Combined effect of voluntary physical exercise and vitamin D supplementation on diet obese C57BL/6J mice

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2.1. But de l'étude

L'accumulation et l'expansion de la masse adipeuse, caractérisant l'obésité, sont possibles grâce à des modifications structurales du tissu adipeux dont font partie les phénomènes d'hypertrophie. Lorsque les capacités de stockage sont atteintes, ceci va provoquer l'apparition de dépôts ectopiques. Dans la prise en charge de l'obésité, il est clairement établi que l'exercice physique joue un rôle majeur dans la perte de poids et la réduction de la masse grasse. Ainsi, comme notre précédente étude (Article n°1) a rapporté des effets bénéfiques de la vitamine D sur les paramètres inflammatoires du tissu adipeux et du foie, nous nous sommes intéressés à l'effet combiné de l'exercice physique volontaire et de la supplémentation en vitamine D en prévention tertiaire de l'obésité chez des souris soumises à un régime riche en graisse et en sucre.

Dans cette étude, nos objectifs étaient d'évaluer dans ce modèle de souris c57/bl6 obèses les effets de l'exercice physique volontaire combiné à une supplémentation en vitamine D, 1/ sur les paramètres morphologiques, l'adiposité et l'homéostasie glucidique, 2/ l'hypertrophie et l'inflammation du tissu adipeux induites par le régime obésogène et 3/ la stéatose hépatique caractérisée par l'infiltration hépatique et la modification du métabolisme hépatique.

2.2. Principaux résultats

L'exercice physique appliqué à ce modèle de souris c57/bl6 durant 15 semaines induit une limitation significative de la prise de poids et une réduction de l'adiposité qui n'étaient pas retrouvées par la seule supplémentation en vitamine D. De plus, l'insulino-résistance induite par le régime obésogène est conservée lorsque l'exercice

physique et la supplémentation en vitamine D sont administrés seuls. Par contre, de façon intéressante, on observe une restauration de la sensibilité à l'insuline dès lors que les deux stratégies sont associées.

Lorsque l'on s'intéresse au tissu adipeux inguinal, l'exercice physique aboutit à une réduction de la taille des adipocytes et coïncide avec la réduction de masse adipeuse observée. Ces observations s'accompagnent d'une baisse de l'inflammation médiée par la diminution d'expression des chimiokines, également retrouvée par la supplémentation en vitamine D. De plus, la vitamine D vient potentialiser les effets de l'exercice physique sur certains médiateurs inflammatoires (*Ccl5, Tgfb*).

Le régime obésogène induit non seulement une augmentation de la masse adipeuse mais également de la masse hépatique. Les 15 semaines d'exercice physique ont engendré une diminution importante de la masse du foie, qui n'est pas observée par la seule supplémentation en vitamine D. Cette diminution s'explique par une réduction de l'infiltration lipidique au niveau hépatique, attestée par histologie et mesurée par dosage des triglycérides. Cette baisse d'infiltration est d'autant plus importante lorsque l'exercice physique est associé à la vitamine D. En parallèle, l'analyse de l'expression génique montre une baisse significative de la lipogenèse par l'exercice physique et la vitamine D avec un retour à la normale lorsque les deux stratégies sont combinées. De plus, le profil inflammatoire hépatique montre une potentialisation des effets de l'exercice physique et de la vitamine D sur l'infiltration macrophagique.

Au vu des résultats bénéfiques obtenus par le couplage exercice physique/supplémentation en vitamine D, il semble pertinent et judicieux de l'appliquer dans les programmes de prise en charge de l'obésité afin d'observer des effets notables au niveau de la perte de poids ainsi qu'une réduction significative de la stéatose hépatique.

2.3. Article

Combined effect of voluntary physical exercise and vitamin D supplementation on diet obese C57BL/6J mice

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Abstract

Purpose: The beneficial effect of association of physical exercise (PE) and nutritional approaches are widely plebiscited to improve metabolic health. Here we took advantage of voluntary PE together with a vitamin D (VD) supplementation, which has already displayed beneficial effects in primary and secondary prevention in obese mice model, to study their combining effects regarding body weight management, glucose homeostasis, metabolic inflammation and liver steatosis, as key markers of metabolic health.

Methods: 10-week-old male C57BL/6J mice were fed with HFS diet during 10 weeks. Then, they were assigned to different conditions for a 15-week period with PE, VD supplementation or both PE and VD supplementation. Morphological, histological, and molecular phenotype were characterized.

Results: The increase body mass, adiposity and adipocyte hypertrophy induced by HFS diet were improved by PE, but not by VD supplementation. HFS-induced inflammation (highlighted by chemokines mRNA levels) in inguinal adipose tissue was decreased by PE and VD supplementation. Interestingly, the intervention combining PE and VD displayed additive effects on insulin sensitivity restoration and limitation of hepatic steatosis, as demonstrated through a normalization of lipid droplets number and size and triglycerides content in the liver. At the molecular level, this result was accompanied by a significant decrease of gene expression coding for key enzymes involved in hepatic de novo lipogenesis.

Conclusion: Taken together, our data show for the first-time beneficial effects of combining PE and VD supplementation on obesity-associated comorbidities such as insulin resistance and hepatic disease. Such strategy could therefore be of particular interest in obesity management programs.

Keywords: obesity, voluntary exercise, vitamin D, tertiary prevention

Introduction

The fundamental cause of obesity and subsequent type 2 diabetes is an energy imbalance. This is classically associated to unhealthy diet (1), characterized by an increased intake of energy-dense foods rich in fat and sugars but poor in micronutrients and a sedentary lifestyle (2).

Obesity, characterised by increased fat accumulation, *i.e*. adiposity, is associated to metabolic and physiological modifications (3) leading to adipocyte structural modifications including increased size (hypertrophy) or increased number (hyperplasia). In this context, many factors including adipokines, cytokines, chemokines and miRNA can be released by adipose tissue (AT), leading to systemic low-grade inflammation and insulin resistance (4–6). In addition, when the maximum capacity of AT expansion is reached, large amount of lipids is stored in ectopic deposit, and notably in the liver (7).

Physical exercise (PE) is known to provide beneficial effects in terms of body weight gain and adiposity (8), and reduction of cardiovascular diseases incidence (9,10). PE also reduces type 2 diabetes associated inflammation (11,12). Indeed, exercise training blunts the systemic inflammation through a downregulation of pro-inflammatory cytokine production in several tissues, including muscles, AT and immune cells (13).

In experimental studies, we and others have previously shown that exercise training reduced subcutaneous and visceral AT masses (14) and prevented the HFS (high fat/ sucrose)-mediated decrease of phospho-AKT / total AKT in the subcutaneous AT (15). Voluntary exercise also reduced hepatic steatosis induced by HFS diet consumption (16) and hepatic inflammation (17). Altogether, it is classically admitted that PE represents evidence-based strategy to promote health.

On the other hand, in murine experimental models, we demonstrated the beneficial impact of vitamin D (VD) supplementation on body weight management, glucose homeostasis, AT and systemic inflammation (cytokines, chemokines and miRNA), and hepatic steatosis (18–23).

Physical activity and nutritional supplementation have been investigated in several interventional studies, but not in the context of obesity. Therefore, we hypothesized that a double intervention combining PE and VD supplementation may potentiate benefits on weight gain, metabolic parameters, inflammation and ectopic fat deposition on obese mice.

Materials and methods

Animal, diets and experiments

Six-week-old male C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France), housed in cages with an enriched environment and maintained in controlled environment conditions (20-23°C; 40% humidity), and on 12 hours light/dark cycle. The mice were fed with 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-of age and weighing 23,85 $\pm$ 5,91g) were randomly assigned into 2 groups: a normal chow group (NC; n=20), a high fat/sucrose diet group (HFS; n=90). The NC group received a normal chow diet (A04, 3.1% Fat, caloric value 3.339 $kcal.kg^{-1}$, Safe, France) and water during the entire protocol of 25 weeks. The HFS group was fed with a fat-enriched-dough (230HF, 60% kcal as fat with caloric value 5.317 $kcal.kg^{-1}$, Safe, France) completed with drink water containing 10% sucrose (D-Saccharose, Fisher Scientific, England) during 10 weeks. After this 10-week period, the HFS group was divided in 4 subgroups, fed with the same HFS diet or HFS supplemented with vitamin D (HFS+D; 15 000 $UI.kg^{-1}$ cholecalciferol; customized HF230, SAFE Diet, Augy, France) randomly assigned to a sedentary subgroup (HFS and HFS+D, n=60) or voluntary physical exercise (HFS+ex and HFS+D+ex, n=30) up to 15-weeks. Voluntary exercise was introduced with a wheel in cages, connected to a sensor.

During the entire protocol, 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. Exercise was measured by daily record of the number of laps being executed in each cage.

At the end of the protocol, mice were fasted overnight and were all kept sedentary. Animals were firstly anesthetized, and blood was collected by intracardiac puncture and plasma was obtained by centrifuging at 3000g for 15 min at 4°C, and stored at -80°C. The animals, under anesthesia, were sacrificed by cervical dislocation and liver, and adipose deposits (epididymal, subcutaneous, retroperitoneal, inguinal) were entirely collected then weighted and snap frozen in liquid nitrogen and stored at -80°C. The adiposity index was calculated by the sum of all adipose tissues (epididymal, subcutaneous, retroperitoneal, inguinal) relative to total body mass.

Evaluation of physical exercise

PE of mice was monitored, first by daily record of the number of laps being executed in each cage during the entire protocol, and second by the maximum aerobic speed (MAS) evaluation. MAS was measured at the beginning of the protocol (T0) and after the 10-week HFS diet. Then, it was evaluated during the exercise protocol at T17 and T24. It consisted on a 6 min treadmill training session at a velocity of 7 $m.min^{-1}$ and increased levels of 4 $m.min^{-1}$ every 1'30 minutes. The MAS was reached when mice were not able to keep up with treadmill speed.

Insulin tolerance test

Mice were subjected to Insulin Tolerance Test (ITT) and were fasted for 6 hours before assessments. Collection of blood (5µl) was realized by using the tail-clip method and fasting glycemia was measured using commercially available glucometer (Accu-Check glucometer, Roche), according to the manufacturer's instructions. Then, ITT were performed after an i.p. injection of insulin solution (1U.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 injection. ITT was realized at T0, T10, T17 and T24.

Biochemical analysis

Adiponectin, calcium (Ca^{2+}), insulin, glucose, non-esterified fatty acids and triglycerides were quantified according to the manufacturer's instructions as previously described (24).

The HOMA-IR index was calculated according to the following formula: fasting insulin (microU.L^{-1}) x fasting glucose (mmol.L^{-1})/22.5 (25).

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, Walthman, United States) according to the protocol previously reported (18,26,27).

Histological analysis

Paraffin embedded tissue sections of liver and inguinal AT (iWAT) were stained with hematoxylin and eosin (H&E) using standard protocols. The images were captured by a light microscope (Zeiss Axio Imager, Germany) and adipocyte area (µm²) was determined using (Image J) software as previously described (24,28,29).

RNA isolation and qPCR

Total RNA from liver, iWAT and epididymal AT (eWAT) were extracted using TRIzol reagent according to the manufacturer's instructions (Thermo Fisher, Courtaboeuf, France). One µg of total RNA was used to synthetize 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, United States) as previously described (30). 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 (31). Primer sequences were used for qPCR determination (see Supplemental Digital Content 1, which lists primer sequences). Data were expressed as relative expression ratio.

Statistical analysis

Data were expressed as the mean ± 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). Correlations between two variables were performed using Prism6 by calculating Pearson correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

Results

Voluntary PE associated to VD limited weight gain

After 10 weeks of HFS diet, mice displayed a significant increased body weight compared with the NC group due to a higher energy intake in the HFS group. In order to assess the effect of a long-term PE and VD supplementation on obese mice, we compared HFS-fed group of mice remained on the HFS diet to 3 other subgroups assigned either to voluntary PE (HFS+ex), VD supplementation (15 000 IU/kg of food) (HFS+D), or both of them (HFS+D+ex) for 15 additional weeks. At the end of the 25-week period, HFS mice continued to gain weight and their final body weight was 1.5-fold higher than NC mice (Figure 1A). Mice assigned to 15 weeks of voluntary exercise alone or together VD supplementation reduced their weight gain (-15%) compared with HFS and HFS+D mice.

Voluntary PE associated to VD supplementation limited adipose tissue mass

HFS diet consumption increased adiposity after 25 weeks (Figure 1B) with a 2.4-fold increase of adiposity index. The adiposity of HFS+D+ex group was reduced (-23%) compared with the HFS and HFS+D groups and was similar to the HFS+ex group. Each AT deposit mass has been measured, in order to estimate AT distribution (Figure 1C). In the HFS group, retroperitoneal, epididymal, inguinal and subcutaneous masses were respectively 4, 2, 3 and 5 times higher compared to NC mice. These masses were significantly reduced in HFS+D+ex and HFS+ex mice (-42% retroperitoneal, -16% epididymal, -31% inguinal and 41% subcutaneous) compared to HFS and HFS+D mice.

Adipocyte morphology of inguinal AT was studied (Figure 1D). Histological analysis showed adipocyte hypertrophy in the HFS group (mean adipocyte area 30% higher) compared to the NC group. In the HFS+D+ex group, adipocyte mean size was significantly reduced by 11%

compared to the HFS group (Figure 1E) and by 16% compared to the HFS+D group. However, no difference was observed between HFS+D+ex and HFS+ex groups.

Voluntary PE associated to VD improved physical performances on obese c57bl6/J mice fed with an HFS diet

Mice in the HFS+ex group and the HFS+D+ex group ran similar distance with respectively 1,314 and 1,429 meters/day (Figure 2A). Cumulative running achieved a total of 11,937 and 10,683 meters in the HFS+ex and HFS+D+ex groups, without any difference between these groups (Figure 2B).

To check if voluntary PE improved physical performances, maximum aerobic speed (MAS) was measured at different time points during the protocol. No modification in MAS was observed between the NC and HFS groups after 10 weeks of protocol. Then, a significant increase in MAS at the middle (17 weeks) as well as at the end (24 weeks) of the protocol was observed in both HFS+ex mice (+40%) and HFS+D+ex (+35%) mice compared to sedentary (NC, HFS and HFS+D) groups of mice (Figure 2C). Moreover, modification observed in MAS was no altered by age because the NC group exhibited same values between the different measures.

Voluntary exercise associated to vitamin D supplementation improved glucose homeostasis and insulin sensitivity

As reported before (Marziou et al. 2020), the 10-week HFS diet protocol induced an insulin resistant pattern in mice. Furthermore, at the end of the 25-week protocol, insulin sensitivity, evaluated by insulin tolerance test (ITT), was 1.5-fold reduced compared to NC mice (Figure 3B). In terms of glycemic response to a single dose of insulin, no difference was observed

between the HFS group and HFS+D or HFS+ex groups. However, in HFS+D+ex group, glycemic response was similar to the NC group (Figure 3A). In addition, calculation of AUC displayed same pattern in HFS+D+ex and NC groups (Figure 3B). Plasma insulin and glucose quantification (Figure 3C) showed that HFS and HFS+D groups displayed hyperglycemia and hyperinsulinemia, while in HFS+D+ex and HFS+ex groups. HOMA-IR also revealed similar response profile (Figure 3D).

Impact of voluntary PE and vitamin D supplementation on plasma parameters

HFS diet supplemented or not with VD for 25 weeks did not modify plasma triglycerides (TG) concentration in comparison to NC diet (Table 1), while PE induced a significant decrease in triglycerides levels in HFS+ex and HFS+D+ex groups compared to HFS and NC groups. Concerning non-esterified fatty acid (NEFA) concentration, a decrease was observed for all HFS groups in comparison with the NC group (Table 1). Adiponectin concentration was slightly increased in the HFS group compared to the NC group and no difference between HFS, HFS+D groups and the NC group was observed. The HFS+ex group showed a decreased adiponectin level in comparison to the HFS group and the HFS+D+ex group showed a significant decreased adiponectin concentration compared with the other HFS+D, HFS+ex and HFS groups.

As expected, 15 weeks of VD supplementation significantly increased cholecalciferol and 25(OH)D plasma levels compared with the HFS and NC groups (Table 1), and resulted in a slight increase of calcemia in HFS and HFS+D fed mice compared with the NC group. Furthermore, in trained mice, a slight but significant decrease of 12% in 25(OH)D level was observed in the HFS+D+ex group as compared with HFS+D (Table 1) and significant decrease of calcemia in comparison to HFS+D, HFS groups but calcemia was similar to HFS+ex group.

Voluntary PE associated to VD supplementation improved inflammatory status mediated by HFS diet on adipose tissue

As voluntary PE coupled to VD supplementation induced significant decrease in iWAT accretion, gene expression analysis was performed to examine consequences on AT inflammatory related genes (Figure 4A). *Mcp1* mRNA level was significantly increased in the HFS group compared with the NC group. VD supplementation (HFS+D) decreased significantly *Mcp1* level in comparison with the HFS group. PE induced a slight but not significant decrease of *Mcp1* mRNA levels in HFS+D+ex and HFS+ex groups, compared to HFS group. The expression of the *Ccl5* mRNA was also increased in the HFS group compared with the NC group, and *Ccl5* mRNA expression was decreased in HFS+D and HFS+ex groups compared to HFS group. The decrease in HFS+D+ex group was more pronounced than in the HFS+D and HFS+ex groups. *Tgfb1* mRNA levels showed an increased expression in the HFS group compared with the NC group, and no major difference was observed between HFS+D and HFS+ex groups compared with the HFS group. However, the HFS+D+ex group showed a significant decrease of *Tgfb1* mRNA expression (-45%) compared with the HFS group. Other cytokines expression such as *Il6*, *Il10* and *Tnfa* expressions were measured and no difference was observed between the HFS group and HFS+D, HFS+ex and HFS+D+ex groups (data not shown).

Visceral and epididymal AT deposits were also studied in terms of gene expression coding for inflammatory proteins. Among the studied chemokines (data not shown), *Ccl5* was 5-fold increase by HS diet in comparison with the NC group and no change was observed in HFS+D and HFS+ex groups. However, in the HFS+D+ex, *Ccl5* mRNA levels were decreased compared with the HFS group.

Voluntary PE improved energetic metabolism in inguinal adipose tissue

mRNA coding for proteins related to metabolic (peroxisome proliferator activator receptor γ coactivator-1α (*Pgc1a*) and carnitine palmitoyltransferase-1 (*Cpt1*) and thermogenic uncoupling protein 1 (*Ucp1*), activities were assessed on iWAT (Figure 4B). HFS diet induced a decrease in *Cpt1* expression on iWAT in comparison with NC diet. This decrease was also observed in all groups (Figure 4B). *Ucp1* mRNA expression was found to be significantly increased (3-fold) in the HFS group compared with the NC group, and significantly decreased in HFS+D+ex and HFS+D but not in HFS+ex group, in comparison with the HFS group.

Pgc1a mRNA levels were significantly decreased in the HFS group as well as in the HFS+D group compared to the NC group, while PE induced a 2-fold increase of *Pgc1a* in HFS+ex and HFS+D+ex groups as compared to HFS group and reached values similar to the NC group.

Voluntary PE associated with VD supplementation decreased ectopic fat liver deposition and inflammatory gene expression

HFS diet induced a significant liver mass increase (2-fold) compared with the NC group (Figure 5A). While VD supplementation did not modify liver mass, exercise reduced it by -32% and -38% in HFS+ex and HFS+D+ex groups respectively in comparison to the HFS group. Moreover, liver mass of HFS+D+ex group was similar to the NC group.

To evaluate hepatic lipid accumulation, intrahepatic triglycerides (TG) were quantified. TG in the HFS group were significantly increased compared to the NC group. In the HFS+D+ex group, a drastic decrease of TG (-70%) was shown compared to the HFS group and a decrease (-37%) compared to the NC group. In HFS+ex and HFS+D groups TG decreased by almost 33% compared to the HFS group and no difference compared to the NC group (Figure 5C). In

agreement with these results, histological sections stained by (H&E) confirmed a reduction of visible lipid droplets (Figure 5B).

At the molecular levels, key genes expression of fat metabolism (de novo lipogenesis versus fatty acid oxidation) were measured in the liver. The evaluation of hepatic de novo lipogenesis through the mRNA expression of Fatty acid synthase (*Fasn*) and Acetyl-CoA carboxylase (*Acaca*) genes was assessed (Figure 5D). As expected de novo lipogenesis was significantly enhanced by HFS diet. In fact, *Fasn* and *Acaca* expression were significantly increased by 2 and 1.8-fold respectively, in the HFS group compared to the NC group. On the contrary, as PE or VD supplementation exerted an inhibitory effect of *Fasn* and *Acaca* gene expression, the association of both, limited significantly the Fasn and Acaca expression in HFS+D+ex group.

Fatty acid oxidation was measured by gene expression of acyl-CoA oxidase (*Acox*) and carnitine palmitoyltransferase-1 (*Cpt1*) (Figure 5D). No difference of *Acox* mRNA levels was observed between HFS, HFS+ex and NC groups whereas groups supplemented with VD (HFS+D and HFS+D+ex groups) showed a significant decrease compared to the HFS group. On the other hand, groups fed with HFS diet (HFS, HFS+D, HFS+ex and HFS+D+ex) induced a significant decrease of *Cpt1* expression compared to the NC group.

The ratio between lipogenesis and lipolysis was also determined and compared with other parameters (Table 2). We observed that this ratio was 3-fold increase in the HFS group compared to the NC group. It was positively correlated with higher liver TG and adiposity index. In HFS+D+ex group, this lipogenic activity was significantly diminished and was associated to decreased liver TG and adiposity index. Coefficient correlation of lipogenesis/lipolysis ratio with liver TG and adiposity index were respectively 0,83 and 0,75.

Peroxisome proliferator-activated receptor gamma (*Ppara* and *Pparg*) expression was measured in the liver (Figure 5E). *Pparg* expression was significantly increased in the HFS group compared to the NC group. On the contrary, with exercise and VD supplementation (HFS+D+ex), *Pparg* mRNA levels were decreased by 38% compared with the HFS group. A decrease was also observed in the HFS+D group (-7%) and in the HFS+ex (-27%) compared to the HFS group. On the other hand, *Ppara* mRNA levels was not affected by any diet or intervention.

Inflammation was also studied in the liver. While *Tnfa* expression was 3-fold increase in the HFS group compared to NC group, no improvement was observed after VD supplementation or PE (Figure 5F). The expression of *Mcp1* mRNA was induced in HFS, HFS+D and HFS+ex compared to the NC group, whereas its expression was decreased in HFS+D+ex group.

Discussion

The aim of this work was to investigate the effects of an intervention associating voluntary PE and VD supplementation during 15 weeks on obesity and metabolic disorders on diet-induced obese male C57bl6/J mouse model.

We implemented a 10-week HFS diet to generate obesity and insulin-resistance in mice, as previously described (24). Interestingly, after the obesity induction, a strategy based on 15-weeks of PE in obese mice resulted in a significant weight limitation gain as reported under shorter PE period (7 weeks; (29)), while VD supplementation alone did not limit HFS-induced weight gain, in accordance with previous data (16,24). The combination of PE with VD supplementation did not enhance the effect obtained with PE alone in terms of limitation of weight gain. Concerning adiposity, only PE alone or in combination with VD improved it significantly. No effect of VD alone was observed. Similar results were observed in the different deposits of AT in visceral (epididymal, inguinal or retroperitoneal) as well as subcutaneous AT, *i.e.* a decrease under PE effect, as previously reported by others (14,32,33). It is noteworthy that histological analysis suggested that increased adiposity was associated to adipocyte hypertrophy under HFS diet. Interestingly, PE not only reduced fat accretion but also limited the adipocyte hypertrophy as previously reported (14).

We evaluated the effect of voluntary PE on HFS-fed mice performance (measured by MAS), and observed that this parameter was improved compared to sedentary mice, while no change was obtained after VD supplementation.

It is well established that increased adiposity promotes a low-grade inflammatory status (4,6), notably characterized by an increase of chemokines expression in AT (34). Whereas HFS diet induced *Mcp1* and *Ccl5* expression in AT, interestingly the combination of PE and VD supplementation significantly reduced the expression of chemokines. VD alone also reduced

such expression whereas it did not modify adiposity, suggesting that VD alone display a specific inhibition of chemokines expression, whereas in the case of PE, the decrease of chemokines expression is not dissociable from the decrease of adiposity. Observations related to the effect of VD are fully consistent with previous reports in several *in vitro* and *in vivo* models, where VD limited cytokines and chemokines expression by adipocytes (22) and leucocyte infiltration in adipose tissue via a deactivation of the NF-κB signaling pathway (19).

Furthermore, a significant decreased in *Tgfb1* in inguinal AT after the combination of PE with VD supplementation was noted, which is in agreement with the correlation between adiposity and TGF-β reported by Yadav and colleagues (35). Indeed, TGF-β has been reported to regulate the differenciation of multipotent stem cells towards adipogenic pathways (36). Moreover, TGF-β is involved in hyperplastic AT expandability, which is related to adipocyte precursor proliferation (37). Thus, our results suggest that reduced *Tgfb1 ex*pression could participate to the reduced adipocyte formation and consequently reduced adiposity.

Adiposity and subsequent AT low-grade inflammation have a major role in systemic insulin resistance development. While, a decreased sensitivity to insulin was observed in HFS fed mice at the end of the protocol, neither PE alone nor VD supplementation improved it. Indeed, insulin sensitivity was similar to NC mice when obese mice were subjected to the combination of PE and VD supplementation. Similar results related to the lack of effect on insulin sensitivity after PE has already been reported by Gehrke et al. (16). In addition, we previously reported that VD supplementation alone was inefficient in similar protocol regarding insulin sensitivity (24). Nevertheless, a major highlight of the present study is the improvement of insulin sensitivity after combining PE and VD supplementation, which normalized insulin sensitivity compared to NC mice. The molecular mechanism involved is presently not identified but will require further investigations.

Aside from exerting beneficial effects on weight loss and cardiovascular system, PE had been shown to improve hepatic disease such as NAFLD (38,39) and is highly recommended by the Clinical Practice Guidelines (40). In our study, 15 weeks of PE reduced significantly liver mass and lipid accumulation, evaluated by TG quantification and histology. Interestingly, when VD supplementation was combined to PE, parameters related to the liver were completely normalized compared to NC group. In order to explain these observations, we evaluated the gene expression profile related to lipogenesis and fatty acid oxidation. We observed that the different strategies decreased the expression of genes coding for key proteins involved in hepatic de novo lipogenesis (*Fasn* and *Acaca*) but did not modify fatty acid oxidation. Therefore, the lipogenesis/lipolysis ratio, which was induced by HFS diet, was reduced under PE and/or VD supplementation. Interestingly, this ratio, which is assumed to play an important role in metabolic homeostasis (41) was positively correlated with both liver TG content and adiposity index. Indeed, HFS diet consumption results in an imbalance between lipid acquisition (*i.e*. de novo lipogenesis) and removal (*i.e.* fatty acid oxidation) and is in favour of lipogenesis which could explain the increased TG accumulation in the liver. Hence, lipogenesis appears as a key event in the development of steatosis (42). Our data demonstrated that lipogenesis/lipolysis ratio was reduced by VD supplementation and PE. Interestingly, such reduction was mainly due to the limitation of lipogenesis, which might explain the liver profile improvement and adiposity.

We next evaluated hepatic inflammation and notably chemokines expression since MCP1 has been reported to be associated with hepatic steatosis (43). Moreover, chemokine expression has been shown to be positively correlated with insulin resistance and visceral obesity (44). Interestingly, *Mcp1* expression was strongly decreased by PE and VD supplementation. Such decrease was associated to the improvement of insulin sensibility in mice, suggesting that this reduced expression of *Mcp1* could have a role in the restoration of insulin sensitivity in HFS+D+ex. Indeed, the contribution of *Mcp1* to insulin resistance had been studied in mice fed

with a high fat/sucrose diet supplemented with an inhibitor of the *Mcp1* receptor, CCR2. The blockage of CCR2 had led to decreased pro-inflammatory macrophage infiltration which both ameliorate insulin resistance and hepatic steatosis (45). Further validations are required to validate that assumption in our study.

To conclude, we reported for the first time a combined effect of PE and VD supplementation. Not all parameters tested were improved by this strategy, but key parameters such as insulin sensitivity and liver TG accumulations were beneficially affected, and the effect of combination was stronger that the effect of PE or VD supplementation alone. Such strategy could therefore be of particular interest to fight obesity and its comorbidities.