Vitamin D Deficiency Increases Lipogenesis and Reduces Beta-Oxidation in the Liver of Diet-Induced Obese Mice

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Summary The study was conducted to understand better the mechanisms involved in liver changes when there is a combination of diet-induced obesity (DIO) and vitamin D deficiency (VDD). After 8 wk of feeding a control diet (C group) or a high-fat diet (HF), both with vitamin D, and counterpart groups without vitamin D (VitD− groups), we found in plasma: higher alanine aminotransferase, and aspartate aminotransferase in the VitD− groups, and more elevated total cholesterol in the HF group. Compared to their counterparts, HF and HF/VitD− showed hyperinsulinemia and higher hepatic triglycerides and steatosis. The protein expressions of markers linked with the vitamin D action were altered by VDD (vitamin D receptor VDR, 25-hydroxyvitamin D-24-hydroxylase CYP24A1, CYP27B1, and CYP2R1). The hepatic lipogenesis and fatty acid synthesis were enhanced by VDD (peroxisome proliferator-activated receptor PPARγ, sterol regulatory element-binding proteins SREBP1c, carbohydrate-responsive element-binding protein ChREBP, and fatty acid synthase FAS), but markers of beta-oxidation were reduced (PPARα and phosphoenolpyruvate carboxykinase PEPCK). In conclusion, the study provides convincing new evidence that there is an additive and adverse effect on the liver caused by the combination of VDD and DIO. The essence of these changes in the liver is an increased lipogenesis and a reduced beta-oxidation, which predisposes to the accumulation of fat in the liver, accompanied by IR. The worsening of the pathogenesis of NAFLD may tilt to more severe stages of liver disease.

Key Words vitamin D deficiency, nonalcoholic fatty liver disease, obesity, lipogenesis, beta-oxidation

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. NAFLD includes a spectrum of liver disease ranging from simple steatosis to nonalcoholic steatohepatitis (1). The insulin resistance (IR) increases the vulnerability of the liver to many factors that promote hepatic injury, inflammation, and fibrosis (2), including the development of NAFLD and hepatocellular carcinoma (3).

Hepatic lipogenesis, inflammation, and beta-oxidation are natural targets in studying NAFLD (4). Peroxisome proliferator-activated receptor (PPAR) gamma is an essential factor of lipogenesis in the liver. Sterol regulatory element-binding protein (SREBP) 1c and its transcript fatty acid synthase (FAS) are regulators of lipogenesis associated with liver lipid accumulation (5). Vitamin D deficiency (VDD) can result from problems related to the absorption of vitamin D, hydroxylation for liver failure, low dietary intake, or inadequate exposure to sunlight (6). Moreover, approximately one billion people worldwide are believed to have deficient 25(OH)D3 levels (7).

Usually, nucleated cells have vitamin D receptors (VDRs), and adequate vitamin D levels may be necessary for reducing the incidence of, or mortality from, some cancers in lowering autoimmune disease and increase insulin responsiveness (8). There is an association between VDD and the presence, severity, and prognosis of liver diseases (7). Obesity, which usually occurs with low levels of 25(OH)D3 (9), also has a strong association with NAFLD (10).

In the study, we hypothesized that the combination of two morbid factors for NAFLD, diet-induced obesity (DIO) and VDD, has the potential to increase liver damage and all the consequences of progression of liver disease.

MATERIALS AND METHODS

Animals and diets. The study was carried out in strict accordance with the recommendations in the current guidelines for experimentation with animals (National Institutes of Health Publication No. 85–23, revised in 1996). The experimental protocol was approved by the Animal Ethics Committee of the University of the State of Rio de Janeiro (Protocol Number CEUA/038/2013), and all efforts were made to minimize suffering.

Twelve-week-old male C57BL/6 mice were maintained under controlled temperature conditions in ventilated cages (NexGen System, Inc., Allentown, PA, 12 h/12 h dark/light cycle, 20±2°C). The study lasted...
for 8 wk and mice had free access to water and food. The control and high-fat diets were manufactured by Pragsolucoses (Jau, São Paulo, Brazil) as detailed in Table 1. The diets were consistent with the commendations of the American Institute of Nutrition Rodent Diets for adult maintenance (AIN-93M) (11).

The animals were randomly divided into four groups (n = 15/group, totalling 60 animals) and were fed the diets for 8 wk: Control diet group (C); High-fat diet group (HF); Control diet group without vitamin D (C/VitD−); and High-fat diet group without vitamin D (HF/VitD−).

**Body mass and feed efficiency.** The food consumption was measured daily, and body mass (BM) was assessed weekly. The energy intake was considered the product of food intake (g/d) and the energy content of the diet (kJ/g). Feed efficiency was designated as the ratio between the energy consumed by each animal divided by its BM (kJ/g).

**Sacrifice and tissue extraction.** At the begin of the ninth week of the experiment, after 6 h of food deprivation, we anesthetized the animals (intraperitoneal sodium pentobarbital, 150 mg/kg) and then they were killed by exsanguination. The blood was collected, and plasma separated by centrifugation (120 × g for 15 min) and stored at −20°C.

The liver was rapidly removed and weighed, and fragments of all lobes were quickly frozen for molecular analyses. Additional fragments were designated for light microscopy and kept in a freshly prepared fixative solution for 48 h (formaldehyde 4% w/v, 0.1 M phosphate buffer, pH 7.2).

<table>
<thead>
<tr>
<th>Nutrients (g/kg)</th>
<th>C</th>
<th>HF</th>
<th>C/VitD−</th>
<th>HF/VitD−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>140</td>
<td>175</td>
<td>140</td>
<td>175</td>
</tr>
<tr>
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<td>347.7</td>
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<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>Soybean oil</td>
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<td>40</td>
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<tr>
<td>Lard</td>
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</tr>
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<td>10</td>
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<tr>
<td>Vitamin D (IU)</td>
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<td>1,000</td>
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<tr>
<td>Energy (%)</td>
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<td>21,000</td>
<td>15,960</td>
<td>21,000</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>76</td>
<td>36</td>
<td>76</td>
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</tr>
<tr>
<td>Protein (%)</td>
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</tr>
<tr>
<td>Fat (%)</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>50</td>
</tr>
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</table>

C, control diet; HF, high-fat diet; C/VitD−, control diet without vitamin D; HF/VitD−, high-fat diet without vitamin D. 1 Vitamin D was added in the vitamin mix.

**Plasma analysis.** The following plasma concentrations were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), and triglycerides (TG) (semiautomatic spectrophotometer using commercial kits. Quibasa, Bioclin System II, Belo Horizonte, MG, Brazil). Other plasma concentrations were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits: 25(OH)D3 (AC-57F1 ELISA kit, Immunodiagnostics Systems, Boldon, UK), and insulin (#EZRMI-13K ELISA kit), all kits from Millipore assay, Darmstadt, Germany.

**Liver biochemistry and microscopy.** Liver TC and TG concentrations were measured in frozen samples (approximately 50 mg) homogenate with isopropanol in an ultrasonor probe (centrifuged at 120 × g for 15 min). The supernatant was analyzed using commercial kits (Quibasa, Bioclin System II, Belo Horizonte). Formalin-fixed fragments were embedded in Paraplast.
supernatant was collected and mixed with protease and Protein Assay Kit (Thermo Scientific, Rockford, IL); the was obtained from 100 mg per group using the BCA groups were used for Western blotting. Total protein approximately 50 mg of liver tissue from mice of the different in the study.

The endogenous control. Table 2 details the primers used between the number of cycles of the target genes and of mRNA was calculated and expressed as the difference a melting curve program. The relative expression ratio activation program for 4 min at 95˚C was followed by the selected genes. A pre-denaturation and polymerase-chain reaction (RT-qPCR) was performed reverse transcriptase (Invitrogen). Quantitative real-time as messenger RNA (mRNA) and Superscript III DNA (cDNA) was performed using Oligo primers (Invitrogen) for messenger RNA (mRNA) and Superscript III DNAse I (Invitrogen). Synthesis of the complementary points (PT): Vv [steatosis, liver] was estimated as the ratio between tem composed of 36 test points in at least ten random atosis. For that, we used point counting with a test sys- hematoxylin and eosin. Digital images were obtained and a DS-Ri1 digital camera, Nikon Instruments, Inc., New York, NY) for stereological estimation of liver steatosis. For that, we used point counting with a test system composed of 36 test points in at least ten random fields per animal. The volume density of hepatic steatosis (Vv [steatosis, liver]) was estimated as the ratio between the points hitting fat drops (Pp) and the number of test points (Pv): Vv [steatosis, liver] = Pp [steatosis]/Pv (12, 13).

Real-time quantitative polymerase chain reaction. Total RNA was extracted from 50 mg of liver tissue using Trizol (Invitrogen, Carlsbad, CA). The amount of RNA was determined, and 1 µg of RNA was treated with DNase I (Invitrogen). Synthesis of the complementary DNA (cDNA) was performed using Oligo primers (Invi-rogen) for messenger RNA (mRNA) and Superscript III reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using the CFX96 cycle Bio-Rad, Hercules, CA) and the SYBR Green mix (Invitrogen). Beta-actin was used as an endogenous control to normalize the expression of the selected genes. A pre-denaturation and polymerase-activation program for 4 min at 95˚C was followed by a melting curve program. The relative expression ratio of mRNA was calculated and expressed as the difference between the number of cycles of the target genes and the endogenous control. Table 2 details the primers used in the study.

Western blot. Proteins extracted from approximately 50 mg of liver tissue from mice of the different groups were used for Western blotting. Total protein was obtained from 100 mg per group using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL); the supernatant was collected and mixed with protease and phosphatase inhibitors. Proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE), and blotted proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) and incubated overnight at 4˚C with primary antibodies with BSA (bovine serum albumin) to block with 3% (wt/vol) in PBS.

Table 3. Feeding behavior, body mass and plasmatic dosages of the animals.

<table>
<thead>
<tr>
<th>Data</th>
<th>C</th>
<th>HF</th>
<th>C/VitD−</th>
<th>HF/VitD−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>28.7±0.3</td>
<td>32.7±0.6†</td>
<td>29.0±0.4</td>
<td>32.2±0.5‡</td>
</tr>
<tr>
<td>Energy intake, kJ/d</td>
<td>47.3±3.2</td>
<td>60.1±4.5†</td>
<td>47.3±3.7</td>
<td>59.8±3.5‡</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D3, nmol/L</td>
<td>71.5±16.4</td>
<td>36.8±13.2†</td>
<td>22.8±7.4†</td>
<td>5.0±1.4‡</td>
</tr>
<tr>
<td>AST, IU/L</td>
<td>30.6±12.6</td>
<td>74.3±24.4</td>
<td>33.4±2.7</td>
<td>180.5±77.2‡</td>
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<tr>
<td>ALT, IU/L</td>
<td>77.5±4.0</td>
<td>112.9±21.6</td>
<td>138.6±34.1†</td>
<td>162.3±59.4</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>1.4±0.4</td>
<td>2.2±0.4†</td>
<td>2.1±0.4‡</td>
<td>2.2±0.3</td>
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<tr>
<td>Insulin, pmol/L</td>
<td>304.4±51.1</td>
<td>1,117.0±272.5†</td>
<td>34.3±199.6</td>
<td>1,460.0±130.0‡</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3±0.3</td>
<td>1.6±0.3</td>
<td>1.4±0.3</td>
<td>1.6±0.2</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Cholesterol, mmol/L/mg</td>
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<td>1.6±0.1</td>
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<td>Triglycerides, mmol/L/mg</td>
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<td>2.2±1.3†</td>
<td>1.0±0.4</td>
<td>2.2±0.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (n=15 for body mass and energy intake, n= 5–8 for other data) and analyzed by one-way ANOVA and the posthoc test of Holm–Sidak. p<0.05 when: † compared to C group; ‡ compared to HF group; § compared to C/VitD− group.

Groups: C, control group; HF, high-fat diet; VitD−, without vitamin D.

Plus (Sigma-Aldrich, St. Louis, MO) and exhaustively sectioned at 5-µm-thick, and sections were stained with hematoxylin and eosin. Digital images were obtained blindly and randomly (Nikon microscope, model 80i, and a DS-Ri1 digital camera, Nikon Instruments, Inc., New York, NY) for stereological estimation of liver steatosis. For that, we used point counting with a test system composed of 36 test points in at least ten random fields per animal. The volume density of hepatic steatosis (Vv [steatosis, liver]) was estimated as the ratio between the points hitting fat drops (Pp) and the number of test points (Pv): Vv [steatosis, liver] = Pp [steatosis]/Pv (12, 13).

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Vitamin D, Hepatic Lipogenesis, and Beta-Oxidation

SC81178, Santa Cruz Biotechnology, 1:1,000) as a loading control to normalize the data.

Statistical analysis. After confirming the data had a normal distribution (Shapiro–Wilk test) and equality of variances (Brown–Forsythe test), the mean and standard deviation were reported. The differences between groups were analyzed with one-way analysis of variance (ANOVA) and the posthoc test of Holm-Sidak. We also reviewed the effects of the vitamin D and diet as independent factors and the possible interactions between them with two-way ANOVA (GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla, CA). A p-value < 0.05 was taken as statistically significant.

RESULTS

Body mass and food intake

The HF diet, but not vitamin D restriction, affected the BM, without interaction between these factors (Table 4). The groups started the study without a difference in their BM. Since the second week of the experimentation, the HF diet led to a BM growth in both the HF and HF/VitD− groups, more significant than in their counterparts. At the sacrifice, the HF group was 14% heavier than the C group, as well as HF/VitD− being 11% heavier than C/VitD− (Table 3). These findings were associated with the energy intake of the animals. Both HF and HF/VitD− groups consumed more energy than their counterparts (26% comparing the groups HF vs. C, and HF/VitD− vs. C/VitD−).

Plasma

The concentrations of ALT were primarily affected by the HF diet, but the HF diet and vitamin D restriction have interaction producing the results. AST and TC were altered by the HF diet and vitamin D restriction. Interestingly, all of the factors have influenced TG levels (Table 4).

ALT was elevated in the HF/VitD− group (140% compared to the HF group; 440% compared to the C group).
C/VitD− group). The levels of AST were higher in the C/VitD− group compared to the C group (+80%). The levels of TC were higher in the HF group (+57%), and higher in the C/VitD− group (+50%) in comparison with the C group. The plasmatic levels of TG did not show difference among the groups (Table 3).

**Insulin and 25(OH)D3**

The HF diet has affected insulin and 25(OH)D3 more than vitamin D restriction (Table 4).

The plasma level of insulin was enhanced by the HF diet and by vitamin D restriction: C vs. HF, −267%; HF vs. HF/VitD−, −31%; C/VitD− vs. HF/VitD−, −325%.

Both the HF diet and vitamin D restriction diminished the plasma level of 25(OH)D3: C vs. HF, −49%; C vs. C/VitD−, −68%; HF vs. HF/VitD− group, −86%. However, no difference was seen when comparing C/VitD− and HF/VitD− (Table 3).

**Liver biochemistry and steatosis**

The HF diet was the primary factor altering the hepatic TG and steatosis (Table 4). TC did not show difference among the groups, while TG was elevated in the liver of the HF group compared to the C group (+214%) (Table 3). Vv [steatosis, liver] varied significantly among the groups: C vs. HF, −89%; C/VitD− vs. HF/VitD−, +63% (Figs. 1 and 2).

**Liver gene expressions**

a) **Lipogenesis and fatty acid synthesis** (Fig. 3). The HF diet and vitamin D restriction affected PPARγ and SREBP1c. The diet changed ChREBP more (Table 4). PPARγ: C vs. C/VitD−, −130%; HF vs. HF/VitD−, +77%.

SREBP1c: C vs. HF, +48%; C vs. C/VitD−, +100%; HF vs. HF/VitD−, +72%.

ChREBP: C vs. HF, −75%; C/VitD− vs. HF/VitD−, −72%.

EAS: C vs. C/VitD−, +170%.

b) **Beta-oxidation** (Fig. 4). The HF diet was a crucial factor modifying PPARα and PEPCK. The vitamin D restriction was the factor that most influenced the changes in CPT1 and IL6. Both the HF diet and vitamin D restriction interacted to affect the results of PPARγ and CPT1 (Table 4).

PPARα: C vs. HF, −90%; C vs. C/VitD−, −50%.

CPT1: HF vs. HF/VitD−, +205%.

PEPCK: C vs. HF, −80%; C/VitD− vs. HF/VitD−, −67%.

IL6: C vs. C/VitD−, +350%; HF vs. HF/VitD−, +250%.

c) **Vitamin D local action** (Fig. 5). The HF diet was a chief factor modifying VDR and CYP24A1. Vitamin D restriction changed CYP2R1 and CYP27B1. Both factors had interaction concerning the results of VDR and CYP24A1 (Table 4).

VDR: C vs. HF, −79%; C/VitD− vs. HF/VitD−, +167%; HF vs. HF/VitD−, +500%.

CYP2R1: C vs. C/VitD−, +299%; HF vs. HF/VitD−, +408%.

CYP27B1: C vs. HF, −79%; C/VitD− vs. HF/VitD−, −59%.
CYP24A1: C vs. C/VitD−, +38%; C/VitD− vs. HF/VitD−, −65%.

Liver protein expressions:

a) Lipogenesis (Fig. 6). The HF diet was an essential factor modifying lipogenesis. Both the HF diet and vitamin D restriction interacted to alter the results (Table 4).

PPARγ: C vs. HF, +175%; HF vs. HF/VitD−, +50%; C/VitD− vs. HF/VitD−, +215%.
SREBP1c: C vs. HF, +225%; C vs. C/VitD−, +200%; HF vs. HF/VitD−, +130%; C/VitD− vs. HF/VitD−, +150%.
ChREBP: HF vs. HF/VitD−, +65%; C/VitD− vs. HF/VitD−, +180%.

b) Beta-oxidation and gluconeogenesis (Fig. 7). The HF diet was the primary factor affecting the hepatic beta-oxidation, although the vitamin D restriction was relevant, changing PPARα and PEPCK. The HF diet and vitamin D restriction interacted to change PEPCK (Table 4).

PPARα: C vs. HF, −50%; C vs. C/VitD−, −40%; C/VitD− vs. HF/VitD−, −55%.
PEPCK: C vs. HF, −50%; C vs. C/VitD−, −50%.
IL6: no differences were observed between groups.

c) Vitamin D local action (Fig. 8). The HF diet was a factor always affecting this panel of protein expressions. VDD, however, also influenced CYP27B1 (Table 4).

VDR: C vs. HF, −50%.
CYP27B1: C vs. HF, −30%; C vs. C/VitD−, −26%; HF vs. HF/VitD−, −35%; C/VitD− vs. HF/VitD−, −38%.
CYP24A1: C vs. HF, +100%; C/VitD− vs. HF/VitD−, +100%.
DISCUSSION

The current original study highlights the combination of VDD and DIO in mice. This combination impairs the hepatic lipid homeostasis, leading to steatosis. Parallelly, there were increased fasting insulin, altered liver enzymes, enhancement of lipogenesis gene and protein expressions, such as PPARγ, SREBP1c, FAS, and declining beta-oxidation protein expressions, such as PPARα. The combination of DIO and VDD (HF/VitD− group) was accompanied by hyperinsulinemia, hypercholesterolemia and elevated levels of hepatic TG, steatosis, ALT, and AST (characterizing hepatocellular involvement or injury) (14).

In the current study, 25(OH)D3 and not 1,25(OH)2D3 was measured. The active form of vitamin D is 1,25(OH)2D3, and it remains at normal concentrations because the hydroxylation of 25(OH)D3 to 1,25(OH)2D3 occurs in several tissues, meeting local needs. Likewise, the half-life of 1,25(OH)2D3 is approximately 6 h, while the half-life of 25(OH)D3 is 2 to 3 wk. In addition, the levels of 1,25(OH)2D3 are approximately 100 times smaller than 25(OH)D3, which often renders their levels undetectable (15).

Previously, a link between VDD and the development of IR in mice was demonstrated, which impaired both glucagon and insulin secretion by pancreatic alpha and beta cells, respectively, and alteration of the pancreatic islet structure (16). Moreover, the maternal vitamin D restriction potentially alters the structure of the liver and pancreas, especially in the F1 generation (17).

Obesity and hypovitaminosis D is associated with
low-grade inflammation characterized by alterations in circulating adipokines involved in the pathogenesis of NAFLD (18). Interestingly, even in the absence of obesity and diabetes, hyperinsulinemia and IR are related to NAFLD and increased activity of ALT and AST (19, 20). The administration of vitamin D has demonstrated an effect against steatosis by stimulating PPARα-mediated lipolysis and lipid clearance, down-regulating inflammatory cytokines, and ameliorating IR (21).

In the liver, PPARγ, SREBP1c, and its transcript FAS are related to lipogenesis and de novo lipogenesis (5). A HF diet induces fatty liver in mice, up-regulating lipogenesis and liver lipid accumulation (22). In this study, VDD was seen as modulating gene expressions that command the lipogenesis, beta-oxidation, and inflammation in the liver.

Twenty-five (OH) D3 is an endogenous inhibitor of SREBP1c (23), and SREBP1c is a well-documented regulator of lipogenesis that is upregulated in obesity (24, 25). The precursor form of SREBP can be accumulated because of the action of 25(OH)D3 with cholesterol and a concomitant decrease in SREBP1c (26). The preventive effect of 1,25(OH)2D3 against diet-induced hepatic steatosis is due to the blockade of lipogenesis and the promotion of fatty acid oxidation in the liver (27). Herein, PPARγ and FAS increased in the HF/VitD− group, corroborating the association of VDD and hepatic steatosis in our animals.

Vitamin D directly modulates the metabolism of free fatty acids (FFA) via its action on PPARγ, thereby relieving FFA-induced IR. Hence, the increased FFA flowing in the bloodstream could promote fat deposition into the hepatocytes and the progression of NAFLD under the condition of VDD (28). Moreover, VDD was proven to stimulate PPARγ expression, which in turn is upregulated in hepatic steatosis (29, 30).
There is an association between PPARγ and VDR, which suggests both signaling pathways are interconnected by the reciprocal effects of the activated receptors (29). Moreover, PPARγ is a primary target of vitamin D, and vitamin D may modulate the metabolisms by modulating PPARγ signaling (30, 31).

In addition to lipogenesis, liver steatosis is also associated with impairment of beta-oxidation (5). Herein, DIO and VDD led to decreased PPARα and its transcript CPT1 (the primary regulators of beta-oxidation), indicating a role of vitamin D signaling on mitochondrial fatty acid oxidation (CPT1 is required for fatty acid beta-oxidation) (32, 33). Furthermore, PPARα is the master regulator of fatty acid beta-oxidation in mitochondria and peroxisomes (34). In the present study, the expression of CPT1 was downregulated in the HF/VitD−/H9251 group, linking obesity and VDD in beta-oxidation.

Twenty-five (OH) D3 represses the transcriptional activity of PPARα in a 1,25(OH)2D3-dependent manner (35). This upregulation can stimulate the expression of other genes involved in mitochondrial and fat oxidation. The decrease of PPARα in the liver impairs the transcription of its target gene CPT1 and fatty acid release (derived from lipolysis, accumulated in the form of TG in the liver) (36). Herein, we have demonstrated that VDD downregulated PPARα, impairing beta-oxidation and contributing to hepatic steatosis. The supplementation of 1,25(OH)2D3 upregulates both PPARα and CPT1 involved in lipid oxidation (37).

CYP24A1 is a cytochrome P450 component that catalyzes the conversion of 25(OH)D3 and 1,25(OH)2D3 into 24-hydroxylated products (the degradation product of vitamin D molecule) (38). CYP27B1 provides instructions for making the enzyme 1-alpha-hydroxylase that carries out the final reaction to convert vitamin D to its active form (1,25(OH)2D3) (39). Vitamin D, through VDR, plays a role in mineralization homeostasis (40). In addition, VDR expression increased in the inflammatory cells of liver biopsies of patients with NASH, suggesting the local activation of vitamin D as a compensatory effect to insults (28). Our HF group showed lower expression of both VDR and CYP27B1. In the HF/VitD− group, CYP24B1 was high (both activation and degradation enzymes) and CYP27B1 was low. Others reported a chronic effect of the HF diet consumption increasing both VDR and CYP27B1 (41).

In conclusion, the study reinforces our knowledge on the subject and provides new convincing evidence that there are an additive and an adverse effect on the liver caused by the combination of VDD and DIO. The essence of these changes in the liver lies in an increased lipogenesis and a reduced beta-oxidation, which predisposes to the accumulation of fat in the liver, accompanied by IR. The worsening of the pathogenesis of NAFLD may tilt to more severe stages of liver disease and hepatocellular injury.

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Author contributions
CCB performed the experiment, animal care, and acquisition of data; AFS helped with the analysis and assisted in animal care and all the techniques involved in the experiments; IB made contributions to the interpretation of the data; CAML made substantial contributions to the conception and design of the work and critically revised the manuscript for important intellectual content; MBA conceived the work and was critically involved in the writing, revising and preparation of the manuscript and approved the final version. All authors read and approved the final manuscript.

REFERENCES
Vitamin D, Hepatic Lipogenesis, and Beta-Oxidation


