcriptase (M-MLV RT) were obtained from Life Technologies (Courtaboeuf, France). SYBR
79 Green reaction buffer was purchased from Eurogentec (Liege, Belgium).

80
81 **Animal, Diets and Experiments** – The protocol was approved by the French Ministry of
82 Research (APAFIS#2595-2016091911217758) after validation by the Aix-Marseille University
83 ethics committee. Six-week-old male C57BL/6J mice were obtained from Janvier Labs (Le
84 Genest-Saint-Isle, France) and fed *ad libitum* with standard chow (maintenance diet A04, Safe
85 diets, Augy France) during a 1-week acclimatization period with *ad libitum* access to drinking
86 water, and maintained at 22°C under a 12h/12h light/dark cycle at 20% relative humidity. The
87 mice were then divided into control-diet group (control: 10% energy from lipids, n=10) or a
88 high-fat diet group (HF: 60% energy from lipids, n=10) (TestDiet, London, UK). Composition of
89 the experimental diet is detailed in Table 1. Weight gain was measured once a week, and dietary
90 intake was measured every two weeks. After 7 weeks or 11 weeks of diet, the mice were fasted
91 overnight, blood was collected by cardiac puncture under anesthesia, and plasma was obtained
92 by centrifuging at 3000 g for 15 min at 4°C, and stored at -80°C. The animals, under anesthesia,
93 were sacrificed by cervical dislocation, and the kidney, liver and epididymal white adipose tissue
94 (eWAT) were collected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C.

95
96 **RNA extraction and real-time PCR** – Total RNA was extracted from the liver, kidney and
97 eWAT using TRIzol reagent (Thermo Fisher, Courtaboeuf, France). One µg of total RNA was
98 used to synthesize cDNA using random primers and -MLV RT (Thermo Fisher). Real-time

99 quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System
100 (Stratagene, La Jolla, CA) as previously described [20]. For each condition, expression was
101 quantified in duplicate, and 18S rRNA was used as endogenous control in the comparative cycle
102 threshold (CT) method [21]. Primer sequences are reported in Supplemental table 1.

103

104 **Protein quantification by ELISA** – Parathyroid hormone (PTH) concentration in mouse plasma
105 was quantified using PTH ELISA (Euromedex, Strasbourg, France). The free form of 25(OH)D
106 was also quantified using ELISA kits from DIAsource ImmunoAssays (DIAsource
107 ImmunoAssays, Louvain-La-Neuve, Belgium). Colorimetric assay kits were used to quantify
108 Ca^{+2} and phosphate concentrations in mouse plasma (Clinisciences, Nanterre, France).

109

110 **Vitamin D3, 25(OH)D and 1,25(OH)₂D quantification in plasma and eWAT** – All
111 quantifications were performed using LC-MS/MS according to the following protocol as
112 previously reported [22].

113 *Preparation of analytical and deuterated standards* – A working solution of deuterated analytes
114 (d3-vitamin D3, d3-25(OH)D and d3-1,25(OH)₂D; internal standards (IS)) was prepared at 0.02
115 ng/mL of each) . They were used to ensure high specificity of the quantification.

116 A primary stock solution of unlabeled vitamin D3, 25(OH)D and 1,25(OH)₂D standards were
117 prepared for calibration curves at concentrations of 100, 50 and 10 ng/mL, respectively, in
118 ethanol and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the
119 3 stock-solution analytes to obtain calibration standards from 0 to 75 ng/mL and by addition of
120 1.5 µL of the working solution of deuterated analytes to each dilution.

121 After complete evaporation of solvent, we proceeded with derivatization. A one-step
122 derivatization was employed to improve the ionization efficiency of the metabolites using
123 Amplifex diene (Amplifex™ Diene Reagent, Sciex Chemistry and Consumables R&D,
124 Framingham, MA) as reagent [23]. Then 30 µL of Amplifex was added to the dried sample
125 above, vortexed for 15 s, and incubated for 30 min at ambient temperature. Next, 30 µL of
126 deionized water was added, vortexed for 15 s, and transferred for LC injection. Calibration
127 curves were plotted with peak area ratio of the vitamin D metabolite to the respective internal
128 standard versus a range of concentrations of the analyte.

129 *Plasma preparation* – Sample preparation was adapted from Wang et al. [24]. The extraction
130 procedure was conducted under low light, as cholecalciferol and its metabolites are light-
131 sensitive. After thawing on ice, mice plasmas were centrifuged at 11,000 g for 15 min at 4°C,
132 and 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated
133 standard working solution. Proteins were precipitated by adding acetonitrile (ACN), then vortex-
134 mixed, and centrifuged at 3,000 g for 10 min. The supernatant was moved to another glass tube,
135 the volume was reduced to half under a nitrogen stream, and 5 mL of ethyl acetate was added to
136 the solution for liquid-liquid extraction. After vigorous shaking, the samples were centrifuged at
137 590 g for 20 min, and the upper organic layer was transferred to a new glass tube and further
138 reduced under a nitrogen stream. The samples were then derivatized as described above.

139 *eWAT preparation* – Sample preparation was adapted from Lipkie et al. [25]. Briefly, 25 µL of
140 deuterated standard working solution was added to tissue homogenates (50 mg of tissue ground
141 into 1 mL of PBS) in a glass test tube. ACN was added, vortexed for 5 min, and centrifuged at
142 6,000 g for 5 min. Then, methyl tert-butyl ether (MTBE) was added, vortexed for 5 min,
143 centrifuged, and the upper organic layer was collected into a new glass tube. The extraction was

144 repeated twice, and the combined supernatants were dried under nitrogen. Oasis HLB SPE
145 cartridges (Waters, Guyancourt, France) were conditioned with ethyl acetate, methanol (MetOH)
146 and H₂O. The sample was reconstituted with 1 mL of MetOH and 1 mL of K₂HPO₄ (0.4 M) then
147 added onto the cartridge. The cartridge was washed with H₂O and 70% MetOH then dried for 2
148 min under vacuum. Tips were washed with ACN, and analytes were eluted with ACN and dried
149 under nitrogen. After complete evaporation of solvent, the samples were derivatized as above.

150 *LC-MS/MS analysis* – Accurate mass measurements were performed on a Q Exactive Plus mass
151 spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray
152 ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for instrument setup,
153 control of the LC-MS system during acquisition, and data processing. The Tune Q Exactive Plus
154 2.5 software was used for direct control of the mass spectrometer.

155 Samples were injected onto a 2.1×100 mm Hypersil GOLD C18 column (Thermo Scientific, Les
156 Ulis, France). Flowrate was 0.4 mL/min and injection volume was 5 µL. The mobile phase was
157 composed of A=ultrapure water with 0.1% formic acid (v/v), and B=ACN with 0.1% formic acid
158 (v/v). Starting conditions were A=70% and B=30% and were held for 4 min. A linear gradient
159 was applied until 10.0 min where A=35% and B=65%, held until 12.0 min, then to 14 min where
160 A=0% and B =100% until 16 min. Starting conditions were re-implemented at 18 min.

161 The Parallel reaction monitoring (PRM) transitions used for quantification of each analyte were:
162 716.5→657.5 (vitamin D3), 719.5→660.5 (d3-vitamin D3), 732.5→673.4 (25(OH)D),
163 735.5→676.4 (d3-25(OH)D), 751.5→692.4 (d3-1,25(OH)₂D) and 748.5→689.4 (1,25(OH)₂D).

164 Validations were performed for linearity and repeatability of the data (Supplemental Table 2).

165

166 **Statistical analysis** – The data are reported as mean \pm SEM. Significant differences were
167 determined using ANOVA followed by the Tukey-Kramer post hoc test and two-way ANOVA
168 using StatView software (SAS Institute, Cary, NC). $p < 0.05$ was considered statistically
169 significant.

170

171 **Results**

172 **Impact of high-fat diet on morphological parameters of the mice.**

173 Mice were fed for 7 or 11 weeks with control or high fat (HF) diet. As expected, body weight,
174 liver weight and adiposity index were increased in the HF group at 7 weeks and at 11 weeks
175 compared to the control diet (Table 2 and Supplemental Figure 1). Body weight showed time and
176 diet effects as well as a significant statistical interaction between time and diet, whereas liver
177 weight and adiposity index only showed time and diet effects (Table 2). Food intakes were
178 quantified. Energy intake was similar between HF group and control group. Vitamin D3 intake
179 was calculated and was not different between groups (Table 3).

180

181 **Impact of high-fat diet on plasma parameters related to vitamin D metabolism of mice and**
182 **amounts of vitamin D3 and metabolites in adipose tissue.**

183 The plasma concentration of various parameters related to vitamin D metabolism was measured
184 in mice fed control or HF diet. After 7 weeks of HF diet, serum cholecalciferol concentration had
185 decreased whereas plasma total 25(OH)D and PTH concentration had increased compared to
186 controls (Table 3). There was no between-group difference in plasma free form of 25(OH)D,
187 1,25(OH)₂D, Ca⁺² and phosphate concentrations. After 11 weeks of HF diet, PTH concentration
188 had increased whereas vitamin D3, total 25(OH)D, 1,25(OH)₂D, Ca⁺² and phosphate plasma
189 concentrations remained unchanged (Table 3). Interestingly, the plasma free form of 25(OH)D
190 had decreased in the HF group compared to controls (from 6.77 ± 0.21 pg/mL to 5.94 ± 0.26
191 pg/mL; *p*<0.05). This plasma free form of 25(OH)D appeared to be inversely correlated with
192 mouse body weight (Fig. 1A), adiposity index (Fig. 1B) and plasma PTH (Fig. 1C).

193 As AT is considered a major storage site for vitamin D and its metabolites, we quantified vitamin
194 D₃, 25(OH)D and 1,25(OH)₂D concentrations by LC-MS/MS in eWAT (Supplemental Table 3)
195 and then calculated quantities as concentration × mass of eWAT (Table 4). After 7 weeks of diet,
196 25(OH)D quantity had increased significantly in the HF group compared to control group (Table
197 4). After 11 weeks of diet, vitamin D₃ and 25(OH)D quantity had increased significantly in the
198 eWAT of HF-fed mice (Table 4). For 25(OH)D quantity in eWAT, two-way ANOVA found
199 time and diet effects as well as a significant interaction between time and diet. Interestingly,
200 25(OH)D quantity in eWAT at 7 and 11 weeks was inversely correlated to plasma free form
201 25(OH)D ($p < 0.01$, Fig. 1). There was no between-group difference in 1,25(OH)₂D quantity in
202 eWAT at both timepoints (Table 4). In terms of concentrations in eWAT, HF diet decreased
203 week-7 and week-11 vitamin D₃ with both diet and time effects, but had no effect on 25(OH)D
204 and 1,25(OH)₂D concentrations (Supplemental Table 3).

205

206 **Impact of high-fat diet on gene expression in liver, kidney and eWAT of mice.**

207 Real-time PCR measured the expression of genes coding for vitamin D metabolic proteins in
208 liver, kidney and eWAT (all data presented in Supplemental Table 4 and 5). After 7 weeks of
209 diet, the genes coding for 3 hepatic enzymes involved in 25-hydroxylation, i.e. Cyp2r1, Cyp27a1
210 and Cyp2j6, were significantly upregulated in the HF group (Fig. 2) whereas after 11 weeks of
211 diet, only Cyp2r1 gene expression remained higher in the HF group compared to control (Fig. 2).
212 Two-way ANOVA found that Cyp27a1 and Cyp2j6 expression was dependent on time, diet and
213 time×diet interaction whereas Cyp2r1 expression was only dependent on time and diet. In
214 kidney, after 7 weeks of diet, mRNA expression of Cyp24a1 was decreased in the HF group
215 compared to controls (Supplemental table 4). After 11 weeks, Cyp27b1 expression was

216 upregulated and Cyp24a1 expression was downregulated in the HF group (Supplemental table
217 5). In eWAT, after 7 weeks of diet, Cyp2r1 and VDR were upregulated in the HF group. After 11
218 weeks of diet, Cyp2r1, Cubilin and Vdr were upregulated whereas Cyp27a1, Cyp2j6 and
219 Cyp27b1 were downregulated in the HF group compared to controls (Fig. 3). Interestingly, the
220 induction of Cyp2r1 observed in the HF group at 7 weeks (2-fold increase *vs* control) was more
221 pronounced at 11 weeks (3-fold increase *vs* control). Furthermore, two-way ANOVA found a
222 time and diet effect as well as a significant interaction between time and diet only for this gene
223 (Fig. 3).

224 Cyp2r1 gene expression in eWAT was found to correlate with mouse body weight, eWAT mass
225 and plasma free 25(OH)D levels (Fig. 4). Cyp2r1 expression was positively correlated with
226 eWAT mass ($r=0.75$, $p < 0.01$) and mice body weight ($r=0.78$, $p < 0.001$) but negatively
227 correlated with concentration of free 25(OH)D ($r= -0.62$; $p < 0.001$).

228

229 .

230 **Discussion**

231 The primary objective of this study was to use a murine model to demonstrate the impact of
232 obesity on vitamin D metabolism, chiefly the effect on plasma free 25(OH)D levels as recently
233 described in humans [14], and bring mechanistic evidence by studying gene expression in the
234 main organs involved in vitamin D metabolism.

235

236 As expected, the HF diet used in our study of weight gain led to a significant increase in total
237 mass of the animals as well as in amount of AT and adiposity index at the two timepoints
238 studied, i.e. at 7 and 11 weeks. These morphological changes were not accompanied by major
239 changes in energy intake or in consumption of vitamin D3. This vitamin D3 parameter was
240 particularly important to control here, since a modification in vitamin D3 intake induced by
241 poorly-balanced diet alone could have led to a vitamin D deficiency. However, despite balanced
242 vitamin D3 throughout the diet, at week 7 plasma vitamin D3 content had decreased and total
243 25(OH)D and PTH concentrations had increased. These are novel findings, apart from the
244 increased PTH as PTH concentration is known to be positively correlated with fat mass [26] and
245 body mass index [14]. The reduction of vitamin D3 is probably due to direct trapping of this
246 hydrophobic molecule in expanded AT. The fact that vitamin D3 decreased whereas total
247 25(OH)D increased is harder to explain, but could be linked to the overall induction of mRNA
248 coding for hepatic 25-hydroxylation. Indeed, even if the expanding AT will store volumetrically
249 more 25(OH)D, the fact that 25-hydroxylation is induced at least partly explains the reduced
250 vitamin D3 levels. It was fairly surprising that these 25-hydroxylation enzymes were upregulated
251 here, since another study in similar experimental conditions found a down-regulation [19]. This
252 discrepancy is hard to explain, but may reflect an adaptive process to high-fat diet that occurs in

253 this time-window, as the increased total 25(OH)D showed no further change at 11 weeks here
254 nor after 18 weeks of HF diet in [19]. Note too that several studies have reported a decrease of
255 total 25(OH)D in mice submitted to high-fat diet [27, 28]. Such discrepancies could be due to the
256 methodology of calcidiol measurement, but could also be related to diet designs that were not
257 adapted to bring similar amounts of vitamin D3 in both the control and high-fat diets tested.

258

259 At 11 weeks, the results for free 25(OH)D, detected with an ELISA kit, were consistent with a
260 recent study reporting similar results in obese subjects [14], but this clinical study also reported a
261 decrease of total 25(OH)D and an increase of 1,25(OH)₂D, which was not the case here. These
262 discrepancies are not presently well understood but could be due to the fact that mice received
263 equal doses of vitamin D3, in contrast to humans whose vitamin D intake and endogenous
264 production are difficult to control. Note too that the decrease of free 25(OH)D was only observed
265 after long-term HF diet (11 weeks) and not after 7 weeks, suggesting a combined effect of time
266 and HF diet. Concerning 1,25(OH)₂D, despite the obesity-associated secondary
267 hyperparathyroidism observed at 7 and 11 weeks, we found no change in 1,25(OH)₂D levels,
268 even with the strong decrease of Cyp24a1 observed in kidney (Supplemental Tables 4 and 5) and
269 the increase of Cyp27b1 at 11 weeks, similarly to a recent report by Park et al. [19].

270 Discrepancies may be related to 1,25(OH)₂D quantification methodologies (LC-MS/MS here *vs.*
271 ELISA kit in Park et al.).

272

273 Plasma levels of free 25(OH)D or total 25(OH)D has not decreased at 7 weeks of HF diet, even
274 though the mice had already gained a lot of fat mass. This observation does not fit with the
275 hypothesis of Wortsmann et al. [16] who suggested that the plasma 25(OH)D decrease observed

276 during obesity is the direct result of AT expansion. Note, however, that the difference in fat mass
277 gain between control mice and HF mice was greater at 7 weeks than at 11 weeks (adiposity index
278 increased by 2.74 at 7 weeks and by 2.09 at 11 weeks) whereas the difference in total body
279 weight gain was greater at 11 weeks than at 7 weeks (total body weight increased by 1.21 at 7
280 weeks and by 1.33 at 11 weeks), suggesting that the decrease in free 25(OH)D content at 11
281 weeks is better correlated with weight gain than with fat mass gain. These observations are
282 therefore more in line with Drincic et al.'s [17] volumetric dilution hypothesis, which states that
283 plasma content is better correlated with total volume than fat mass. Indeed, we found a better
284 correlation between free 25(OH)D and body weight ($r = -0.53$) than free 25(OH)D and adiposity
285 index ($r = -0.38$). Remember that these hypotheses were advanced for total 25(OH)D, not free
286 25(OH)D, so extrapolability to free 25(OH)D remains an issue that warrants further
287 investigation.

288

289 Free 25(OH)D concentration was strongly correlated with the various morphological parameters
290 tested (total body weight, PTH level, adiposity index) at 11 weeks, whereas these relationships
291 were non-existent at 7 weeks (data not shown). This makes it tempting to speculate that plasma
292 free 25(OH)D could a better marker of vitamin D status than total 25(OH)D during obesity, in
293 agreement with the “free hormone hypothesis” [29]. Interestingly, we also found that plasma free
294 25(OH)D level was inversely correlated to the amount of 25(OH)D in the AT, suggesting as AT
295 expands during obesity, it increasingly stores more 25(OH)D, thus reducing the free fraction of
296 25(OH)D in the plasma. In addition to a potential dilution effect, these data suggest that the
297 stability of free 25(OH)D at 7 weeks could be the result of a balance between clearance and
298 synthesis. Indeed, the decrease in free 25(OH)D does not appear solely due to an increase in

309 body volume of the animals but also due to a blunting of an adaptive process leading to a loss of
300 homeostasis. However, this kinetic evolution in plasma level of free 25(OH)D during obesity
301 between 7 weeks and 11 weeks clearly supports a causal role of obesity in this decrease, as
302 previously asserted [10, 30]. However, , this decrease in free 25(OH)D content could in turn go
303 on to amplify obesity and/or metabolic inflammation, since we have previously shown that
304 vitamin D3 supplementation limits the occurrence of HF diet-induced obesity by decreasing lipid
305 oxidation [31, 32] and metabolic inflammation [33-35].

306

307 In order to provide some mechanistic explanations for our observations, we undertook a study of
308 gene expression, including in AT. The results show an increase in the expression of Cyp2r1 in
309 HF mice at week 7 and week 11, which was 2.4-fold higher at 11 weeks than at 7 weeks, as well
310 as a decrease in the expression of Cyp27b1 which encodes the enzyme involved in 1,25-
311 hydroxylation at 11 weeks. A decrease in Cyp27b1 expression in the subcutaneous AT of obese
312 subjects compared to normal subjects has already been reported by Wamberg et al. [18], and in
313 mice [19]. Nevertheless, this novel data on the expression of Cyp2r1, which encodes a major
314 enzyme of 25-hydroxylation [36], suggests an increased ability of AT to store vitamin D3 as
315 25(OH)D. In addition, this Cyp2r1 expression is inversely correlated with total body weight and
316 epididymal fat mass, suggesting that obesity leads to enhanced AT production of 25(OH)D and a
317 subsequent reduction of free 25(OH)D (as stated above). To validate this hypothesis, we
318 quantified vitamin D3 and 25(OH)D in AT. Under the HF diet, vitamin D3 concentration
319 decreased but quantity increased in a similar way at 7 and 11 weeks (1.93 and 2-fold
320 respectively). Carrelli et al. quantified vitamin D2 and vitamin D3 in subcutaneous and omental
321 AT of lean and obese women by LC-MS/MS [37], and found that obese subjects have greater

322 adipose stores of vitamin D2 and D3, supporting the hypothesis that the large amount of AT in
323 obese individuals serves as a reservoir for vitamin D.
324 The increase in 25(OH)D in eWAT did not change in time in concentration terms but was
325 stronger in quantity terms at 11 weeks than at 7 weeks (3.2 and 2.7-fold increase, respectively),
326 suggesting that increase of 25(OH)D quantity in eWAT resulted from an induction of production
327 between week 7 and week 11 weeks, consistent with the induction of Cyp2r1.
328 Despite decreased expression of Cyp27b1 and increased expression of Vdr, which suggested a
329 local activation of vitamin D signaling, we observed no change in concentration and quantity of
330 1,25(OH)₂D in eWAT, possibly due to the very low level of 1,25(OH)₂D in AT, which was
331 really near the limit of quantification.

332

333 To conclude, in our model of HF diet-induced obesity with constant vitamin D3 intake, we
334 observed a transient increase of total 25(OH)D together with a decrease of vitamin D3 in plasma
335 that could be due to the upregulation of 25-hydroxylase genes in the liver. Over a longer period,
336 we found a decrease of free 25(OH)D, strongly associated to the induction of Cyp2r1 in adipose
337 tissue, which could be responsible for the active production and storage of 25(OH)D highlighted
338 in adipose tissue by direct quantification. Taken together, our data suggest that adipose tissue
339 plays an important active role in the modulation of vitamin D metabolism observed during
340 obesity.

341

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344

345 **Figure legends**

346

347 **Figure 1: Relationship between biochemical and morphological parameters and free**
348 **25(OH)D in high-fat diet-fed mice.**

349 Correlation between plasma free 25(OH)D concentration and final body weight (A) and
350 adiposity index (B) of control or high-fat diet-fed mice (HF) at 7 and 11 weeks (control n=10,
351 HF n=10). Correlation between plasma free 25(OH)D concentration and plasma PTH
352 concentration (C) of control or high-fat diet-fed mice (HF) at 11 weeks (control n=10, HF n=10).
353 Correlation between quantity of 25(OH)D in eWAT and plasma free 25(OH)D (D) of control or
354 high-fat diet-fed mice (HF) at 7 and 11 weeks (control n=10, HF n=10).

355

356 **Figure 2: Effect of high-fat diet on hepatic vitamin D metabolism of mice.**

357 Expression of genes coding 25-hydroxylases (Cyp27a1, Cyp2j6 and Cyp2r1) relative to 18S
358 ribosomal RNA in the liver of mice fed a control diet or a high-fat diet (HF) for 7 weeks (control
359 n=10, HF n=10). mRNA levels were measured by quantitative rt-PCR. Values are reported as
360 means \pm SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer
361 *post hoc* test at $p < 0.05$. T, time effect in two-way ANOVA ($p < 0.05$); D, diet effect in two-way
362 ANOVA ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA ($p < 0.05$).

363

364 **Figure 3: Effect of high fat diet on adipose tissue vitamin D metabolism of mice.**

365 Expression of genes coding for proteins involved in vitamin D metabolism (Cyp2r1, Cyp2j6,
366 Cyp27a1, cubilin, Vdr and Cyp27b1) relative to 18S ribosomal RNA in epididymal adipose

367 tissue (AT) of mice fed a control diet or a high-fat diet (HF) for 7 weeks (control n=10, HF
368 n=10). mRNA levels were measured by quantitative rt-PCR. Values are reported as means \pm
369 SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer *post hoc*
370 test at $p < 0.05$. T, time effect in two-way ANOVA ($p < 0.05$); D, diet effect in two-way ANOVA
371 ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA ($p < 0.05$).

372

373 **Figure 4: Correlation between Cyp2r1 expression in adipose tissue and biochemical and**
374 **morphological parameters in mice.**

375 Correlation between adipose tissue Cyp2r1 mRNA levels and mice body weight (A), epididymal
376 white adipose tissue mass (B) and plasma free 25(OH)D (C) of mice fed a control diet or a high-
377 fat diet (HF) at 7 and 11 weeks (control n=10, HF n=10).

378

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477

Table 1: Experimental diets composition

Item (g)	Control diet	High fat diet
Sucrose	33.1290	8.8470
Dextrin	29.8560	0
Casein –Vitamin, Tested	18.9560	25.8450
Powdered Cellulose	4.7390	6.40610
Maltodextrin	3.3170	16.1530
Soybean oil	2.3700	3.2310
Lard	1.8960	31.6600
Potassium Citrate, Tribasic Monohydrate	1.5640	2.1320
Calcium Phosphate	1.2320	1.6800
DIO Mineral Mix	0.9480	1.2920
AIN-76A Vitamin mix	0.9480	1.2920
Calcium Carbonate	0.5210	0.7110
L-Cystine	0.2840	0.3880
Choline Bitartrate	0.1900	0.2580
FD&C Yellow 5 Lake	0.0500	0.0500

Total	100	100
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Table 2: Mice morphologic parameters

	7 weeks		11 weeks		Two-way ANOVA
	Control	HF	Control	HF	
Body weight at start (g)	22.4 ± 0.22 ^a	22.2 ± 0.13 ^a	22.4 ± 0.22 ^a	22.2 ± 0.13 ^a	
Body weight at the end (g)	29.2 ± 0.44 ^a	35.4 ± 1 ^b	31.5 ± 0.5 ^b	42.2 ± 1.32 ^c	T, D and TxD
Liver weight (mg)	0.87 ± 0.03 ^a	0.97 ± 0.02 ^a	0.96 ± 0.02 ^a	1.21 ± 0.09 ^b	T, D
Adiposity index	3.12 ± 1.02 ^a	8.56 ± 2.9 ^b	4.9 ± 1.35 ^a	10.25 ± 0.86 ^b	T, D

Adiposity index is the sum of epididymal, retroperitoneal and inguinal adipose tissue, divided by the total body weight. Values are presented as means ± SEM. n = 10 for each group. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$ between control group (control) and high fat group (HF) for 7 and 11 weeks and each condition. T, time effect in two-way ANOVA analysis ($p < 0.05$); D, diet effect in two-way ANOVA analysis ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0.05$).

Table 3: Food intake parameters

	Food intake (g)	Energy intake (kJ/day)	Vitamin D intake (UI/day)
Control	3.8 ± 0.27	60.26 ± 3.39	3.44 ± 0.19
7 and 11 weeks			
HF	2.78 ± 0.18 *	59.30 ± 1.92	3.61 ± 0.18

Dietary intake was assessed one week on two. Values are presented as means ± SEM. Student's t-test was used, p values: *. $p < 0.05$ between control group (control) and high fat group (HF) for 7 and 11 weeks and each condition.

Table 4: Biochemical parameters

	7 weeks		11 weeks		
	Control	HF	Control	HF	Two-way ANOVA
Plasma vitamin D3 concentration (ng/mL)	2.0 ± 0.3 ^a	0.6 ± 0.1 ^b	2.5 ± 0.1 ^a	1.2 ± 0.1 ^{a,b}	D

Plasma total 25(OH)D concentration (ng/mL)	34.8 ± 1.1 ^a	40.5 ± 1.0 ^b	38.4 ± 0.8 ^{a,b}	42.7 ± 1.4 ^b	T, D
Plasma free 25(OH)D concentration (pg/mL)	6.6 ± 0.1 ^{a,b}	6.3 ± 0.1 ^{a,b}	6.7 ± 0.2 ^a	5.9 ± 0.2 ^b	D
Plasma 1,25(OH)₂D concentration (pg/mL)	374 ± 20 ^a	376 ± 27 ^a	240 ± 21 ^b	239 ± 9 ^b	T
Plasma PTH concentration (pg/mL)	75.54 ± 7.16 ^a	127.61 ± 6.91 ^b	122.76 ± 11.74 ^{a,b}	196.76 ± 19.20 ^c	T, D
Plasma Ca²⁺ concentration (mmol/L)	0.093 ± 0.002 ^a	0.097 ± 0.002 ^a	0.099 ± 0.003 ^a	0.1 ± 0.002 ^a	
Plasma Phosphate concentration (mmol/L)	0.045 ± 0.003 ^a	0.042 ± 0.002 ^a	0.045 ± 0.003 ^a	0.046 ± 0.002 ^a	
eWAT vitamin D3 quantity (ng)	12.7 ± 1.0 ^a	26.3 ± 5.0 ^a	28.3 ± 2.8 ^a	54.7 ± 6.0 ^b	T, D
eWAT 25(OH)D quantity (ng)	2.7 ± 0.2 ^a	7.4 ± 0.9 ^b	4.3 ± 0.5 ^a	14.0 ± 1.1 ^c	T, D and TxD
eWAT 1,25(OH)₂D quantity (ng)	1.6 ± 0.3 ^a	3.7 ± 0.8 ^a	4.0 ± 0.6 ^{a,b}	6.0 ± 1.3 ^b	T, D

Plasma and epididymal white adipose tissue (eWAT) concentrations of vitamin D₃, total 25(OH)D and 1,25(OH)₂D were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Concentrations of free 25(OH)D and parathyroid hormone (PTH) were quantified by ELISA. Phosphate and Ca²⁺ plasma concentrations were quantified by colorimetric assay kits. Values are presented as means ± SEM Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$ between control group (control) and high fat group (HF) for 7 and 11 weeks and each condition (control n=10, HF n=10). T, time effect in two-way ANOVA analysis ($p < 0.05$); D, diet effect in two-way ANOVA analysis ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0.05$).

Figure 1

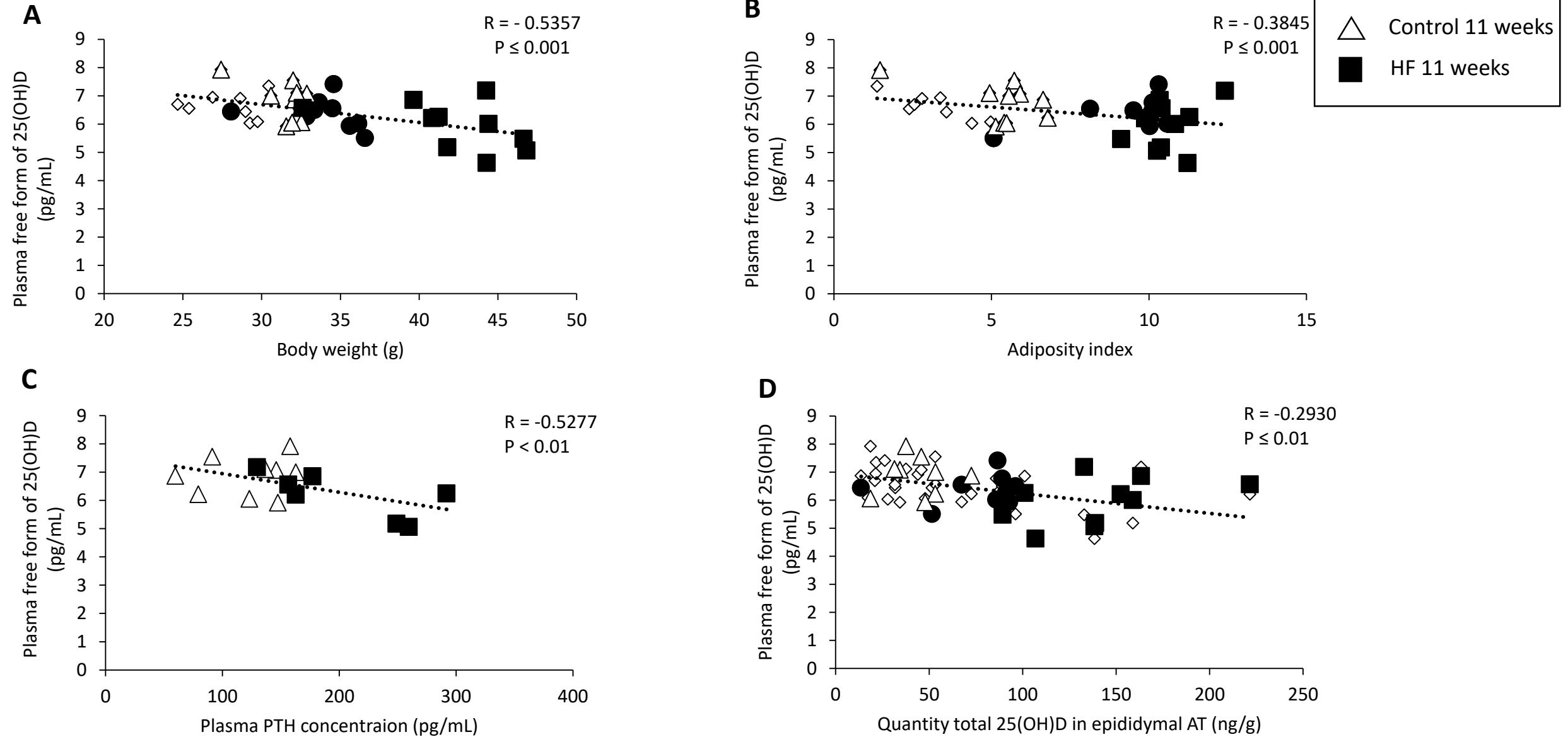


Figure 2

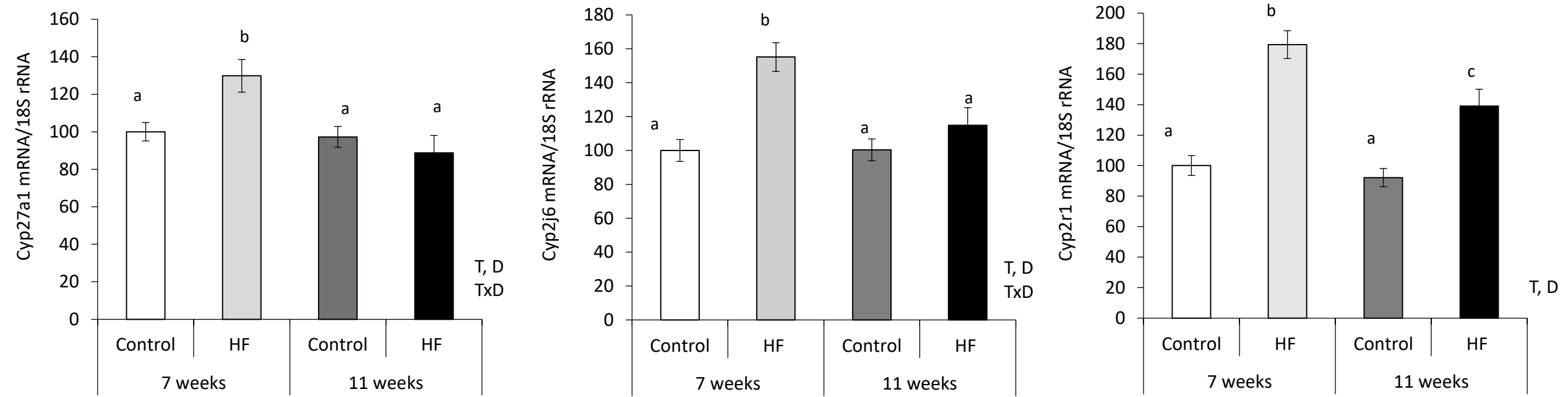


Figure 3

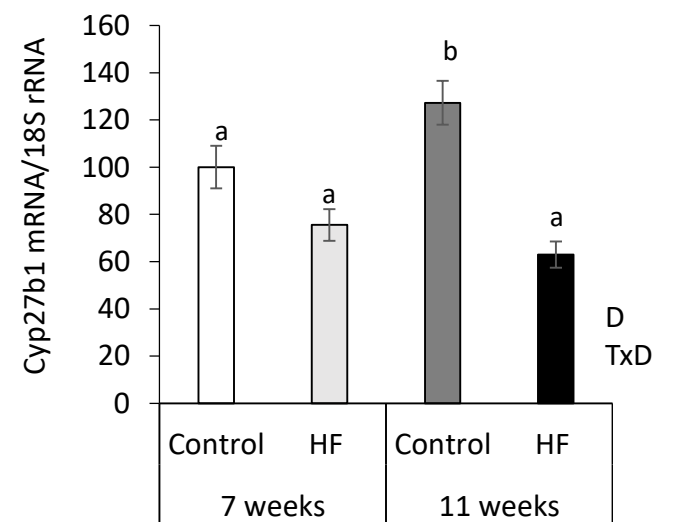
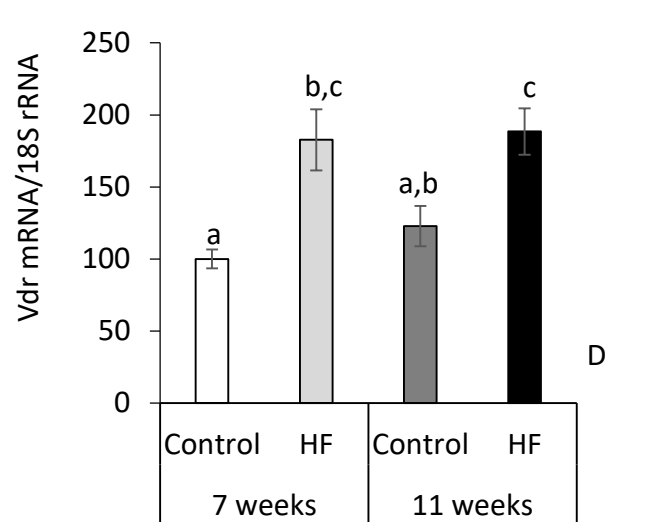
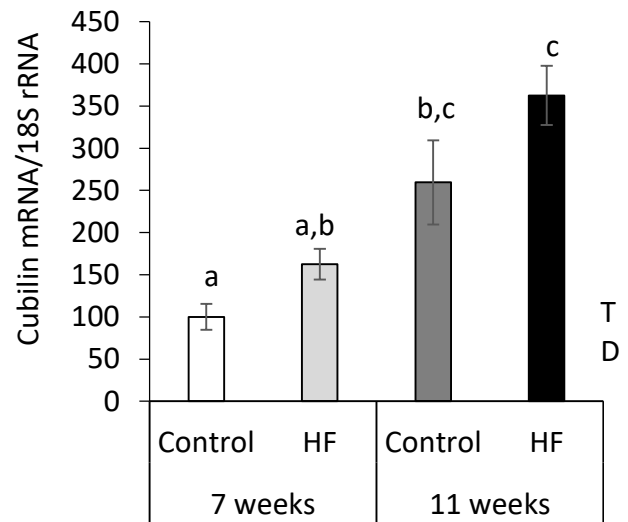
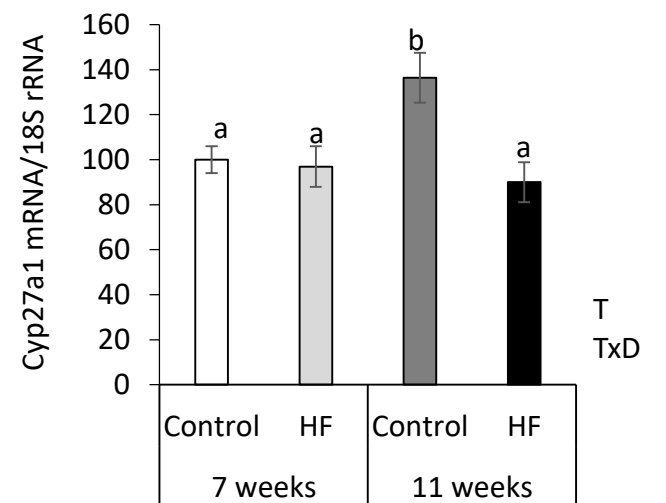
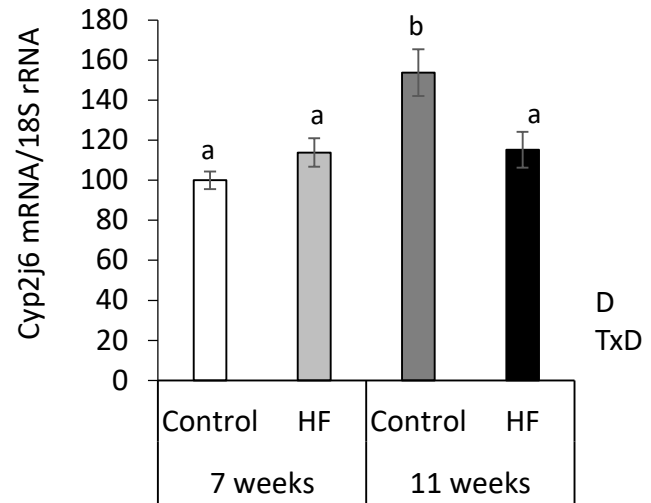
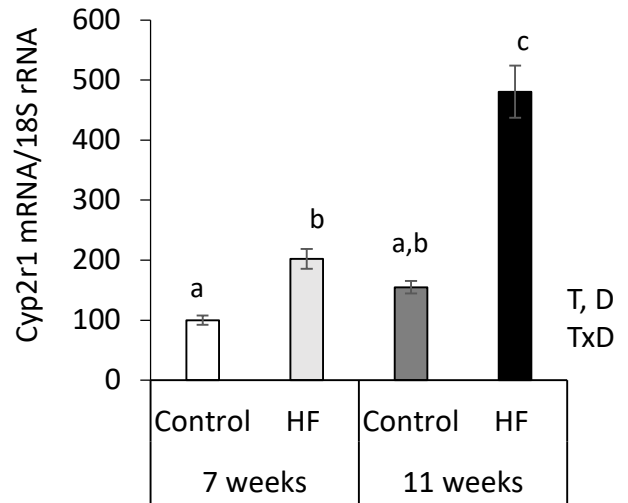


Figure 4

