Effect of Vitamin D₃ on the Postprandial Lipid Profile in Obese Patients: A Non-Targeted Lipidomics Study

Salvador Fernández-Arroyo¹,²,* , Anna Hernández-Aguilera³, Marijke A. de Vries⁴, Benjamin Burggraaf⁴, Ellen van der Zwan⁵, Nadine Pouw⁵, Jorge Joven¹,²,³ and Manuel Castro Cabezas⁴

¹ Universitat Rovira i Virgili, Departament de Medicina i Cirurgia, Unitat de Recerca Biomèdica, C/de l’Escarxador S/N, 43003 Tarragona, Spain; jorge.joven@urv.cat
² Campus of International Excellence Southern Catalonia, 43003 Tarragona, Spain
³ Institut d’investigació Sanitària Pere Virgili, C/de l’Escreixador S/N, 43003 Tarragona, Spain; anna.hernandez@gmail.com
⁴ Department of Internal Medicine, Center for Diabetes and Vascular Medicine, Franciscus Gasthuis & Vietland, 3045 PM Rotterdam, The Netherlands; M.deVries@Franciscus.nl (M.A.d.V.); B.Burggraaf@Franciscus.nl (B.B.); M.CastroCabezas@Franciscus.nl (M.C.C.)
⁵ Department of Clinical Chemistry, Franciscus Gasthuis & Vietland, 3045 PM Rotterdam, The Netherlands; E.vanderZwan@Franciscus.nl (E.v.d.Z.); N.Pouw@Franciscus.nl (N.P.)
* Correspondence: salvador.fernandez@urv.cat; Tel.: +34-977-310-300

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Abstract: Postprandial lipemia can lead to an accumulation of atherogenic lipoproteins in the circulation associated with systemic low-grade inflammation and an increased risk of cardiovascular disease. Lifestyle and pharmacological treatments are usually prescribed for prevention. Vitamin D₃ (cholecalciferol), as an anti-atherogenic agent, is being taken into consideration due to its potential beneficial effects in lipid metabolism and its anti-inflammatory potency. To assess the effects of vitamin D₃ in the postprandial lipid profile in obese, vitamin D-deficient women, a non-targeted lipidomics approach using liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer was used to identify and quantify a wide-range of circulating lipid species, including diglycerides, lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins and triglycerides. The most important changes were found in plasmatic sphingomyelins levels, which experience a decrease after vitamin D₃ intake. Our results suggest a turnover of sphingomyelins, probably due to an increased activity of neutral sphingomyelinases, and, therefore, with implications in the clearance of chylomicrons, LDL and VLDL, decreasing postprandial inflammation and macrophage adherence to endothelia, potentially improving cardiovascular disease risk.

Keywords: cholecalciferol; lipid absorption; lipidomics; obesity; postprandial inflammation; sphingomyelin

1. Introduction

Several forms of evidence suggest that the postprandial period is closely associated to a situation of low-grade systemic inflammation and oxidative stress (1–4). These pathophysiological changes have been linked to diseases like type 2 diabetes mellitus and atherosclerosis. Postprandial lipemia is characterized by the secretion of chylomicrons (CM), VLDL and their remnants. Accumulation of these lipoproteins in the circulation can result in systemic leukocyte activation in addition to impaired endothelial cell function [1,2]. The leukocyte activation starts a signaling cascade where several pro-inflammatory cytokines (IL-1β, IL-6, MCP-1, TNF-α), adhesion molecules (VCAM-1, ICAM-1), integrins (CD11b, CD66b) and the complement system (C3) are involved [2–4]. This may lead to
migration of leukocytes into the subintimal space of the vessel wall, resulting in foam cell formation and development of the atherosclerotic plaque.

Numerous lifestyle and pharmacological interventions have been described to reduce (postprandial) inflammation and cardiovascular disease (CVD) risk [5]. The intake of dietary polyphenols with antioxidant and anti-inflammatory properties [6–8] or a monounsaturated or polyunsaturated fatty acid rich-diet [9,10] seems to diminish postprandial inflammation, to increase insulin sensitivity and to decrease the CVD risk. Likewise, the use of lipid-lowering drugs (such as statins, fenofibrate, rosiglitazone or metformin) have similar effects [11–14].

Vitamin D₃ (cholecalciferol), belonging to the family of secosteroids, has received special attention in the last years due to its potential pleiotropic effects on lipid metabolism [15], the modulation of cell proliferation [16], cytokine production [17], immune system function [18–20], arterial stiffness [21] and inflammation [22]. In particular, the effects of vitamin D₃ on mitochondrial oxidation and phospholipid metabolism have received much attention [23,24]. Since phospholipids are major components of the lipoprotein membranes, we were interested in the changes occurring in the human plasma after vitamin D₃ ingestion. So far, no data on this subject have been published.

The present study describes a double-blind randomized study showing the effects of different doses of vitamin D₃ on postprandial lipid profile in obese, pre-menopausal, vitamin D-deficient women by means of a non-targeted lipidomics approach, including diglycerides (DG), lysophosphatidylcholines (LPC), phosphatidylethanolamines (PE), sphingomyelins (SM) and triglycerides (TG).

2. Material and Methods

2.1. Reagents

Grade MS methanol (MeOH) and 2-propanol, ammonium formate, formic acid, acetic acid, methyl tert-buthyl ether (MTBE) and standards for calibration curves (1,3-dilinoleoyl-rac-glycerol [DG 36:4], 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol [TG 52:3], 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine [LPC 18:1], 1,2-dioleoyl-sn-glycero-3-phosphocholine [PC 36:2], 1,2-dipalmitoyl-sn-glycero-phosphoethanolamine [PE 32:0] and N-palmitoyl-d-erythro-sphingosylphosphorylcholine [SM 34:1]) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Water (milliQ grade) was obtained from a Milli-Q integral water purification system (Millipore Corp., Burlington, MA, USA). For internal standards, SPLASH Lipidomix was purchased from Avanti Polar lipids (Alabaster, AL, USA).

2.2. Subjects and Study Design

A 1:1 randomized, double-blind trial was designed to compare the lipid profile on postprandial inflammation in obese, pre-menopausal, vitamin D-deficient women (n = 24) after a single high (300,000 IU) (n = 12) or low (75,000 IU) (n = 12) dose of cholecalciferol supplementation. The study was carried out following the rules of the Declaration of Helsinki of 1975 (revised in 2013) and was approved by the Institutional Review Board of the Franciscus Gasthuis, Rotterdam, the Netherlands, and the regional independent medical research ethics committee (TWOR), Rotterdam, the Netherlands. All participants provided written informed consent. The study was registered at clinicaltrials.gov under the number NCT01967459. Inclusion and exclusion criteria and clinical characteristics have been published previously [25]. Blood samples were drawn from the participants after a 10-h overnight fast (baseline). Then, 50 g of fat (commercial fresh cream, Albert Heijn, Zaandam, the Netherlands) per m² body surface was ingested (the composition of fresh cream consisted of 35% of fat, of which 23% were saturated, 10% monounsaturated and 0.9% polyunsaturated). Blood samples were collected at 2, 4, 6 and 8 h after ingestion. During the fat load test, patients were only allowed to drink water. At the end of the first oral fat loading test, participants received a single dose of cholecalciferol (double-blinded for high and low dose). The oral fat loading test was repeated after 7 days following the same protocol.
2.3. Samples, External Calibration Curve and Quality Control Preparation

Lipid extraction was performed with a modification of the Bligh and Dyer protocol [26]. Briefly, 750 µL of a solution consisting of MTBE:MeOH (1:2 v/v) with 0.5% acetic acid and containing 1:100 (v/v) internal standard mixture (SPLASH Lipidomix) were added to 20 µL of plasma and vortexed for 10 min. Afterwards, 250 µL of MTBE and 350 µL of water were added, with a vortex step of 1 min between both additions. After centrifugation at 15000 rpm during 10 min at 4 °C, 350 µL of organic phase were collected, dried in a Savant SPD2010 SpeedVac rotatory vacuum system (Thermo Fischer, Waltham, MA, USA), reconstituted in 100 µL of MeOH:MTBE (9:1 v/v) and placed into glass vials for LC-MS analysis.

For calibration curves, stock standards were dissolved in MeOH. Then, 10 seriated concentrations (range from 0.2 to 100 µM) containing 1:100 (v/v) internal standard mixture were prepared dissolving stocks in MeOH:MTBE (9:1 v/v) and placed into glass vials for LC-MS analysis. Calibration curves were plotted using the relative response (ordinate axis) and relative concentration (abscisse axis) to the internal standard. Slope and linearity for each standard are detailed in Supplementary Table S1.

For quality control, a pool of different samples included in the study was made, lipids were extracted and injected twice a day during the analysis to ensure the reproducibility of the experiment. Overlaid chromatograms of quality controls are available in Supplementary Figure S1.

2.4. UHPLC-ESI-QTOF-MS Conditions

Samples (2 µL) were injected directly into a 1290 Infinity ultra-high-pressure liquid chromatograph (UHPLC) coupled with a dual Agilent jet stream electrospray ionization (ESI) source to a 6550 quadrupole-time-of-flight mass spectrometer (QTOF) (Agilent Technologies, Santa Clara, USA). The UHPLC system was equipped with a binary pump (G4220A), an autosampler (G4226A) thermostatized at 4 °C, and a Kinetex EVO C18 column 2.6 µm, 2.1 mm × 100 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of A: water, B: MeOH and C: 2-propanol containing 10 mM ammonium formate +0.1% formic acid, at a flow rate of 0.6 mL/min. The gradient used was as follows: 0 min, 10% B, 40% C; 0.5 min, 10% B, 50% C; 1.5 min, 9.5% B, 52.5% C; 1.6 min, 7.5% B, 63.5% C; 5 min, 7% B, 66.5% C; 5.1 min, 4% B, 82.5% C; 7.5 min, 3.5% B, 85% C; 9 min, 3.5% B, 85% C; 9.5 min, 0% B, 100% C; 11.5 min, 0% B, 100% C; 11.6 min, 10% B, 40% C. A post run of 2 min in initial conditions was used for column conditioning. For the ESI source, the optimized parameters were as follows: gas temperature 225 °C, gas flow 11 L/min, nebulizer 35 psi, sheath gas temperature 300 °C, and sheath gas flow 12 L/min. For the QTOF-MS, running in positive mode, the capillary, nozzle and fragmentor voltages were set at 3500 V, 500 V and 380 V, respectively.

2.5. Data Analysis and Statistics

Prior to the injection of the samples into the LC-MS system, a pool of samples was injected and the raw chromatogram was deconvoluted using “Find by Molecular Feature” algorithm from MassHunter Qualitative Analysis B.07.00 software (Agilent Technologies, Santa Clara, CA, USA). Lipid characterization was performed by matching their accurate mass and isotopic distribution to Metlin-PCDL database (Agilent Technologies, Santa Clara, CA, USA) allowing a mass error of 10 ppm and a score higher than 80 for isotopic distribution. To ensure the tentative characterization, chromatographic behavior of pure standards and corroboration with Lipid Maps database (www.lipidmaps.org) was carried out. Afterwards, compound match entities were selected to perform a targeted MS/MS acquisition on LC-QTOF-MS instrument to corroborate the identification. It is important to note that previous identification of compounds before statistical analysis allows us to use the most appropriate internal standards based on the lipid family to correct the instrumental deviations and increase the statistical power of the study. Supplementary Table S2 provides m/z and retention time values of each lipid species characterized.
Selected lipid species were indirectly quantitated across all the samples using MassHunter Quantitative Analysis B.07.00 (Agilent Technologies, Santa Clara, CA, USA) attending to the calibration curve of its corresponding standard.

Outliers were detected using the interquartile range method. Missing values and outliers were replaced by the median of the group and data was normalized using the log $2$. Paired or unpaired t-tests with Bonferroni correction were used to compare groups using SPSS 25 (IBM Corp., Armonk, NY, USA) with a significance threshold of 0.05. Partial least square discriminant analysis (PLS-DA) and heatmaps were performed in Metaboanalyst 4.0 (www.metaboanalyst.ca) [27] using the already normalized database (thus, without further steps of data processing in Metaboanalyst).

3. Results

3.1. Characterization, Quantitation and Absorption Dynamics of Lipid Species

A total of 128 lipids belonging to 6 different families were characterized, including 8 DG, 20 LPC, 47 PC, 4 PE, 24 SM and 25 TG. Concentrations of all lipid species can be found in the Supplementary Table S3. The most important baseline variables, as well as lipid concentrations (grouped by family), at the beginning of the study for the low and high vitamin D$_3$ supplementation groups are listed in Table 1. Interestingly, some specific lipids (DG 36:1, DG 36:2, TG 48:0, TG 50:0 and TG 52:1) were statistically different at the beginning of the study (Supplementary Table S3). Lipidomics experiment at day 1 (before vitamin D$_3$ treatment) revealed a maximum absorption of DG, PC, SM and TG at 4 h after the fat intake, 2 h for LPC and between 4 and 6 h for PE. A similar pattern of absorption dynamics was found at 7 days after vitamin D$_3$ supplementation (Figure 1), with some differences depending to the dose administrated (Figures 2 and 3), as detailed below.

### Table 1. Baseline characteristics of the participants. Data are expressed as mean ± SEM.

|                      | Low Dose Vit. D$_3$ ($n = 12$) | High Dose Vit D$_3$ ($n = 12$) |
|----------------------|--------------------------------|-------------------------------|
| Age (years) $^a$     | 29 ± 3                         | 27 ± 2                        |
| BMI (Kg/m$^2$) $^a$  | 31.2 ± 1.3                     | 33.0 ± 1.0                    |
| Glucose (mmol/L) $^a$| 5.2 ± 0.1                      | 5.2 ± 0.1                     |
| Total cholesterol (mmol/L) $^a$ | 5.1 ± 0.2             | 5.3 ± 0.3                     |
| HDL-cholesterol (mmol/L) $^a$ | 1.5 ± 0.1            | 1.4 ± 0.1                     |
| LDL-cholesterol (mmol/L) $^a$ | 3.1 ± 0.2            | 3.4 ± 0.3                     |
| Leukocyte count (10$^9$/L) $^a,*$ | 6.6 ± 0.3         | 8.1 ± 0.6                     |
| Monocyte count (10$^9$/L) $^a$ | 0.5 ± 0.06        | 0.5 ± 0.03                    |
| Neutrophil count (10$^9$/L) $^a,*$ | 3.4 ± 0.3        | 4.4 ± 0.4                     |
| 25-OH vitamin D (nmol/L) $^a$ | 27.3 ± 4.5        | 26.8 ± 3.6                    |
| DG (µg/dL)           | 20,312 ± 2095                 | 23,425 ± 2467                 |
| LPC (µg/dL)          | 4158 ± 482                    | 3935 ± 403                    |
| PC (µg/dL)           | 139,244 ± 7972                | 147,992 ± 11,465              |
| PE (µg/dL)           | 765 ± 50                      | 744 ± 82                      |
| SM (µg/dL)           | 20,325 ± 793                  | 20,647 ± 1115                 |
| TG (µg/dL)           | 355,163 ± 54,657              | 427,374 ± 55,708              |

HDL, high-density lipoprotein; LDL, low-density lipoprotein; DG, diglycerides; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; SM, sphingomyelins; TG triglycerides. $^a$ Data extracted from de Vries et al. [17]. $^*$ p-value < 0.05.

3.2. Low Dose of Vitamin D$_3$ Decreases Total SM Levels and Specific PC and PE Species

Comparing the lipid values before and after vitamin D$_3$ supplementation, we observed statistically significant changes in the circulating lipids (Supplementary Table S3). Even if the total concentration of PC were not significantly reduced, we found a decrease in several lipids belonging to this family, mainly in the alkyl ether species (Figure 2). In the same way, absorption of PE was also decreased after vitamin D$_3$ supplementation at 2 and 4 h after a fat loading test (Figures 1F and 2F). Of note, the most important differences were found in the SM family, including a decrease at day 7 in almost
the entirety of the SM species quantitated (Figures 1C and 2C). No significant changes were found in levels of DG, TG or LPC before and after vitamin D$_3$ treatment (Figure 1A,B,E and Figure 2A,B,E). Differences between day 1 and day 7 after vitamin D$_3$ supplementation at time 0 h allow us to separate both groups in a PLS-DA analysis (Figure 4A). However, no characteristic patterns can be deduced in the heatmap (Supplementary Figure S2A).

Figure 1. Concentrations (in µg/dL) of lipid species grouped by families (expressed as mean ± SEM) during the postprandial fat intake before and after vitamin D3 supplementation. (A) diglycerides; (B) triglycerides; (C) sphingomyelins; (D) phosphatidylcholines; (E) lysophosphatidylcholines; (F) phosphatidylethanolamines. HDL, high-density lipoprotein; LDL, low-density lipoprotein; DG, diglycerides; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; SM, sphingomyelins; TG triglycerides. $p$-value < 0.05 between day 1 and day 7 in low dose (blue asterisc) and high dose (red asterisc) groups.
Figure 2. Fold-change (after/before) at time 0 h of lipid species (expressed as mean) in the low dose of vitamin D₃ group. (A) diglycerides; (B) triglycerides; (C) sphingomyelins; (D) phosphatidylcholines; (E) lysophosphatidylcholines; (F) phosphatidylethanolamines. * p-value < 0.05.

Figure 3. Fold-change (after/before) at time 0 h of lipid species (expressed as mean) in the high dose of vitamin D₃ group. (A) diglycerides; (B) triglycerides; (C) sphingomyelins; (D) phosphatidylcholines; (E) lysophosphatidylcholines; (F) phosphatidylethanolamines. * p-value < 0.05.
3.3. High Dose of Vitamin D₃ Increases Specific PC and PE Species

Similar to the low dose group, the total PC concentration remained unchanged before and after vitamin D₃ supplementation (Figures 1D and 3D), but, in contrast, several specific PC species were increased (Supplementary Table S3) after the oral fat intake, especially those with high-degree of unstaturations. PE levels were only significantly increased at 2 h after the fat loading test (Figure 1F). No significant changes were found in total levels of DG, TG, SM or LPC (Figure 1A–C,E and Figure 3A–C,E). In the same way than patients with a low dose of vitamin D₃, PLS-DA separates both groups (Figure 4B), but any characteristic pattern can be observed in the heatmap (Supplementary Figure S2B).
4. Discussion

CVD, obesity and diabetes (closely related metabolic diseases) are becoming one of the most important epidemics of the 21th century, with alarming growth occurring in the developed societies. Dyslipidemia, insulin resistance or inflammation, among other factors, due to a sedentary lifestyle and unhealthy diet promote a mitochondrial impairment and, thus, an imbalance between energy intake and expenditure leading to metabolic disturbances and disease [28]. In the last decade, several studies focused on the actions of vitamin D, either directly or via its receptor. The impact of cholecalciferol in lipid metabolism is well known, but the underpinned mechanisms still remain unclear [15,29–31].

In our study, we analyzed different species of DG, LPC, PC, PE, SM and TG families. To the best of our knowledge, this is the first time that an interventional study explored the postprandial fatty acid profile while including this wide range of lipids in relation to vitamin D. Only a significant decrease of total SM and specific PC and PE levels were found after a low dose of vitamin D₃ supplementation. SM, PC and PE are related through the same biosynthetic pathway, being phosphorylcholine from PC or phosphorylethanolamine from PE precursors of SM synthesis [32]. However, our results are not sufficient to support the idea that a decrease in SM levels is due to a lack of precursors, because only 4 PE species were detected and quantitated (we cannot ensure that PE levels globally decrease) and only a reduction in specific PC species, but not in the total PC content, was found.

This SM turnover by vitamin D₃ was already observed in vitro using HL-60 cells, for the first time, by Okazaki et al. in 1989 [33]. It is important to note that intracellular changes are not necessarily reflected in plasma. Moreover, an inter-organ metabolic crosstalk should be considered in these patients [34,35]. However, according to the authors, and analogous to our results, we can speculate that cholecalciferol decreases SM levels while PC levels remain similar, with an increase in ceramide and phosphorylcholine units, suggesting that the hydrolysis of SM induced by vitamin D₃ could be through activation of neutral SMases. Similar results were found in the human keratinocytes HaCaT cell line [36,37] and in glioblastoma cells [38].

SM in plasma is mainly associated to apo B-containing lipoprotein particles, including CM and VLDL [39]. SM are also important components of cell membranes and regulate cell growth, differentiation and apoptosis [40], being also involved in the adherence during macrophage differentiation and adherence [41], cholesterol distribution and homeostasis [42] and in the development of Niemann-Pick disease [43] and increased CVD risk [44].

In contrast to our expectations, a high dose of vitamin D₃ reverted the effects observed in the low dose group. These data suggest a possible loss of effectiveness when exceeding the recommended dose. The increasing use of vitamin D, inappropriately prescribed, or due to the abuse of new nutraceuticals is a subject of major debate to ensure efficacy and safety in dose and administration guidelines [45–48].

In conclusion, we describe, for the first time in humans, the effects of vitamin D₃ in the profile and absorption dynamics of a wide range of lipid species after a fat intake using a non-targeted lipidomics approach. Our results show that a low dose (75,000 IU) of cholecalciferol induces an SM turnover, probably due to the activation of neutral SMases, which can be of interest due to its implications in the clearance of CM, LDL and VLDL, mitigating the postprandial inflammation, the macrophage adherence to endothelia and ameliorating CVD risk. In contrast, high doses of vitamin D₃ can have undesirable effects in the lipid profile.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/5/1194/s1. Experimental m/z and retention time of each lipid species quantitated are available on Table S1; slope and linearity of standards used for the external calibration curves are available on Table S2; concentration of each lipid species analyzed is available on Table S3; overlaid chromatograms of quality controls are available on Figure S1; heatmap of patients before and after vitamin D₃ intake on Figure S2.

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