



## Diet induced obesity modifies vitamin D metabolism and adipose tissue storage in mice

Lauriane Bonnet, Julien Astier, Mohammed Amine Hachemi, Esma Karkeni,  
Charlène Couturier, Catherine Defoort, Ljubica Svilar, Jean-Charles Martin,  
Franck Tourniaire, Jean-Francois Landrier

### ► To cite this version:

Lauriane Bonnet, Julien Astier, Mohammed Amine Hachemi, Esma Karkeni, Charlène Couturier, et al.. Diet induced obesity modifies vitamin D metabolism and adipose tissue storage in mice. Journal of Steroid Biochemistry and Molecular Biology, 2019, 185, pp.39-46. 10.1016/j.jsbmb.2018.07.006 . hal-01998526

**HAL Id: hal-01998526**

**<https://amu.hal.science/hal-01998526>**

Submitted on 29 Jan 2019

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

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



Distributed under a Creative Commons Attribution 4.0 International License

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

Lauriane Bonnet<sup>1</sup>, Mohammed Amine Hachemi<sup>1</sup>, Esma Karkeni<sup>1</sup>, Charlene Couturier<sup>1</sup>, Julien Astier<sup>1</sup>, Catherine Defoort<sup>1,2</sup>, Ljubica Svilar<sup>1,2</sup>, Jean-Charles Martin<sup>1,2</sup>, Franck Tourniaire<sup>1,2</sup>, Jean-François Landrier<sup>1,2</sup>

<sup>1</sup> NORT, Aix-Marseille Université, INRA, INSERM, 13000 Marseille, France

<sup>2</sup> CriBioM, Criblage Biologique Marseille, Faculté de Médecine de la Timone, Marseille, France.

Abbreviated title: Obesity impacts vitamin D metabolism.

*Address correspondence and reprint requests to:*

Jean-François Landrier, UMR 1260 INRA/1062 INSERM/Université d'Aix-Marseille, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France.

Phone: +33 4 91 32 42 75; Fax: +33 4 91 78 21 01; e-mail: [jean-francois.landrier@univ-amu.fr](mailto:jean-francois.landrier@univ-amu.fr)

Disclosure statement: The authors have nothing to disclose.

## Abstract

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

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

## Introduction

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

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

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

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

## Material and methods

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

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

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

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

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

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

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

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

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

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

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



repeated twice, and the combined supernatants were dried under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with ethyl acetate, methanol (MetOH) and H<sub>2</sub>O. The sample was reconstituted with 1 mL of MetOH and 1 mL of K<sub>2</sub>HPO<sub>4</sub> (0.4 M) then added onto the cartridge. The cartridge was washed with H<sub>2</sub>O and 70% MetOH then dried for 2 min under vacuum. Tips were washed with ACN, and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of solvent, the samples were derivatized as above.

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

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

The Parallel reaction monitoring (PRM) transitions used for quantification of each analyte were:

716.5→657.5 (vitamin D3), 719.5→660.5 (d3-vitamin D3), 732.5→673.4 (25(OH)D), 735.5→676.4 (d3-25(OH)D), 751.5→692.4 (d3-1,25(OH)<sub>2</sub>D) and 748.5→689.4 (1,25(OH)<sub>2</sub>D).

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

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

## Results

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

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

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

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

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

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

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

## Discussion

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

As expected, the HF diet used in our study of weight gain led to a significant increase in total mass of the animals as well as in amount of AT and adiposity index at the two timepoints studied, i.e. at 7 and 11 weeks. These morphological changes were not accompanied by major changes in energy intake or in consumption of vitamin D3. This vitamin D3 parameter was particularly important to control here, since a modification in vitamin D3 intake induced by poorly-balanced diet alone could have led to a vitamin D deficiency. However, despite balanced vitamin D3 throughout the diet, at week 7 plasma vitamin D3 content had decreased and total 25(OH)D and PTH concentrations had increased. These are novel findings, apart from the increased PTH as PTH concentration is known to be positively correlated with fat mass [26] and body mass index [14]. The reduction of vitamin D3 is probably due to direct trapping of this hydrophobic molecule in expanded AT. The fact that vitamin D3 decreased whereas total 25(OH)D increased is harder to explain, but could be linked to the overall induction of mRNA coding for hepatic 25-hydroxylation. Indeed, even if the expanding AT will store volumetrically more 25(OH)D, the fact that 25-hydroxylation is induced at least partly explains the reduced vitamin D3 levels. It was fairly surprising that these 25-hydroxylation enzymes were upregulated here, since another study in similar experimental conditions found a down-regulation [19]. This 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)<sub>2</sub>D, 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)<sub>2</sub>D, despite the obesity-associated secondary  
267 hyperparathyroidism observed at 7 and 11 weeks, we found no change in 1,25(OH)<sub>2</sub>D 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)<sub>2</sub>D 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 Wortsman et al. [16] who suggested that the plasma 25(OH)D decrease observed

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

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



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

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

adipose stores of vitamin D2 and D3, supporting the hypothesis that the large amount of AT in obese individuals serves as a reservoir for vitamin D.

The increase in 25(OH)D in eWAT did not change in time in concentration terms but was stronger in quantity terms at 11 weeks than at 7 weeks (3.2 and 2.7-fold increase, respectively), suggesting that increase of 25(OH)D quantity in eWAT resulted from an induction of production between week 7 and week 11 weeks, consistent with the induction of Cyp2r1.

Despite decreased expression of Cyp27b1 and increased expression of Vdr, which suggested a local activation of vitamin D signaling, we observed no change in concentration and quantity of 1,25(OH)<sub>2</sub>D in eWAT, possibly due to the very low level of 1,25(OH)<sub>2</sub>D in AT, which was really near the limit of quantification.

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

**Acknowledgments:** This study was funded through grants from the INRA, the Inserm, and Aix-Marseille University (to JFL).

## Figure legends

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

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

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

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

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

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

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

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

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

**References**

- [1] M.F. Holick, Vitamin D deficiency, *N Engl J Med*, 357 (2007) 266-281.
- [2] H.F. DeLuca, Vitamin D: Historical Overview, *Vitam Horm*, 100 (2016) 1-20.
- [3] A. Schmid, B. Walther, Natural vitamin D content in animal products, *Adv Nutr*, 4 (2013) 453-462.
- [4] J.F. Landrier, J. Marcotorchino, F. Tourniaire, Lipophilic micronutrients and adipose tissue biology, *Nutrients*, 4 (2012) 1622-1649.
- [5] I. Schuster, Cytochromes P450 are essential players in the vitamin D signaling system, *Biochim Biophys Acta*, 1814 (2011) 186-199.
- [6] C. Carlberg, S. Seuter, A genomic perspective on vitamin D signaling, *Anticancer Res*, 29 (2009) 3485-3493.
- [7] A.S. Dusso, A.J. Brown, E. Slatopolsky, Vitamin D, *Am J Physiol Renal Physiol*, 289 (2005) F8-28.
- [8] D.D. Bikle, E. Gee, B. Halloran, M.A. Kowalski, E. Ryzen, J.G. Haddad, Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein, *J Clin Endocrinol Metab*, 63 (1986) 954-959.
- [9] J.G. Haddad, D.R. Fraser, D.E. Lawson, Vitamin D plasma binding protein. Turnover and fate in the rabbit, *J Clin Invest*, 67 (1981) 1550-1560.
- [10] C.P. Earthman, L.M. Beckman, K. Masodkar, S.D. Sibley, The link between obesity and low circulating 25-hydroxyvitamin D concentrations: considerations and implications, *Int J Obes (Lond)*, 36 (2012) 387-396.

- [11] N. Vilarrasa, J. Maravall, A. Estepa, R. Sanchez, C. Masdevall, M.A. Navarro, P. Alia, J. Soler, J.M. Gomez, Low 25-hydroxyvitamin D concentrations in obese women: their clinical significance and relationship with anthropometric and body composition variables, *J Endocrinol Invest*, 30 (2007) 653-658.
- [12] O.P. Garcia, K.Z. Long, J.L. Rosado, Impact of micronutrient deficiencies on obesity, *Nutr Rev*, 67 (2009) 559-572.
- [13] S. Cheng, J.M. Massaro, C.S. Fox, M.G. Larson, M.J. Keyes, E.L. McCabe, S.J. Robins, C.J. O'Donnell, U. Hoffmann, P.F. Jacques, S.L. Booth, R.S. Vasan, M. Wolf, T.J. Wang, Adiposity, cardiometabolic risk, and vitamin D status: the Framingham Heart Study, *Diabetes*, 59 (2010) 242-248.
- [14] J.S. Walsh, A.L. Evans, S. Bowles, K.E. Naylor, K.S. Jones, I. Schoenmakers, R.M. Jacques, R. Eastell, Free 25-hydroxyvitamin D is low in obesity, but there are no adverse associations with bone health, *Am J Clin Nutr*, 103 (2016) 1465-1471.
- [15] N.H. Bell, S. Shaw, R.T. Turner, Evidence that 1,25-dihydroxyvitamin D<sub>3</sub> inhibits the hepatic production of 25-hydroxyvitamin D in man, *J Clin Invest*, 74 (1984) 1540-1544.
- [16] J. Wortsman, L.Y. Matsuoka, T.C. Chen, Z. Lu, M.F. Holick, Decreased bioavailability of vitamin D in obesity, *Am J Clin Nutr*, 72 (2000) 690-693.
- [17] A.T. Drincic, L.A. Armas, E.E. Van Diest, R.P. Heaney, Volumetric dilution, rather than sequestration best explains the low vitamin D status of obesity, *Obesity (Silver Spring)*, 20 (2012) 1444-1448.
- [18] L. Wamberg, T. Christiansen, S.K. Paulsen, S. Fisker, P. Rask, L. Rejnmark, B. Richelsen, S.B. Pedersen, Expression of vitamin D-metabolizing enzymes in human adipose tissue -- the effect of obesity and diet-induced weight loss, *Int J Obes (Lond)*, 37 (2013) 651-657.
- [19] J.M. Park, C.Y. Park, S.N. Han, High fat diet-Induced obesity alters vitamin D metabolizing enzyme expression in mice, *Biofactors*, 41 (2015) 175-182.
- [20] J.F. Landrier, C. Malezet-Desmoulins, E. Reboul, A. Marie Lorec, M. Josephe Amiot, P. Borel, Comparison of different vehicles to study the effect of tocopherols on gene expression in intestinal cells, *Free Radic Res*, 42 (2008) 523-530.
- [21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(-Delta Delta C(T)) Method, *Methods*, 25 (2001) 402-408.
- [22] L. Bonnet, E. Karkeni, C. Couturier, J. Astier, J. Dalifard, C. Defoort, L. Svilar, J.C. Martin, F. Tourniaire, J.F. Landrier, Gene expression pattern in response to cholecalciferol supplementation highlights cubilin as a major protein of 25(OH)D uptake in adipocytes and male mice white adipose tissue, *Endocrinology*, (2017).
- [23] C.J. Hedman, D.A. Wiebe, S. Dey, J. Plath, J.W. Kemnitz, T.E. Ziegler, Development of a sensitive LC/MS/MS method for vitamin D metabolites: 1,25 Dihydroxyvitamin D<sub>2&3</sub> measurement using a novel derivatization agent, *J Chromatogr B Analyt Technol Biomed Life Sci*, 953-954 (2014) 62-67.
- [24] Z. Wang, T. Senn, T. Kalhorn, X.E. Zheng, S. Zheng, C.L. Davis, M.F. Hebert, Y.S. Lin, K.E. Thummel, Simultaneous measurement of plasma vitamin D(3) metabolites, including 4beta,25-dihydroxyvitamin D(3), using liquid chromatography-tandem mass spectrometry, *Anal Biochem*, 418 (2011) 126-133.
- [25] T.E. Lipkie, A. Janasch, B.R. Cooper, E.E. Hohman, C.M. Weaver, M.G. Ferruzzi, Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography-tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci*, 932 (2013) 6-11.
- [26] M.B. Snijder, R.M. van Dam, M. Visser, D.J. Deeg, J.M. Dekker, L.M. Bouter, J.C. Seidell, P. Lips, Adiposity in relation to vitamin D status and parathyroid hormone levels: a population-based study in older men and women, *J Clin Endocrinol Metab*, 90 (2005) 4119-4123.
- [27] I.N. Sergeev, Q. Song, High vitamin D and calcium intakes reduce diet-induced obesity in mice by increasing adipose tissue apoptosis, *Mol Nutr Food Res*, 58 (2014) 1342-1348.
- [28] Q. Song, I.N. Sergeev, High vitamin D and calcium intakes increase bone mineral (Ca and P) content in high-fat diet-induced obese mice, *Nutr Res*, 35 (2015) 146-154.

- [29] M.S. Johnsen, G. Grimnes, Y. Figenschau, P.A. Torjesen, B. Almas, R. Jorde, Serum free and bio-available 25-hydroxyvitamin D correlate better with bone density than serum total 25-hydroxyvitamin D, *Scand J Clin Lab Invest*, 74 (2014) 177-183.
- [30] K.S. Vimalaswaran, D.J. Berry, C. Lu, E. Tikkanen, S. Pilz, L.T. Hiraki, J.D. Cooper, Z. Dastani, R. Li, D.K. Houston, A.R. Wood, K. Michaelsson, L. Vandenput, L. Zgaga, L.M. Yerges-Armstrong, M.I. McCarthy, J. Dupuis, M. Kaakinen, M.E. Kleber, K. Jameson, N. Arden, O. Raitakari, J. Viikari, K.K. Lohman, L. Ferrucci, H. Melhus, E. Ingelsson, L. Byberg, L. Lind, M. Lorentzon, V. Salomaa, H. Campbell, M. Dunlop, B.D. Mitchell, K.H. Herzig, A. Pouta, A.L. Hartikainen, G.C. Genetic Investigation of Anthropometric Traits, E.A. Streeten, E. Theodoratou, A. Jula, N.J. Wareham, C. Ohlsson, T.M. Frayling, S.B. Kritchevsky, T.D. Spector, J.B. Richards, T. Lehtimäki, W.H. Ouwehand, P. Kraft, C. Cooper, W. Marz, C. Power, R.J. Loos, T.J. Wang, M.R. Jarvelin, J.C. Whittaker, A.D. Hingorani, E. Hyppönen, Causal relationship between obesity and vitamin D status: bi-directional Mendelian randomization analysis of multiple cohorts, *PLoS Med*, 10 (2013) e1001383.
- [31] J. Marcotrichino, F. Tourniaire, J. Astier, E. Karkeni, M. Canault, M.J. Amiot, D. Bendahan, M. Bernard, J.C. Martin, B. Giannesini, J.F. Landrier, Vitamin D protects against diet-induced obesity by enhancing fatty acid oxidation, *J Nutr Biochem*, 25 (2014) 1077-1083.
- [32] J.F. Landrier, E. Karkeni, J. Marcotrichino, L. Bonnet, F. Tourniaire, Vitamin D modulates adipose tissue biology: possible consequences for obesity?, *Proceedings of the Nutrition Society*, 75 (2016) 38-46.
- [33] E. Karkeni, L. Bonnet, J. Marcotrichino, F. Tourniaire, J. Astier, J. Ye, J.F. Landrier, Vitamin D limits inflammation-linked microRNA expression in adipocytes in vitro and in vivo: A new mechanism for the regulation of inflammation by vitamin D, *Epigenetics*, (2017) 0.
- [34] E. Karkeni, J. Marcotrichino, F. Tourniaire, J. Astier, F. Peiretti, P. Darmon, J.F. Landrier, Vitamin D limits chemokine expression in adipocytes and macrophage migration in vitro and in male mice, *Endocrinology*, 156 (2015) 1782-1793.
- [35] J. Marcotrichino, E. Gouranton, B. Romier, F. Tourniaire, J. Astier, C. Malezet, M.J. Amiot, J.F. Landrier, Vitamin D reduces the inflammatory response and restores glucose uptake in adipocytes, *Mol Nutr Food Res*, 56 (2012) 1771-1782.
- [36] J.G. Zhu, J.T. Ocholek, M. Kaufmann, G. Jones, H.F. Deluca, CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo, *Proc Natl Acad Sci U S A*, 110 (2013) 15650-15655.
- [37] A. Carrelli, M. Bucovsky, R. Horst, S. Cremers, C. Zhang, M. Bessler, B. Schroppe, J. Evanko, J. Blanco, S.J. Silverberg, E.M. Stein, Vitamin D Storage in Adipose Tissue of Obese and Normal Weight Women, *J Bone Miner Res*, 32 (2017) 237-242.

**Table 1:** Experimental diets composition

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

<b>Total</b>	100	100
--------------	-----	-----

**Table 2: Mice morphologic parameters**

	<b>7 weeks</b>		<b>11 weeks</b>		
	<b>Control</b>	<b>HF</b>	<b>Control</b>	<b>HF</b>	<b>Two-way ANOVA</b>
<b>Body weight at start (g)</b>	22.4 ± 0.22 <sup>a</sup>	22.2 ± 0.13 <sup>a</sup>	22.4 ± 0.22 <sup>a</sup>	22.2 ± 0.13 <sup>a</sup>	
<b>Body weight at the end (g)</b>	29.2 ± 0.44 <sup>a</sup>	35.4 ± 1 <sup>b</sup>	31.5 ± 0.5 <sup>b</sup>	42.2 ± 1.32 <sup>c</sup>	T, D and TxD
<b>Liver weight (mg)</b>	0.87 ± 0.03 <sup>a</sup>	0.97 ± 0.02 <sup>a</sup>	0.96 ± 0.02 <sup>a</sup>	1.21 ± 0.09 <sup>b</sup>	T, D
<b>Adiposity index</b>	3.12 ± 1.02 <sup>a</sup>	8.56 ± 2.9 <sup>b</sup>	4.9 ± 1.35 <sup>a</sup>	10.25 ± 0.86 <sup>b</sup>	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)
7 and 11 weeks	Control	3.8 ± 0.27	60.26 ± 3.39	3.44 ± 0.19
	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		Two-way ANOVA
	Control	HF	Control	HF	
Plasma vitamin D3 concentration (ng/mL)	2.0 ± 0.3 <sup>a</sup>	0.6 ± 0.1 <sup>b</sup>	2.5 ± 0.1 <sup>a</sup>	1.2 ± 0.1 <sup>a,b</sup>	D

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

Plasma and epididymal white adipose tissue (eWAT) concentrations of vitamin D<sub>3</sub>, total 25(OH)D and 1,25(OH)<sub>2</sub>D 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 Ca<sup>2+</sup> 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**

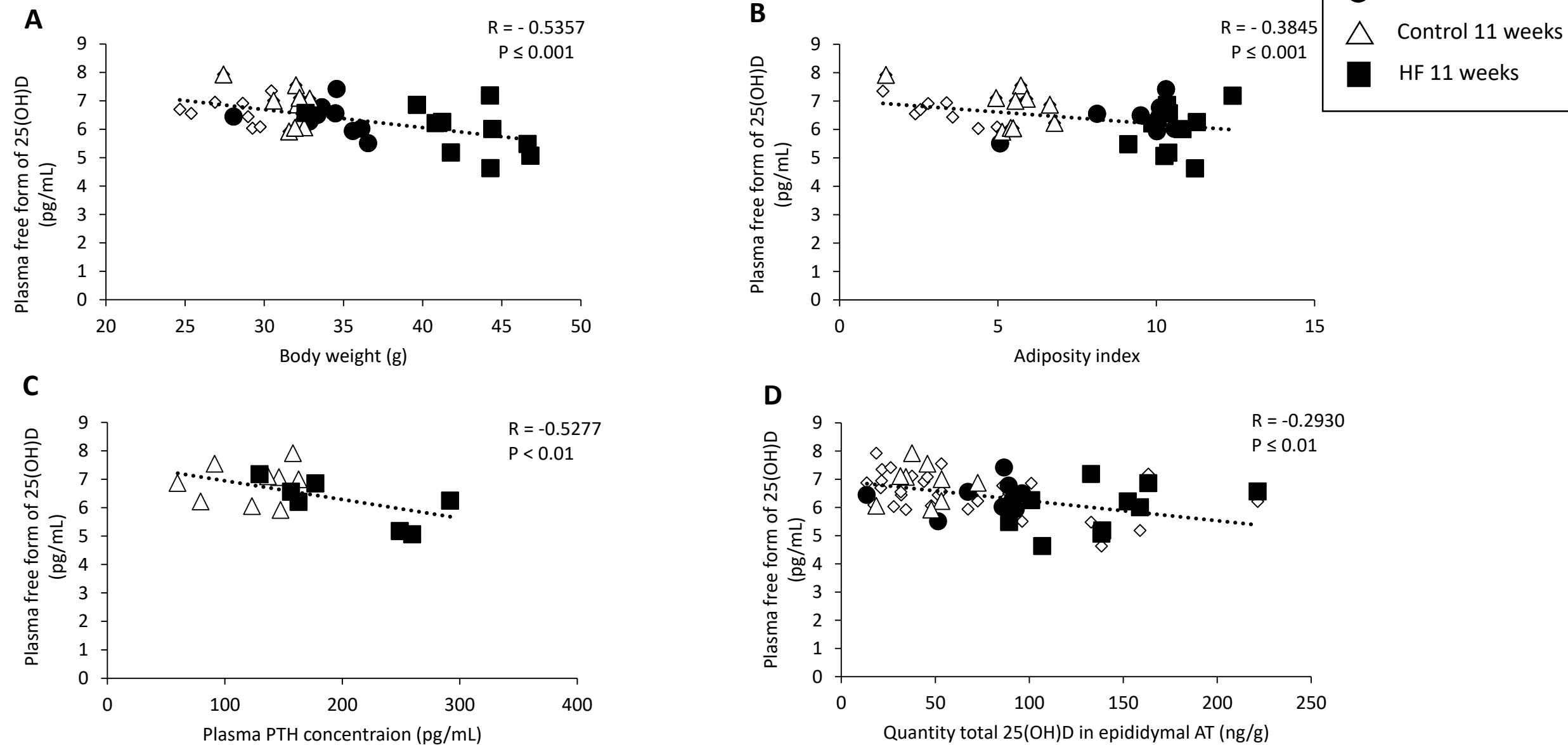


Figure 2

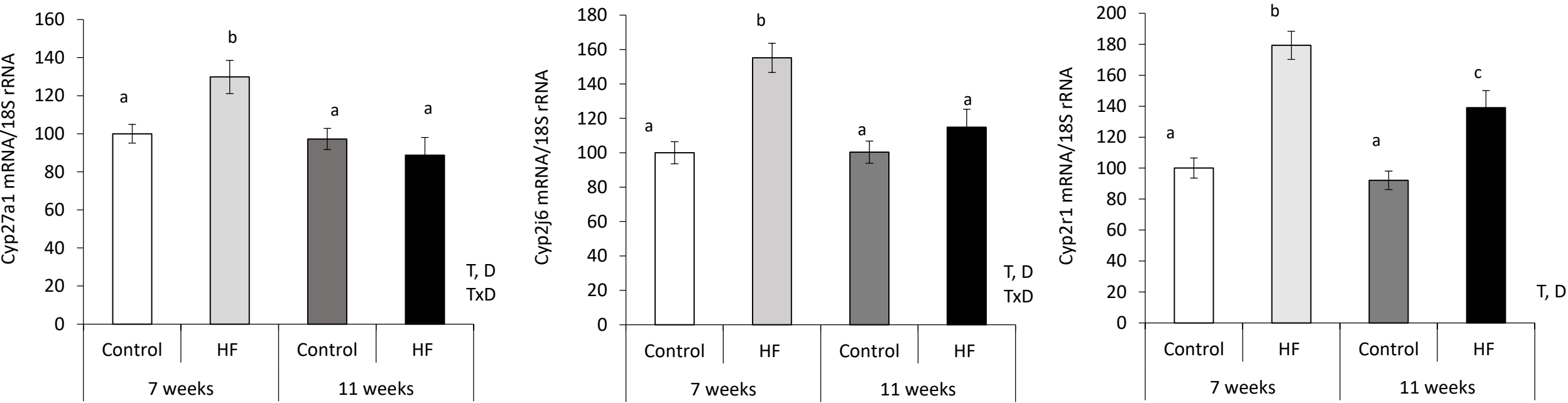


Figure 3

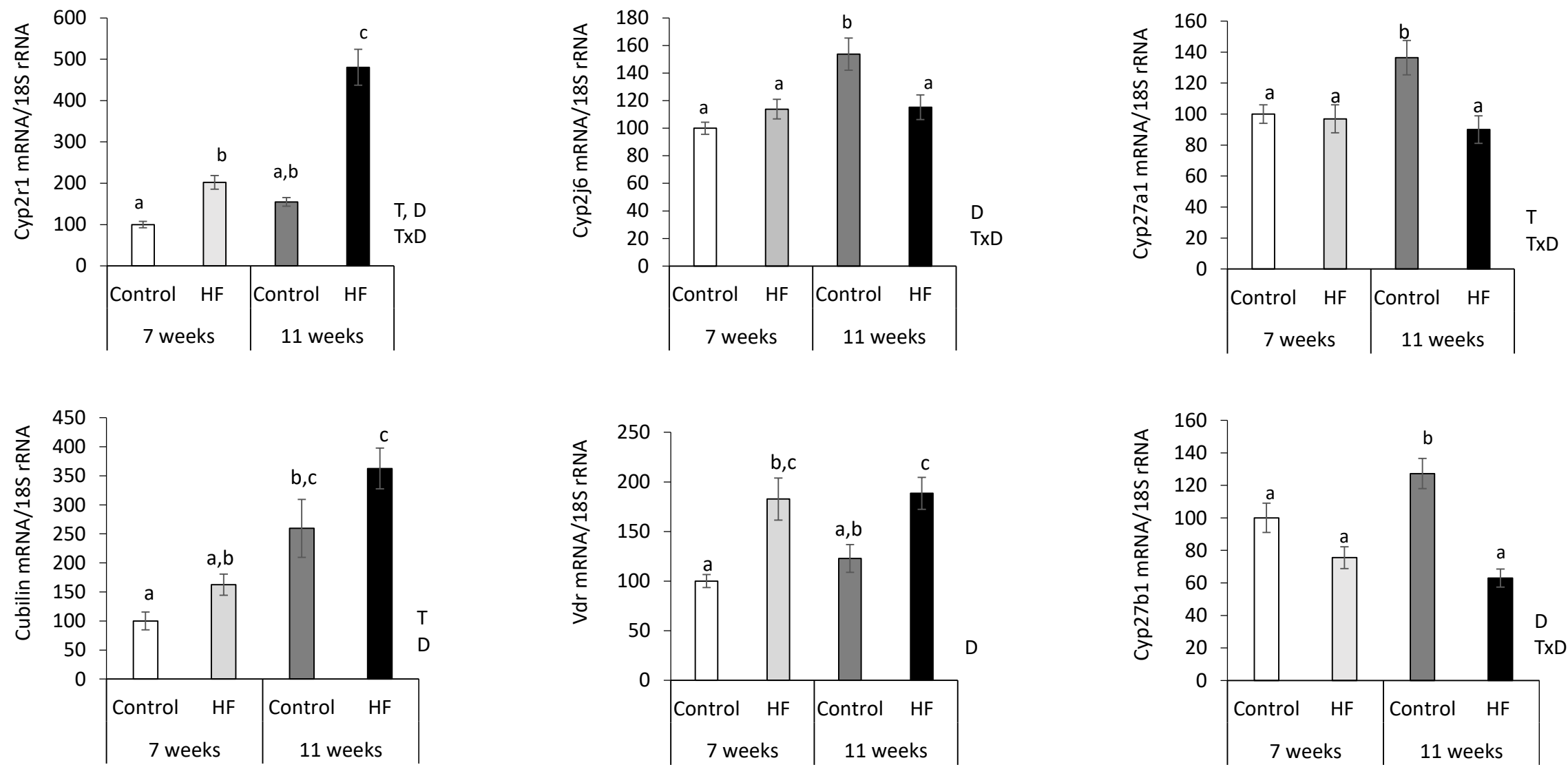


Figure 4

