

Expérimentation animale

Le modèle expérimental choisi pour la réalisation des études a été la souris c57bl/6j. C'est un modèle couramment utilisé en laboratoire susceptible de développer à la fois le diabète et l'obésité par des interventions nutritionnelles (Loustau et al., 2020; Petro et al., 2000; Shafrir and Ziv, 2009).

Les expérimentations ont été réalisées sur des souris c57bl/6j âgées de 8 semaines (n=110) et ont été hébergées en animalerie dans des conditions contrôlées avec un cycle de 12 heures lumière-obscurité, avec une humidité avoisinant les 60% ainsi qu'une température d'environ 21 °C. Les souris ont eu un accès *ad libitum* à la nourriture et à l'eau. Les souris ont été acclimatées 2 semaines afin de débiter les protocoles à l'âge de 10 semaines. Le protocole d'étude est résumé sur le schéma présenté ci-dessous (Figure 19) :

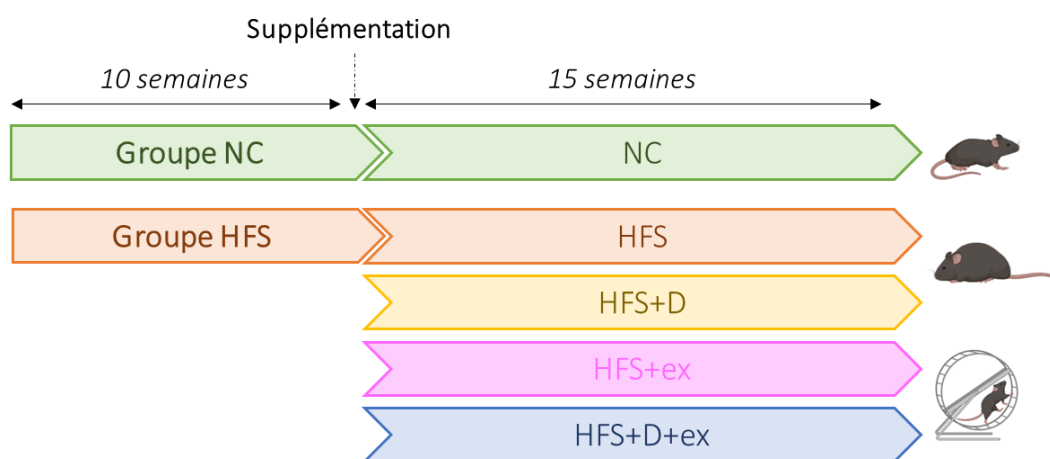


Figure 19 : Schéma expérimental. Groupe NC (Normal Chow) : n=20 ; groupe HFS (High fat high sucrose) : n=30 ; groupe HFS+D (HFS supplémentation en vitamine D) : n=30 ; HFS+ex (HFS + exercice) : n=15 ; HFS+D+ex (HFS supplémentation en vitamine D + exercice) : n=15.

Dans un premier temps, les souris ont été réparties aléatoirement en deux groupes de régime :

- Le groupe « normal chow » (NC) correspondant au régime standard (croquettes A04 (SAFE Diets, Augy, France) et de l'eau.
- Le groupe « High Fat high Sucrose » (HFS) correspondant au régime hypercalorique riche en graisse et en sucre (pâte HF230 (SAFE Diets, Augy France) et de 10% de saccharose contenu dans l'eau de boisson) (Figure 20).

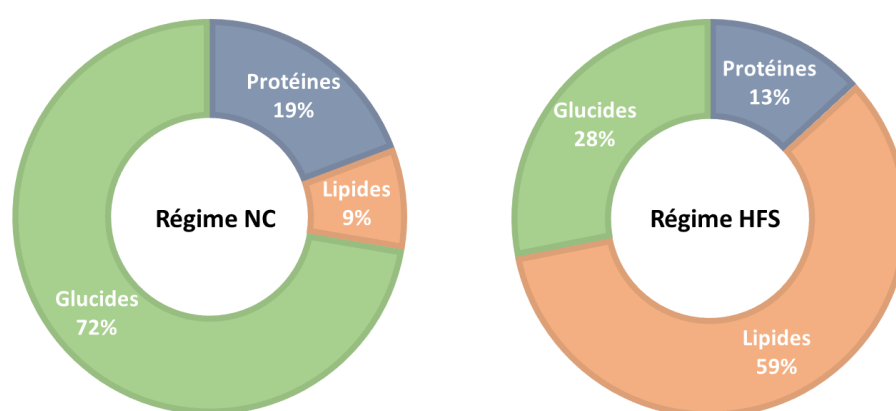


Figure 20 : Composition des différents régimes. NC : Normal Chow ; HFS : High Fat high Sucrose diet.

Ce régime obésogène a été appliqué durant 10 semaines afin d'induire un phénotype obèse chez les souris c57bl/6j. S'en est suivie, une randomisation des souris HFS dans différents groupes où la supplémentation en vitamine D et l'exercice physique vont être ajoutés à la nourriture HFS :

- Le groupe HFS supplémenté en vitamine D₃ (HFS+D) correspondant au régime HFS enrichie en VD à 15 000 UI/kg (SAFE Diets, Augy, France).
- Le groupe HFS+ex correspondant au régime HFS couplé à de l'exercice physique volontaire, où une roue a été installée dans la cage.
- Le groupe HFS+D+ex correspondant au régime HFS+D couplé à l'exercice physique volontaire.

2. Mesures longitudinales

Des mesures longitudinales de suivi des paramètres d'apport et de dépense énergétique ainsi que des paramètres morphologiques ont été réalisées tout au long du protocole.

2.1. Suivi des apports alimentaires

L'apport alimentaire, que ce soit de la nourriture ou de la consommation de boisson, a été évalué à chaque renouvellement dans les cages, à raison de 3 fois par semaine. Grâce aux mesures de la prise alimentaire, le contrôle de la supplémentation en vitamine D a pu être réalisé. Les résultats sont présentés en Annexe.

2.2. Mesure des distances parcourues

Les roues placées dans les cages des souris étaient munies d'un capteur, connectées à des compteurs permettant le relevé du nombre de tours effectués permettant d'évaluer quotidiennement la distance parcourue par les souris.

2.3. Suivi morphologique

Les animaux ont été pesés de manière hebdomadaire à partir du début du protocole (T0), à l'âge de 10 semaines et ce jusqu'à la fin du protocole (T25), à l'âge de 35 semaines afin d'avoir une évolution de leur masse corporelle au cours du protocole.

Les mesures du suivi de l'homéostasie glucidique et de la vitesse maximale aérobie ont été réalisées : avant la mise en place du régime obésogène (T0), avant la mise en place des supplémentations (T10), au milieu des supplémentations (T17) et à la fin du protocole (T24).

2.4. Suivi de l'homéostasie glucidique

Le suivi de l'homéostasie glucidique a été réalisé par le test de tolérance à l'insuline sur des souris mises à jeun 6 heures avant le début des mesures. Par une incision en bout de queue des animaux, les prélèvements sanguins ont pu être réalisés afin d'obtenir la glycémie avec le lecteur à bandelettes (CareSens N™, Dinno Santé,

Bussy Saint-Georges, France). Une fois la glycémie à jeun relevée, une injection intrapéritonéale d'insuline à 1 UI.kg^{-1} a été réalisée et de nouvelles mesures de glycémie ont été relevées à 10, 30, 60, 90 et 120 minutes post-injection.

Des mesures plasmatiques de glucose et d'insuline ont également été réalisées sur les échantillons de plasma recueillis après le sacrifice des animaux, à l'aide d'un test colorimétrique et d'un dosage immuno-enzymatique.

2.5. Mesure de la vitesse maximale aérobie

La vitesse maximale aérobie (VMA) a été réalisée sur des tapis de courses où les souris ont eu un échauffement de 6 minutes à une vitesse de 7 m.min^{-1} puis de niveaux croissants de 4 m.min^{-1} toutes les 1'30 minutes. La VMA est atteinte lorsque les souris n'étaient plus capables de suivre la vitesse du tapis. Le palier précédent est alors considéré comme maximal.

3. Sacrifice des animaux

A la fin des 25 semaines de protocole, les souris ont été mises à jeun d'environ 12 heures puis anesthésiées par inhalation d'un mélange à 4% d'isoflurane (IsoFlo™, Zoetis, Parsippany, Etats-Unis) afin de réaliser un prélèvement sanguin par ponction intracardiaque sur seringue héparinée (S-Monovette®, Sarsted, Nümbrecht, Allemagne). Après centrifugation, le plasma a été aliquoté et congelé à -80°C avant analyse.

Les souris ont ensuite été mises à mort par dislocation cervicale et les différents organes, cœur, foie, et rein ont été prélevés et pesés. L'ensemble des tissus adipeux épидидymal, sous-cutané, inguinal, périrénal et brun ont été prélevés et pesés. Les muscles soléaire et gastrocnémien ont également été prélevés. En guise d'indicateur, la rate a été prélevée et pesée afin d'évaluer le statut inflammatoire des souris. Si celui-ci est trop élevé, la souris est exclue.

Ces différents tissus ont été soit fixés dans la formaline puis rincés dans du tampon phosphate alcalin et conservés dans l'éthanol à 70% à $+4^{\circ}\text{C}$ pour analyse histologique soit congelés dans l'azote liquide et conservés à -80°C pour analyse biochimique et moléculaire.

4. Mesure des concentrations plasmatiques de vitamine D₃

Les échantillons de plasma ont été utilisés afin de mesurer les concentrations plasmatiques de cholécalciférol et de vitamine D totale et de confirmer l'effet de la supplémentation en vitamine D₃. Ceci a été réalisé par chromatographie liquide couplée à la spectrométrie de masse (LC-MS/MS) (Bonnet et al., 2019b).

5. Extraction des ARN totaux

Les ARN totaux ont été extraits à partir de tissus congelés. Le tissu a tout d'abord été homogénéisé à l'aide d'un robot à billes (Retsch GmbH, Haan, Allemagne) dans 1 mL de TRI Reagent (Invitrogen) permettant la lyse des cellules tout en préservant l'intégrité des molécules d'ADN et d'ARN. Les échantillons ont ensuite été mélangés à 200 µL de chloroforme afin de solubiliser les lipides et centrifugés pendant 15 minutes à 1500g et à +4 °C. La solution triphasée obtenue a permis de différencier la phase organique (ADN, protéines) de la phase aqueuse contenant l'ARN délimitée par une interface. La phase supérieure a été transférée dans un nouveau tube en présence de 500 µL d'isopropanol afin de précipiter les ARN. Ce mélange a été incubé 30 minutes à -20 °C et ensuite centrifugé pendant 15 minutes à 12000g et à +4 °C. Le surnageant obtenu a été éliminé puis le culot d'ARN est lavé deux fois avec 1 mL d'éthanol à 80% et centrifugés pendant 5 minutes à 7500g à +4 °C. Après évaporation complète, les culots ont été séchés avant d'être repris dans de l'eau RNase free. La quantité et la pureté des ARN extraits ont été mesurées par spectrophotométrie (BioDrop µLITE, Isogen Life Science, Utrecht, Pays-Bas).

6. Transcription inverse

La transcription inverse a été réalisée à partir d'1µg d'ARN totaux et dans un volume final ajusté en fonction des concentrations en ARN précédemment dosées. Les ARN ont été incubés avec un mix réactionnel préparé à partir du kit M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase, Invitrogen) et comprenant 4 µL de tampon 5X, 2 µL de dithiothréitol (0,1 M), 2 µL de dNTP (5 mM), 1 µL d'hexamères (0,3 µg/µL) et 1 µL de M-MLV RT (200 U/µL). La synthèse a été réalisée à 37 °C pendant 60 min puis les ADNc obtenus sont dilués dans l'eau bidistillée à 2,5 ng/mL et conservés à -20 °C jusqu'à utilisation.

7. PCR quantitative en temps réel

L'amplification des gènes d'intérêt a été réalisée avec 4 µL de la solution d'ADNc préalablement préparée dans un volume final de 10 µL contenant 5 µL de SYBR Green (Eurogentec, Liège, Belgique), 0,5 µL de chacun des deux amorces sens et antisens à 10 pmol/ µL. La réaction a été effectuée par le système AriaMx (Agilent Technologies, Santa Clara, Etats-Unis) et a débuté après 2 minutes à 50 °C puis 10 minutes à 95 °C. L'amplication a été effectuée en 40 cycles comprenant une étape de dénaturation de 15 secondes à 95 °C et une étape d'hybridation des amorces d'une minute à 60 °C. A chaque fin de cycle, la fluorescence a été mesurée et les résultats ont été exprimés par rapport à l'ARN ribosomal 18S, ayant une expression ubiquitaire quelques soient les conditions expérimentales.

8. Réalisation et coloration des coupes histologiques

Les échantillons préalablement fixés et conservés ont été déshydratés avant l'enrobage à la paraffine. Une fois les échantillons inclus, des coupes de 5 à 8µm des différents tissus (foie, tissus adipeux), ont été réalisées puis fixées sur lame/lamelle afin de procéder ensuite à l'étape de coloration. Les lames ont été plongées dans du xylène puis progressivement dans de l'éthanol 100% à 70%, s'en suivaient des bains d'eau distillée puis la coloration dans l'hémaroxyline à 0,1%. Après rinçage à l'eau distillée, la coloration à l'éosine a été réalisée puis rinçée. Ensuite, des bains successifs d'éthanol allant de 70% à 100% ont été réalisés. Une fois l'étape de séchage à température respectée, les lames ont été analysées au microscope Zeiss à l'objectif x10 et x40 et 15 à 20 images ont été prises sur chaque lame afin d'être finalement analysées sur le logiciel image J (Image J Software, NIH, USA).

9. Analyses statistiques

Dans cette étude, toutes les données ont été exprimées en moyenne $\pm$ erreur standard à la moyenne (SEM). L'ensemble des données statistiques a été effectuée à l'aide du logiciel GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). La signification statistique a été déterminée par une analyse de variance (ANOVA) suivie d'une analyse post-ANOVA PLSD Fisher pour analyser les différences intergroupes. Concernant les différences entre deux groupes, l'évaluation statistique a été réalisée par le test t de Student.

Pour l'ensemble des analyses, une valeur de $p < 0,05$ a été considérée comme statistiquement significative.

Résultats

1. Article n°1 :

Vitamin D supplementation improves adipose tissue inflammation and reduces hepatic steatosis on obese c57bl/6J mice

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1.1. But de l'étude

A ce jour, le rôle de la vitamine D dans les études interventionnelles du traitement de l'obésité ne semble pas clairement établi. Des études précliniques expérimentant la supplémentation en vitamine D en prévention primaire ont en revanche rapporté de nombreux effets bénéfiques avec notamment une limitation de la prise de poids et une restauration de l'homéostasie glucidique sur un modèle murin soumis à un régime riche en graisse et en sucre. Une supplémentation en vitamine D induit une amélioration du statut inflammatoire du tissu adipeux par la limitation d'expression de chimiokines et cytokines ainsi qu'une diminution de l'infiltration leucocytaire. De plus, il a été récemment démontré que la supplémentation en vitamine D a des effets protecteurs sur la stéatose hépatique. Ses effets en prévention tertiaire restent néanmoins très peu documentés. Notre objectif était donc d'étudier sur une longue durée les effets de la supplémentation en vitamine D en prévention tertiaire de l'obésité sur un modèle de souris obèse induit par la consommation d'un régime obésogène.

Préalablement rendues obèses grâce à un régime riche en graisse et en sucre durant 10 semaines, les souris ont été supplémentées en vitamine D durant 15 semaines. Nos objectifs principaux étaient d'évaluer 1/ les effets de cette supplémentation sur les paramètres morphologiques et la répartition des différents tissus adipeux, 2/ la sensibilité à l'insuline par un test de tolérance à l'insuline ainsi que des dosages plasmatiques ; de caractériser 3/ le tissu adipeux à l'échelle histologique et génique par l'analyse de l'expression de gènes impliqués dans l'inflammation et 4/ l'infiltration lipidique au niveau hépatique ainsi que le profil métabolique sur le plan génique.

1.2. Principaux résultats

Les 10 semaines de régime riche en graisse et en sucre ont conduit au développement de l'obésité dans un modèle de souris c57/bl6. Cette obésité est caractérisée par une augmentation de l'adiposité, une hypertrophie adipocytaire, une accumulation de lipides ectopiques au niveau hépatique ainsi qu'à la mise en place d'une résistance à l'insuline et de l'inflammation.

La supplémentation en vitamine D associée au régime riche en graisse et en sucre, durant 15 semaines, n'a pas permis de limiter la prise de masse, l'adiposité et de corriger l'insulino-résistance. Toutefois, même si la masse adipeuse reste inchangée et l'hypertrophie adipocytaire conservée, la vitamine D diminue significativement l'inflammation du tissu adipeux par la limitation d'expression des chimiokines. En outre, l'infiltration lipidique au niveau hépatique, retrouvée après les 10 semaines de régime obésogène, est diminuée par la supplémentation de 15 semaines en vitamine D. Celle-ci s'accompagne d'une baisse d'expression des gènes impliqués dans la lipogenèse hépatique.

D'après nos résultats, 15 semaines de supplémentation ne semblent pas suffisantes pour induire une perte de poids chez les souris obèses. En revanche, la vitamine D permet d'améliorer le statut inflammatoire et joue un rôle considérable dans la réduction de la stéatose hépatique. Etant donné l'importance de la perte de poids dans l'amélioration du statut métabolique, il semblerait judicieux de coupler cette supplémentation en vitamine D à des programmes d'exercice physique.

1.3. Article



Vitamin D Supplementation Improves Adipose Tissue Inflammation and Reduces Hepatic Steatosis in Obese C57BL/6J Mice

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Abstract: The beneficial effect of vitamin D (VD) supplementation on body weight gain limitation and inflammation has been highlighted in primary prevention mice models, but the long-term effect of VD supplementation in tertiary prevention has never been reported in obesity models. The curative effect of VD supplementation on obesity and associated disorders was evaluated in highfat- and high-sucrose (HFS)-fed mice. Morphological, histological, and molecular phenotype were characterized. The increased body mass and adiposity caused by HFS diet as well as fat cell hypertrophy and glucose homeostasis were not improved by VD supplementation. However, VD supplementation led to a decrease of HFS-induced inflammation in inguinal adipose tissue, characterized by a decreased expression of chemokine mRNA levels. Moreover, a protective effect of VD on HFS-induced hepatic steatosis was highlighted by a decrease of lipid droplets and a reduction of triglyceride accumulation in the liver. This result was associated with a significant decrease of gene expression coding for key enzymes involved in hepatic de novo lipogenesis and fatty acid oxidation. Altogether, our results show that VD supplementation could be of interest to blunt the adipose tissue inflammation and hepatic steatosis and could represent an interesting nutritional strategy to fight obesity-associated comorbidities.

Keywords: vitamin D; inflammation; obesity; adipose tissue; steatosis; tertiary prevention

¹. Introduction

Obesity is a worldwide health issue. It increases cardiovascular morbidity and impairs quality of life and consequently represents one of the leading causes of mortality. Obesity, defined by an excessive fat accumulation in adipocytes, results from an imbalance between energy intake and energy expenditure. It is caused by changes in food consumption behaviors and lifestyle [1,2]. Nowadays, according to the World Health Organization (WHO), over 1.9 billion adults are overweight and among them 650 million are obese [3]. This pathology is strongly related to metabolic disorders such as arterial hypertension, chronic low-grade inflammation, and insulin resistance that could lead to type 2 diabetes [4,5]. Obesity is also strongly linked to ectopic fat accumulation, particularly in the liver, contributing to an emergence of non-alcoholic fatty liver disease (NAFLD) [6].

Interestingly, from an epidemiologic standpoint obesity and NAFLD are commonly associated with low 25-hydroxyvitamin D (25(OH)D) plasma levels. Indeed, in prospective studies, low 25(OH)D plasma levels were associated with higher incidence of obesity in adults, children, and elderly women, it was also correlated to a higher five-year waist circumference [7]. Several crosssectional studies have reported an inverse relationship between plasma concentrations of 25(OH)D and body mass index (BMI) [8–10]. However, the causality between these two parameters remains unclear [11,12]. Moreover, recent epidemiological data have also enabled the linkage of hypovitaminosis D with both obesity and NAFLD [13].

As of today, intervention studies have remained ambiguous, because some of them reported a beneficial effect on body weight management [14,15] and other studies observed no effect [16,17]. Nevertheless, preclinical studies on VD supplementation have demonstrated benefits for weight management in primary prevention in mice fed with a high-fat diet [18,19]. This was associated with the improvement of the inflammatory status in adipose tissue, characterized by a limitation of cytokines and chemokine expression by adipocytes [20], leukocyte infiltration in adipose tissue [21], and a reduction of the expression of pro-inflammatory miRNA in adipocytes and adipose tissue [22].

Nevertheless, the impact of VD supplementation on body weight reduction and associated pathologies remains poorly documented in obese mice models. A recent study reports that the body weight of obese mice was not affected by VD supplementation [23]. It is important to consider that the supplement was administered over a short period of time. The aim of this study is to evaluate the consequences of administering VD to obese mice over a longer period.

2. Materials and Methods

2.1. Animal, Diets, and Experiments

Six-week-old male C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). They were kept in cages with an enriched environment maintaining controlled environmental conditions (20–23 °C; 40% humidity), and a 12-hour light/dark cycle. The mice were fed water and food ad libitum. All procedures were performed in accordance with the local research ethics committee (2017110611453051-RIVA) and the agreement of European and French Ministry of Agriculture about the care and use of laboratory animals 2010/63/EU (N°CEEA—00322.03). Mice (10-week-old age and weighing 41.55 ± 0.64 g) were randomly placed into two groups: a normal chow group (NC; $n = 20$) or a high-fat/high-sucrose diet group (HFS; $n = 60$). The NC group was fed a normal chow diet (A04, 3.1% fat, caloric value $3.339 \text{ kcal}\cdot\text{kg}^{-1}$, Safe, France) and water during the entire protocol of 25 weeks. The HFS group was fed a fat-enriched-dough (230HF, 60% kcal from fat with a caloric value of $5.317 \text{ kcal}\cdot\text{kg}^{-1}$, Safe, France) completed with drinking water containing 10% sucrose (D-Saccharose, Fisher Scientific, England) over a 10-week period to induce obesity and type 2 diabetes. After this 10-week period, the HFS group was divided in two subgroups. They were either fed with the same HFS diet or with an HFS diet supplemented with vitamin D (HFS + D; $15,000 \text{ IU}\cdot\text{kg}^{-1}$ cholecalciferol; customized HF230, SAFE Diet, Augy, France) over an additional 15-week period. During the experiment, body weight was measured once a week and dietary intake was assessed daily. Energy intake was calculated per cage from the amount of food and drink consumed by the animals and its caloric equivalence. At the end of the protocol, mice were fasted overnight. Animals were anesthetized, before the intracardiac puncture blood collection. The plasma was obtained by centrifuging at $3000\times g$ for 15 min at 4 °C, it was then stored at -80 °C. The animals, still under anesthesia, were then sacrificed by cervical dislocation. Liver and adipose deposits (epididymal, subcutaneous, perirenal, inguinal) were entirely collected, weighed, and immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. Insulin Tolerance Test

Seven weeks and 12 weeks after starting VD supplementation, mice were subjected to an insulin tolerance test (ITT), after they had been fasted for 6 hours. Blood was collected (5 μL) using the tailclip method and fasting glycemia was measured using a commercially available glucometer (AccuCheck glucometer, Roche, Basel, Switzerland), in accordance with the manufacturer's instructions. ITT were then performed after an intraperitoneal injection of insulin solution ($1 \text{ U}\cdot\text{kg}^{-1}$), and blood glucose levels were measured from tail blood taken at the indicated times after injection: 10, 30, 60, 90, 120 minutes after the injection.

2.3. Biochemical Analysis

Plasma glucose and calcium (Ca^{2+}) were analyzed using colorimetric methods (BIOLABO, Maizy, France) as non-esterified fatty acids (NEFA) (RANDOX, Crumlin, Co., Antrim, United Kingdom). Plasma insulin was measured using an enzyme-linked immunosorbent assay ELISA (ALPCO Diagnostics, New Hampshire, USA) and adiponectin was quantified using the ELISA kit (R&D Development, Minneapolis, USA) in accordance with the manufacturer's instructions. Triglycerides (TG) were quantified both in plasma samples and in the liver using colorimetric methods (BIOLABO, Maizy, France). The Homeostasis Model Assessment of insulin resistance (HOMA-IR) index was calculated according to the following formula: fasting insulin ($\text{microU}\cdot\text{L}^{-1}$) $\times$ fasting glucose ($\text{mmol}\cdot\text{L}^{-1}$)/22.5 [24].

2.4. 25(OH)D Quantification in Plasma

All quantifications were performed using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Hypersil Gold® C18 column, Orbitrap™ Q Exactive™ Plus system and Xcalibur™ software, Thermo Fisher Scientific, Waltham, USA) according to the protocol previously mentioned [25].

2.5. Histological Analysis

Paraffin-embedded tissue sections of liver and inguinal adipose tissue (iWAT) were stained with hematoxylin and eosin (H&E) using standard protocols [26]. The images were captured by a light microscope (Zeiss Axio Imager, Oberkochen, Germany) and the adipocyte area (μm^2) was determined using (Image J) software as previously described [24].

2.6. RNA Isolation and qPCR

Total RNA from liver and iWAT were extracted using TRIzol reagent according to the manufacturer's instructions (Thermo Fisher, Courtaboeuf, France). One microgram of total RNA was used to synthesize cDNA in 20 μL using random primers and Moloney murine leukemia virus reverse transcriptase (Thermo Fisher, Courtaboeuf, France). Real-time quantitative RT-PCR analyses were performed using the AriaMx System (Agilent, Santa Clara, USA) as previously described [27]. All PCR reactions were using a SYBR Green Master mix (PowerUp™ SYBR®, Thermo Fisher, Courtaboeuf, France). For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [28]. Data were expressed as a relative expression ratio. Primers sequences are presented in Supplementary Table S1.

2.7. Statistical Analysis

Data were expressed as the mean $\pm$ SEM. Significant differences between control and treated groups were determined using ANOVA, followed by the PLSD Fischer post hoc test using Prism6 (GraphPad Software Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Impact of High-Fat/High-Sucrose Diet and Vitamin D Supplementation on Morphological Parameters

After 10 weeks of high-fat/high-sucrose (HFS) diet, body weight of HFS mice was considerably increased compared with that of the NC group (Figure 1A). In fact, energy intake was higher in the HFS group compared with that in the control group (Figure 1B). In regard to body composition, HFS consumption significantly increased adipose tissues (peri-renal, epididymal, inguinal, and subcutaneous) absolute (Figure 1D) and relative masses (Supplementary Figure S1). The adiposity index calculated by the sum of all adipose tissues relative to total body mass was increased by a factor 2 in the HFS group compared with that in the NC group. To evaluate the metabolic impact of VD supplementation, half of the mice were fed with HFS diet supplemented with VD (15,000 UI $\cdot\text{kg}^{-1}$ of food) (HFS + D), during an additional 15-week period. The other HFS-fed mice remained on the HFS diet. Mice that were on the HFS diet continued to gain body weight compared with those in the NC group. At the end of the 25-week protocol, the body weight as well as the adiposity index of mice supplemented with VD was not different from that of HFS-fed mice as shown in

Figure 1C,F. These results were consistent with the higher energy intake in the HFS or HFS + D groups compared with that in the NC group, and there was no difference between HFS and HFS + D groups (Figure 1B).

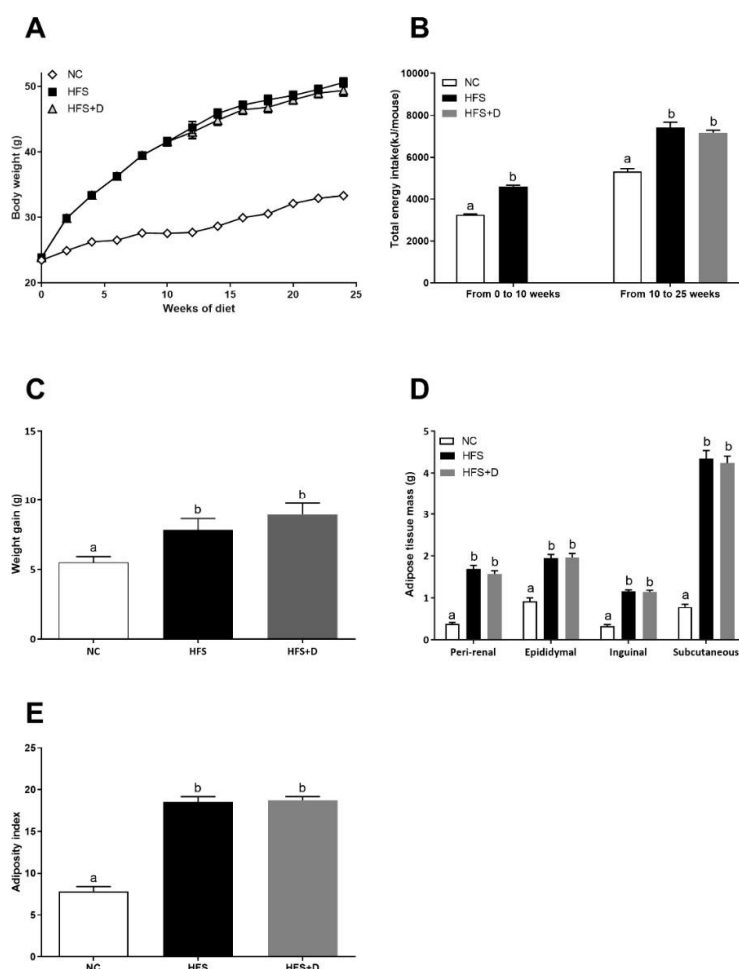


Figure 1. Morphological parameters of mice fed with high-fat/high-sucrose diet and vitamin D supplementation. **(A)** Body weight evolution curve of mice fed with a controlled diet (NC), highfat/high-sucrose diet (HFS) during the first 10 weeks and then supplemented with vitamin D over a 15-week period (HFS + D). **(B)** The energy intake was quantified by measuring food intake and sucrose water every day for a period of 25 weeks following respective diet consumption. **(C)** Animal weight gain was established; it corresponds to the difference between the body weight at end of the protocol (25th week) and the body weight at the end of the 10-week HFS diet. **(D)** During the sacrifice, adipose tissues were weighed, and body composition was expressed in terms of adipose tissues' absolute mass. **(E)** An adiposity index was calculated by calculating the ratio between the sum of perirenal, epididymal, inguinal, and subcutaneous adipose masses and body weight; values are presented as means $\pm$ SEM; values not sharing the same letter were significantly different, $p < 0.05$.

3.2. Impact of High-Fat/High-Sucrose Diet and Vitamin D Supplementation on Glucose Homeostasis

Then, we evaluated the effect of VD supplementation on glucose homeostasis. At the beginning of VD supplementation, insulin resistance of mice was confirmed by ITT (Supplementary Figure S2). As expected, the HFS diet reduced insulin sensitivity (Figure 2A–D), increased glycaemia, insulinemia (Figure 2E), and HOMA-IR (Figure 2F). After 7 and 15 weeks of VD supplementation, no difference was observed in either the HFS or the HFS + D groups in terms of glycemic response. Quantification of plasma glucose and insulin at the end of the 25-week protocol (Figure 2E, Table 1) confirmed the fasting hyperglycemia and increased plasma insulin concentrations in both HFS and HFS + D groups compared with those in the NC group (Figure 2E) and HOMA-IR increased, in both HFS and HFS + D groups compared with that in the NC group (Figure 2F).

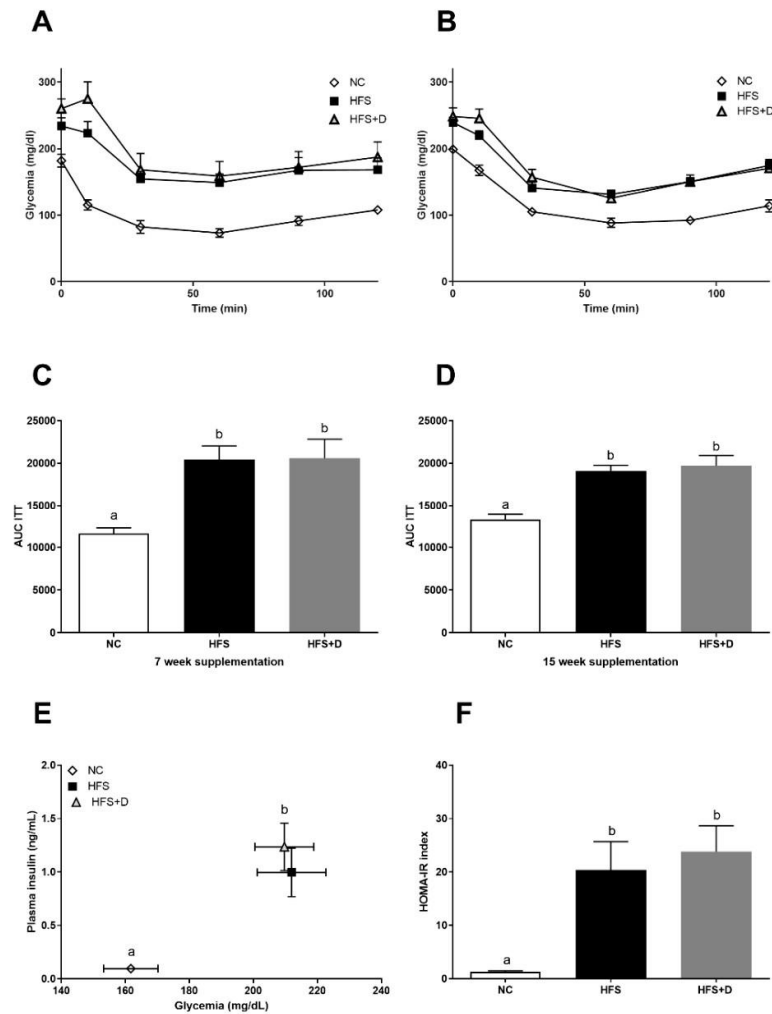


Figure 2. Effects of vitamin D (VD) supplementation on glucose homeostasis. (A) Glycemic response to insulin injection after 7 weeks of VD supplementation. (B) Glycemic response to insulin injection after 15 weeks of VD supplementation. (C) Area under the curve (AUC) calculated from the glycemic response curve after 7 weeks of VD supplementation. (D) AUC calculated from the glycemic response curve after 15 weeks of VD supplementation. (E) Quantification of plasma glucose and insulin at the end of the 25-week protocol. (F) HOMA-IR was calculated according to the following formula: (fasting insulin ($\mu\text{mol}\cdot\text{L}^{-1}$) $\times$ fasting glucose ($\text{nmol}\cdot\text{L}^{-1}$)/22.5); insulin sensitivity was measured by ITT; values are presented as mean $\pm$ SEM; values not sharing the same letter were significantly different, $p < 0.05$.

3.3. Impact of High-Fat/High-Sucrose Diet and Vitamin D Supplementation on Plasma Parameters

After 25 weeks, no modification of the triglyceride (TG) concentration was observed in the three groups (Table 1). The NEFA concentration was decreased in both HFS and HFS + D groups compared with that in the NC group. Adiponectin concentration had a predictable decrease in HFS-fed mice compared with that in NC-fed mice whereas unexpectedly HFS-fed mice supplemented with VD had a lower adiponectin level than both NC and HFS-fed mice. As expected, 15 weeks of VD supplementation significantly increased cholecalciferol and 25(OH)D plasma levels compared with the HFS and NC groups. Moreover, there was a slight increase of calcemia in HFS- and HFS + D-fed mice compared with that in the NC group.

Table 1. Effect of vitamin D on mice biological parameters.

Biological Parameters	NC	HFS	HFS + D
Triglycerides (mmol·L ⁻¹)	1.01 ± 0.05 ^a	1.02 ± 0.06 ^a	1.02 ± 0.08 ^a
Non-esterified fatty acid (mmol·L ⁻¹)	0.96 ± 0.05 ^a	0.70 ± 0.04 ^b	0.66 ± 0.04 ^b
Adiponectin (µg·mL ⁻¹)	6.44 ± 0.47 ^a	5.15 ± 0.28 ^b	1.54 ± 0.22 ^c
Calcium (mmol·L ⁻¹)	2.32 ± 0.07 ^a	2.49 ± 0.02 ^b	2.59 ± 0.07 ^b
Glucose (mmol·L ⁻¹)	8.98 ± 0.47 ^a	11.76 ± 0.60 ^b	11.64 ± 0.51 ^b
Insulin (mmol·L ⁻¹)	0.01 ± 0.01 ^a	1.00 ± 0.23 ^b	1.24 ± 0.22 ^b
Vitamin D ₃ (ng·mL ⁻¹)	0.71 ± 0.20 ^a	4.52 ± 0.81 ^a	77.64 ± 5.86 ^b
25(OH)D ₃ (ng·mL ⁻¹)	117.10 ± 3.43 ^a	154.10 ± 5.00 ^b	240.20 ± 12.56 ^c

NC: normal chow, HFS: high-fat/high-sucrose diet, HFS + D: high fat/high sucrose diet supplemented with vitamin D. Values are presented as mean ± SEM. Values not sharing the same letter were significantly different, $p < 0.05$.

3.4. Impact of High-Fat/High-Sucrose Diet and Vitamin D Supplementation on Inguinal White Adipose Tissue (iWAT) Biology

Histological analysis was performed to assess the effect of regimens on iWAT cellularity (Figure 3A). HFS diet induced significant enlargement of the adipocyte area compared with the NC group, while no significant change of cellularity was observed in HFS + D compared with the HFS-fed mice (Figure 3B). Furthermore, inflammatory status in iWAT evaluated by monocyte chemoattractant protein 1 (*Mcp1*) and chemokine C-C motif ligand 5 (*Ccl5*) mRNA levels revealed that their expressions were drastically increased by the HFS diet as compared with the NC group (Figure 3C), and VD supplementation significantly decreased *Mcp1* and *Ccl5* mRNA levels (41% and 36%, respectively).

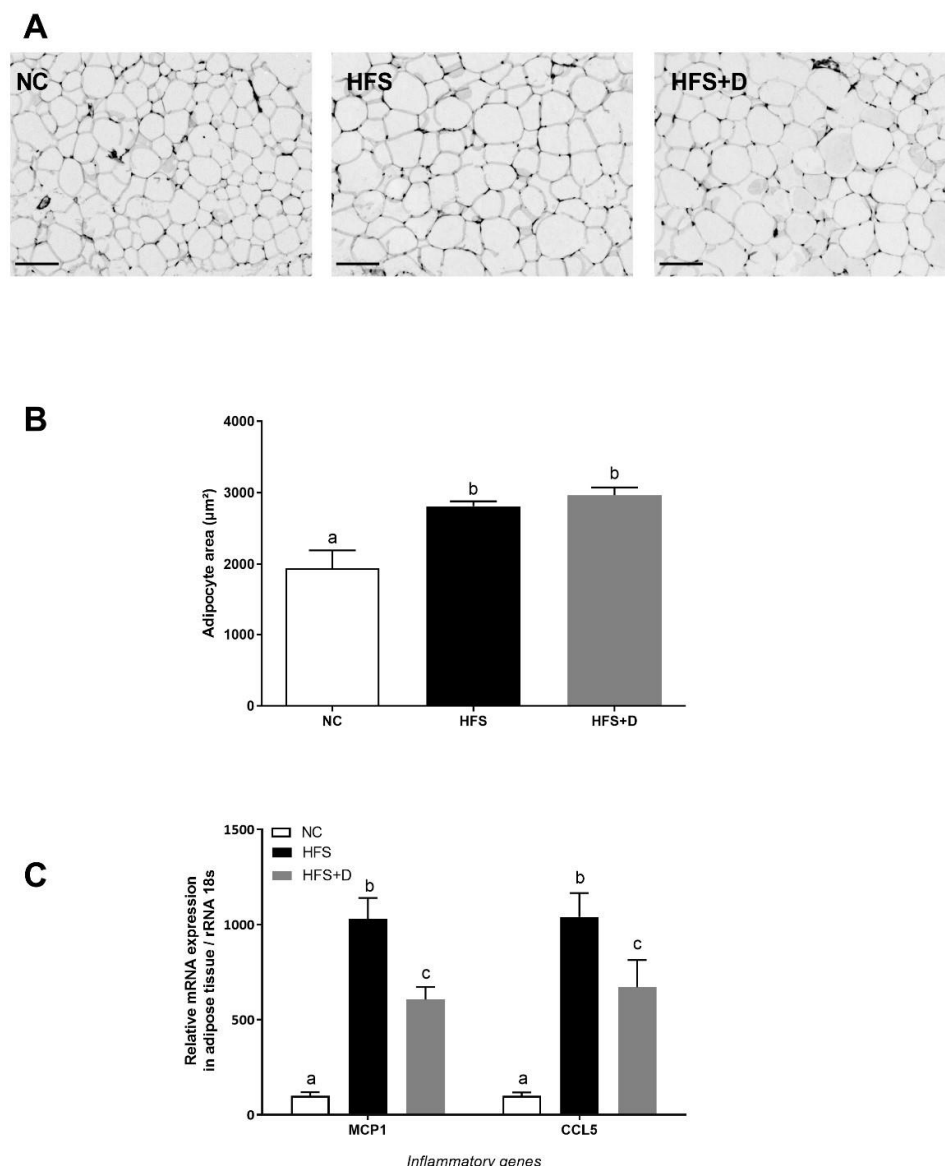


Figure 3. Effects of high-fat/high-sucrose diet and vitamin D supplementation on different parameters of inguinal adipose tissue. (A) Representative histological images of inguinal fat pads after H&E staining, taken at 40 $\times$ magnification (scale bar represents 100 μm). NC: normal chow, HFS: highfat/high-sucrose diet, HFS + D: high-fat/high-sucrose diet supplemented with vitamin D. (B) Adipocyte area, determined using Image J software. (C) Relative expression of mRNA inflammatory genes measured through qPCR and expressed relative to 18S ribosomal RNA. Values are presented as mean $\pm$ SEM. Values not sharing the same letter were significantly different, $p < 0.05$.

3.5. Impact of High-Fat/High-Sucrose Diet and Vitamin D Supplementation on the Liver

Triglycerides (TGs) were quantified in the liver to estimate hepatic lipid accumulation. Our results showed that the HFS diet significantly increased TGs compared with the NC diet and that VD supplementation limited TG accumulation in the liver by 33% (Figure 4A). Histological sections stained by (H&E) confirmed this result by a reduction of visible lipid droplets in VD-supplemented mice compared with that in the HFS-fed mice (Figure 4B).

The effect of VD supplementation on hepatic lipid metabolism was also studied at the molecular level. In particular, the hepatic de novo lipogenesis was evaluated by the mRNA expression of the fatty acid synthase (*Fasn*) and acetyl-coA carboxylase 1 (*Acaca*) genes (Figure 4C). Their mRNA levels were significantly increased in the HFS mice, whereas VD supplementation induced a significant decrease, by 30% and 35% for

Fasn and *Acaca* respectively in comparison with the HFS mice. Moreover, gene expression involved in fatty acid oxidation such as the acetyl-coA oxidase (*Acox*) gene was not modified in the HFS mice whereas this level was downregulated in VD-supplemented mice (Figure 4D), while carnitine palmitoyl transferase (*Cpt1*) mRNA level was equally decreased in the HFS group and in the HFS + D group in comparison with that in NC mice.

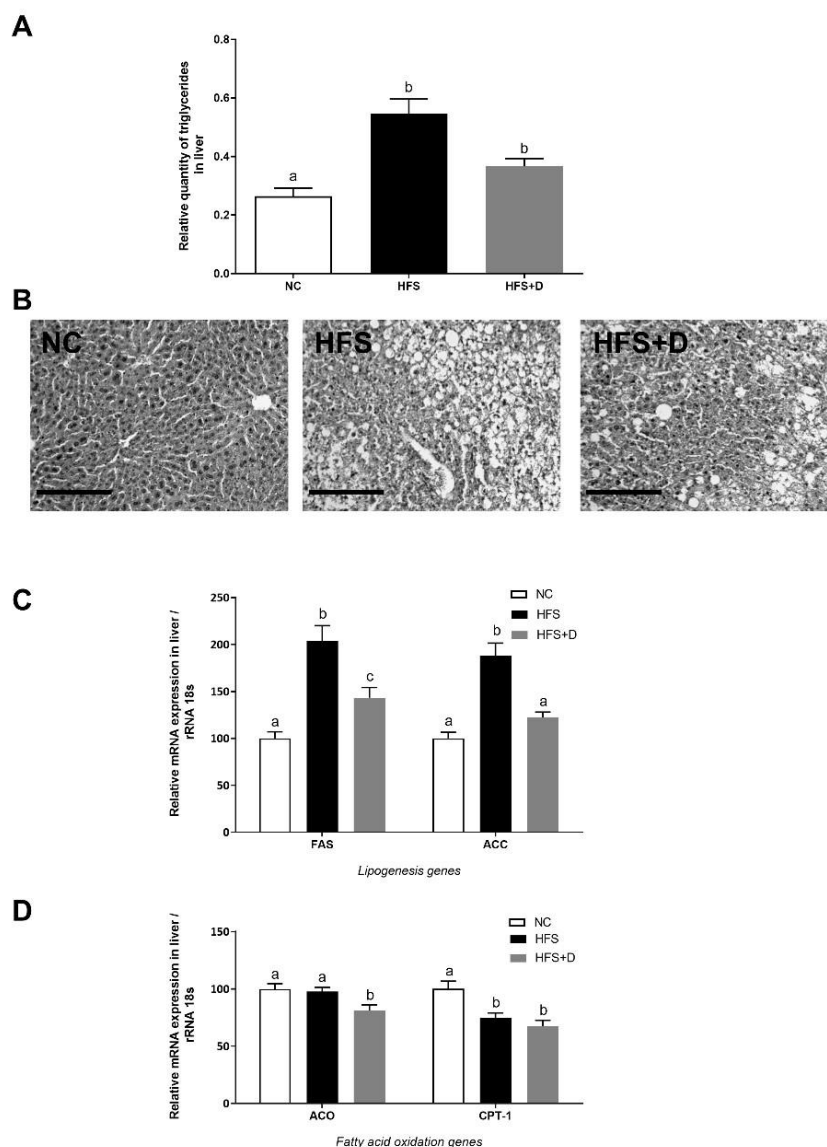


Figure 4. Effects of high-fat/high-sucrose diet and vitamin D supplementation on different parameters in the liver. (A) Quantification of triglycerides in liver tissue, related to liver mass. (B) Representative histological images of liver tissue after H&E staining, taken at 40× magnification (scale bar represents 200 μ m). NC: normal chow, HFS: high-fat/high-sucrose diet, HFS + D: high-fat/high-sucrose diet supplemented with vitamin D. (C) Relative expression of mRNA genes related to lipogenesis and (D) fatty acid oxidation measured through qPCR and expressed relative to 18S ribosomal RNA. Values are presented as mean $\pm$ SEM. Values not sharing the same letter were significantly different, $p < 0.05$.

4. Discussion

In the present study, we investigated the effect of VD supplementation for 15 weeks on obese C57BL/6J mice. The mice were obese as a result of an imposed high-fat/high-sucrose diet over a 10week period prior to being given VD supplementation. We highlighted that VD supplementation did not modify the obese phenotype, adiposity, and insulin resistance in the HFS mice, but exerted an anti-inflammatory effect on iWAT and an improvement of hepatic steatosis.

The protocol implemented to generate obesity was a 10-week HFS diet, which led to a significant increase in the total body mass and adiposity index of mice and as expected was associated with hyperglycemia and insulin resistance. This diet-induced obesity and diabetes model was preferred to transgenic models for its comparison with the etiology of insulin resistance and its associated risk factors in a more physiological process. In these obese mice, 15 weeks of VD supplementation did not improve disrupted glucose homeostasis and had no impact on obesity. This observation was consistent with a recently published study that did not find evidence that body weight could be managed in obese mice via VD supplementation [23]. This is also in accordance with several clinical trials that did not report beneficial effects of VD on weight loss for overweight or obese subjects [16,29,30] and was recently meta-analyzed [31,32]. However, it is noteworthy that a study, performed on obese mice injected with 1,25(OH)₂D reported a limited weight gain compared with control obese mice [33]. Such discrepancy could be due to the impact of diet-induced obesity on VD metabolism [34]; the direct administration of 1,25(OH)₂D, could bypass the metabolism of cholecalciferol, and therefore be more efficient. As stated previously, it has recently been reported that obesity in humans reduced the effect of VD supplementation [35]. In the present study, we did not measure adipose tissue concentration of 1,25(OH)₂D, but based on our previously reported data [34], we can speculate that this metabolite is present at a lower concentration in obese mice compared with the levels in the NC-fed mice. We can also speculate that the VD supplementation was not sufficient to reach a 1,25(OH)₂D concentration able to modulate fatty acid oxidation, which is considered as a major driving force of weight loss [18,36,37].

As increased adiposity contributes to insulin resistance, insulin sensitivity was evaluated during the 25-week protocol and as expected the HFS diet induced a decreased sensitivity. Glucose metabolism parameters (glycemia, insulinemia, and HOMA-IR) also highlighted negative effects of the HFS diet, corresponding to an insulin-resistant profile [38,39]. No improvement of those parameters as well as insulin sensitivity were observed under VD supplementation. Such observation could appear to be in contradiction with other studies that demonstrate the beneficial effects of VD supplementation in patients with insulin-resistance and low VD plasma levels [40]. Indeed, the beneficial effects of VD supplementation were mainly obtained on a population with a clear disruption of glucose homeostasis on one hand [15], and on the other hand a population with VD insufficiency [40]. We have to keep in mind that our mice were not deficient in VD, as highlighted by the 25(OH)D plasma level. Thus, it is not surprising that no beneficial effects of VD supplementation were observed on glucose homeostasis parameters.

The role of adipose tissue is now not only considered as a storage site for triglycerides but also as a major endocrine organ via adipokine secretion [41]. In case of obesity, adipocyte biology is deeply modified and characterized by hypertrophy and adipokine secretion profile modification, resulting in a pro-inflammatory environment [4,42]. In agreement, our results showed that the HFS diet induced hypertrophy of adipocytes and increased mRNA levels coding for pro-inflammatory chemokines. If the VD supplementation had no effect on adipocytes morphology, it is noteworthy that VD supplementation improved the inflammatory status within the adipose tissue. Indeed, the rise of inflammatory markers (*Mcp1* and *Ccl5* mRNA levels) induced by HFS diet consumption, was reduced by VD supplementation. Such observation is clearly in line with previous demonstrations of the anti-inflammatory effect of VD, particularly at the chemokine level [20,21,43–45]. Even though the molecular mechanism has not been investigated in the present study, based on our previous results, we can easily hypothesize that NF- κ B and/or p38 MAPK signaling are disrupted by the VD supplementation [20,21].

Interestingly, adipose MCP-1 overexpression has been reported to be associated with hepatic steatosis [46], thus special attention was given to the effect of VD on lipid accumulation and metabolism in the liver. Our results showed a protective effect of VD on HFS-induced hepatic steatosis, highlighted by a decrease of lipid droplets in the liver and a reduction of TG content. At a molecular level, VD supplementation decreased the expression of genes (*Acaca* and *Fasn*) coding for key proteins involved in hepatic de novo lipogenesis, that were induced by the HFS diet. In addition, we have also reported an induction of gene coding for proteins involved in fatty acid oxidation (*Acox*). Taken together, such modulations of genes expression could explain the limitation of TG accumulation observed in VD-supplemented mice. Similarly, a recent study depicted the role of VD supplementation on liver steatosis in a mouse model of obesity [23] and demonstrated that VD intervention significantly reduced hepatic steatosis. No effect on lipogenesis was reported in this study, and

the origin of such a discrepancy is not understood at this time but could be related to the duration of the intervention.

In the human population, the role of VD in non-alcoholic fatty liver disease has recently emerged. Several observational studies reported a link between low 25(OH)D plasma levels and fatty liver diseases [13,47–49]. If the causality is still not clearly demonstrated in humans [50], observations on animal models, including the present study, tend to confirm such an assumption.

To conclude, even though VD supplementation did not improve body weight and insulin sensitivity, interesting data were generated in regard to the impact of VD supplementation on hepatic steatosis and adipose tissue inflammation. It is highly probable that these two occurrences are strongly linked, based on the role of adipose MCP-1 [46]. Such an assumption will require further investigations to be established. Nevertheless, VD supplementation could represent an interesting therapeutic strategy to blunt non-alcoholic fatty liver diseases.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Adipose tissue relative mass after 25-weeks protocol; Figure S2: AUC insulin tolerance test (ITT) values of mice fed with 10 weeks of high-fat/high-sucrose diet; Table S1: Primers sequences.

Author Contributions: A.M. conceived the experiments/researched data/wrote/edited manuscript; C.P., C.C. and J.A., researched data, P.O. edited the manuscript; J.F.L. and C.R. conceived the experiments/researched data/wrote/edited manuscript and are guarantors of this work and, as such, have full access to all the study data and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read and agreed to the published version of the manuscript.

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Table S1. Primers sequences.

Gene	Primer sequences	
m18S	Forward	CGCCGCTAGAGGTGAAATTCT
	Reverse	CATTCTTGGCAAATGCTTTCG
mMCP1	Forward	FCATCCACGTGTTGGCTCA
	Reverse	RGATCATCTTGCTGGTGAATGAGT
mCCL5	Forward	TGCAGAGGACTCTGAGACAGC
	Reverse	GAGTGGTGTCCGAGCCATA
mFAS	Forward	CATGACCTCGTGATGAACGTGT
	Reverse	TCGGGTGAGGACGTTTACAAA
mACC	Forward	GGAGTGGATGATGGTCTGA
	Reverse	GGCCTTGATCATCACTGGAT
mACO	Forward	GCTGGCCGTGTCCATAGC
	Reverse	TTATCCGTGGGTCCAAACTGA
mCPT1a	Forward	TGCCTTTACATCGTCTCCAA
	Reverse	GGCTCCAGGGTTCAGAAAGT

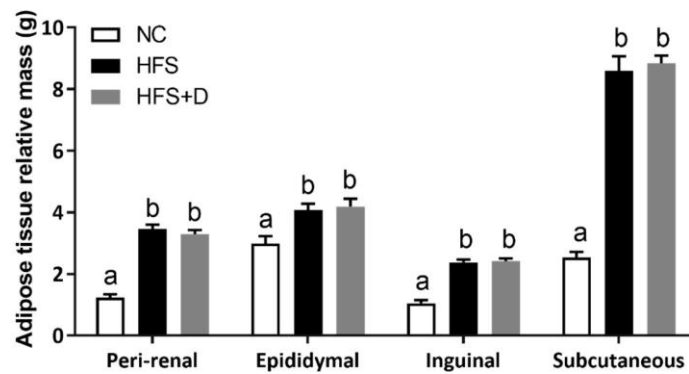


Figure S1. Adipose tissue relative mass after 25-weeks protocol. Values are presented as mean $\pm$ SEM. Values not sharing the same letter were significantly different, $p < 0.05$.

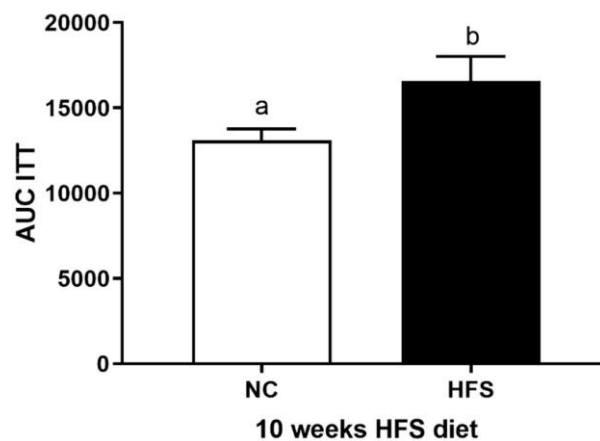


Figure S2: AUC ITT values of mice fed with 10 weeks of high fat/high sucrose diet. Values are presented as mean $\pm$ SEM. Values not sharing the same letter were significantly different, $p < 0.05$.

