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Original Citation:
Impaired release of Vitamin D in dysfunctional adipose tissue: New cues on Vitamin D supplementation in obesity / DI NISIO, Andrea; DE TONI, Luca; Sabovic, Iva; Rocca, MARIA SANTA; DE FILIPPIS, Vincenzo; Opocher, Giuseppe; Azzena, Bruno; Vettor, Roberto; Plebani, Mario; Foresta, Carlo. - In: THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM. - ISSN 0021-972X. - STAMPA. - 102:7(2017), pp. 2564-2574.

Availability:
This version is available at: 11577/3231985 since: 2017-05-20T23:26:05Z

Publisher:

Published version:
DOI: 10.1210/jc.2016-3591

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The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: November 02, 2016
Accepted: February 07, 2017
First Online: February 10, 2017

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Vitamin D trafficking and supplementation in obese

Impaired release of vitamin D in dysfunctional adipose tissue: new cues on vitamin D supplementation in obesity

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Received 2 November 2016. Accepted 07 February 2017.

Context: Vitamin D accumulates in adipose tissue (AT) and vitamin D deficiency is frequent in obesity.

Objective: We hypothesize that trafficking of vitamin D is altered in dysfunctional AT.

Design, Patients, Settings: 54 normal-weight and 67 obese males were recruited in a prospective study and randomly assigned to supplementation with 50 µg/week 25-hydroxyvitamin-D3 (25(OH)D) or 150 µg/week vitamin D3 for 1 year, raising dosage by 50% if vitamin D-sufficiency (serum 25(OH)D>50 nomol/l), was not achieved at 6 months; 97 subjects completed the study.

Methods: Vitamin D3 (D3) and 25(OH)D were quantified by HPLC-MS in control and insulin-resistant (IR) 3T3-L1 cells and subcutaneous AT (SAT) from lean and obese subjects, incubated with or without adrenaline; expression of 25-hydroxylase (CYP27A1), 1α-hydroxylase (CYP27B1) and vitamin D receptor (VDR) were analysed by real-time PCR;

Results: In IR adipocytes the uptake of D3 and 25(OH)D was higher, but after adrenaline stimulation, the decrement in D3 and 25(OH)D was stronger in control cells, which also showed increased expression of CYP27A1 and CYP27B1 and higher levels of 25(OH)D. In SAT from obese subjects, the adrenaline-induced release of D3 and 25(OH)D was blunted; in both IR cells and obese SAT, protein expression of β2-adrenergic receptor was reduced. Supplementation with 25-hydroxyvitamin-D3 was more effective in achieving vitamin D sufficiency in obese, but not in normal weight subjects.

Conclusion: Dysfunctional AT shows a reduced catecholamine-induced release of D3 and 25(OH)D, and altered activity of vitamin D-metabolizing enzymes, for these reasons supplementation with 25-hydroxyvitamin-D3 is more effective in obese individuals.

Introduction

Vitamin D is a lipophilic hormone playing a key role in bone metabolism and calcium homeostasis(1), and it is also involved in many other pathological processes(2) commonly linked with obesity. In order to achieve its biological function, 1,25-dihydroxyvitamin D3 (1,25(OH)D) requires two sequential hydroxylation steps from vitamin D3 (D3) catalysed by 25-hydroxylase and 1α-hydroxylase. The concentration of the pre-activated vitamin D, 25-hydroxyvitamin D (25OHD), is considered the most reliable marker of vitamin D status.
The high prevalence of vitamin D deficiency in obese subjects(3) suggests that adipose tissue (AT) has a major role in the body distribution of vitamin D, but the causal relationship between obesity and low levels of circulating 25(OH)D has not been completely elucidated yet. To this regard, as suggested from studies in humans and animal models receiving high doses of oral vitamin D3(4,5), it has been proposed that vitamin D, being fat-soluble, could be sequestered in body fat depots, leading to lower bioavailability in the obese state(5,6). An alternative hypothesis considers low serum 25(OH)D in obese as a result of volumetric dilution of vitamin D in the large adipose stores(7).

Taken together, these results suggest that BMI should be taken into account when determining the vitamin D intake required for optimal status(8,9). Moreover, while fat depots mainly consist of cholecalciferol, 25(OH)D is widely distributed in many tissues, with the highest concentrations in serum(10). On these bases, we can understand why orally-administrated vitamin-D3 has a lower impact on 25(OH)D levels in obese subjects(5,11), as it is markedly stored in AT(12).

This scenario is further complicated by the altered molecular phenotype featuring AT in obesity-induced inflammation and insulin resistance(13). The catecholamine and natriuretic peptide lipolytic resistance in both obese patients(14) and insulin-resistant adipocytes(15), may lead to a reduced release of vitamin D from AT. Indeed, not only vitamin D is stored in AT, but it is slowly released into circulation(16). However, the mechanisms controlling the deposition and release of vitamin D from AT are still unknown(17). It is likely that, since vitamin D is a fat soluble secosteroid, it is subjected to the same pathway of catecholamine-induced lipolysis observed for cholesterol(18).

Moreover, both human and murine adipocytes express vitamin D-metabolizing enzymes (reviewed by Ding et al.(19)), suggesting that AT not only passively accumulates vitamin D, but also changes its metabolism in obesity.

Despite the well-established relationship between obesity and low vitamin D, few experimental studies have investigated the biological processes involved in vitamin D metabolism in AT, with conflicting results(10,20).

We hypothesized that AT is capable of vitamin D uptake, but in the pathological state the lipolysis-mediated release of vitamin D is impaired, concomitantly with an altered enzymatic machinery. On these bases, we would expect that 25-hydroxyvitamin-D3 rather than vitamin D3 is a more adequate supplementation in obese subjects. Thus we investigated (1) whether D3 and 25(OH)D are differentially accumulated and released after adrenaline-induced lipolysis in control and insulin-resistant (IR) 3T3-L1 adipocytes; (2) whether vitamin D metabolizing enzymes are impaired in IR adipocytes; (3) the concentrations of vitamin D3 and 25(OH)D in ex-vivo subcutaneous adipose tissue (SAT) from normal weight and obese subjects before and after lipolytic stimulation; (4) whether supplementation with 25(OH)D is more effective in achieving vitamin D-sufficiency in obese subjects, compared with vitamin D3.

Methods

Cell culture

3T3-L1 cells (ATCC, Manassas, VA, USA) were cultured in growth medium (GM) consisting of DMEM high glucose supplemented with 10% FBS (Sigma-Aldrich, Milan, Italy), 1% glutamine and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and 1% amphotericin B (Euroclone, Milan, Italy). Media was changed every other day. The cells were seeded in 35 mm dishes (Corning Life Sciences, Durham, NC, USA) at a density of 6x10^5 cells/dish and differentiated as previously described(21). Adipocytes were used for experiments on 9th day of the initiation of differentiation. Insulin resistance (IR) was induced in 3T3-L1 adipocytes as previously described with slight modifications(22,23). Fully differentiated 3T3-L1 adipocytes were treated with growth medium supplemented with 20 nM dexamethasone and 10
µg/ml insulin for 4 days, media was changed every other day. 3T3-L1 cells were incubated at 37 °C with 5% CO₂ in each culture step.

2-Deoxy glucose uptake assay
Control and IR 3T3-L1 adipocytes in 12-well plates were washed twice and were starved for glucose by pre-incubating in Krebs-Ringer-Phosphate-Hepes (KRP) buffer containing 2% BSA for 50 min. Following starvation, 2-deoxy glucose (2-DG) uptake assay was carried with Abcam glucose uptake colorimetric assay kit (Abcam, Milan, Italy) according to the manufacturer’s instructions, as previously described(23). Briefly, cells were stimulated with insulin (10 mg/mL) for 20 min and treated with 2-DG, mixed and incubated for 20 min. Each condition was performed in triplicate.

Glycerol release
3T3-L1 adipocytes and SAT samples were serum-starved in low-glucose DMEM for 2 hours. Experiments were conducted in 4% BSA Krebs-Ringer phosphate (KRP) buffer containing 136 mM NaCl, 4.7 mM KCl, 10 mM NaPO₄ (pH 7.4), 0.9 mM MgSO₄, and 0.9 mM CaCl₂ with 10⁻⁶ M adrenaline (Monico, Venice, Italy) for 1 h at 37°C. Reactions were stopped on ice, and media were collected and assayed for glycerol content by using free glycerol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. Glycerol release was normalized to cellular protein content. All values are normalized to the adrenaline-stimulated release from normal adipocytes for 3T3-L1 cells and from lean subjects for SAT.

Uptake and release of vitamin D₃ and 25-hydroxyvitamin-D
Fully-differentiated control and IR 3T3-L1 cells were starved in serum-free medium for 2 hours and then incubated overnight with or without 10⁻⁸ M D₃ (SUPELCO, Bellefonte, PA) and 10⁻⁸ M 25(OH)D (Sigma-Aldrich) in DMEM with 3% bovine serum albumin (BSA). After loading with D₃ and 25(OH)D, lipolysis experiments with 3T3-L1 cells were conducted with adrenaline as previously described(24,25). In brief, after incubation with vitamin D forms, the cells were washed and incubated for 4 hours at 37 °C in DMEM supplemented with 3% BSA with and without 10⁻⁶ M adrenaline. All experiments were repeated three times for each test condition.

Samples of SAT were obtained from five obese (BMI> 30 kg/m²) and five normal weight male patients attending the Plastic and Aesthetic Surgery Clinic and the Internal Medicine Clinic of the Hospital of Padova, under informed consent. Subjects were free from any vitamin D supplementation. Serum concentrations of 25(OH)D and anthropometric measurements were obtained pre-operatively as described later. Surgical samples were weighted and processed under sterile conditions as previously described(26).

Western blot for β₂-adrenergic receptor
SAT and differentiated 3T3-L1 cells were homogenized in RIPA Lysis Buffer with protease inhibitors (Sigma–Aldrich), centrifuged at 800 g for 10 min at 4 °C and then the middle layer was collected. Total protein content was measured by BCA Protein assay kit (Sigma–Aldrich). Equal amounts (40 µg) of proteins were denatured in O-solution, boiled for 10 minutes and then separated on 10% SDS-polyacrylamide gel. Proteins were then transferred on a nitrocellulose membrane and detected by primary antibodies, respectively rabbit polyclonal anti-human/mouse β₂-adrenergic receptor (1:100, predicted band size: 46 kD; Abcam) and mouse monoclonal anti-human β-actin (1:1000, Abcam). Primary immune-reaction was finally detected by peroxidase-conjugated secondary antibodies were followed by HRP Substrate (KPL, Gaithersburg, MA, USA). Protein signals were visualized the Chemidoc XRS System (Bio-Rad, Milan, Italy).

HPLC-MS
The total lipid fraction from cell pellet of differentiated adipocytes from each well (approximately 6x10⁵ cells) and 0.2-0.4 g ex vivo AT was extracted with a chloroform/methanol mixture(27). After lyophilization by Vacufuge®-Concentrator plus (Eppendorf, Milan, Italy), the extract was resuspended in 200 µl of chloroform. The sterol fraction was isolated by solid-phase extraction on 1 mL silica column. The column was preconditioned with methanol-chloroform
solution, the phospholipid component was discarded by chloroform elution while the sterol fraction was eluted with acetone. The lyophilized sterol fraction was dissolved in methanol and 20 µl were injected in the LC-MS system. A Zorbax eclipse plus C18 column (2.1mm×50mm, 1.8-µm particle size) (Phenomenex, CA, USA) was used at the temperature of 40°C for the chromatographic step. Isocratic elution was performed by a mixture of mobile phase A: H2O milliQ+0.1% formic acid (A) and methanol +0.3% formic acid (B) in equal part (v/v). The positive ionization of the eluted simple was performed by Electrospray ionization with a capillary potential of 1.50 kV. Source temperatures was 100 °C. Quantification of D3 and 25(OH)D was performed using the corresponding calibration curves prepared by serial dilution of each analyte (Supplementary Figure 1). The data were acquired by the Mass-Lynks software using a data pitch of 0.5 sec and integration of mass peaks was performed with the BioPharmaLynks suite (Waters). Monoisotopic mass values were determined with an accuracy <2ppm. Data were analysed with MASS-LYNX Software (Waters, MA, USA) and total amount of both D3 and 25(OH)D was normalized on cell count for 3T3-L1 cells and on specimens weight for SAT.

Expression of vitamin D metabolizing enzymes
Control and IR fully-differentiated adipocytes were cultured and stimulated for 4 hours with vitamin D3 and 25(OH)D as described above. After centrifugation, cell pellet was collected and stored at -80°C for gene expression analysis of vitamin D metabolizing enzymes (CYP27A1, CYP27B1 and VDR). Total RNA was isolated with RNaseasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. The quality and amount of RNA isolated was determined on a NanoDrop spectrophotometer (Thermo, Wilmington, DE, USA). 500 ng of total RNA was used for first-strand cDNA synthesis using the Superscript III RT kit (Invitrogen) according to the manufacturer's instructions. Total cDNA was amplified by PCR using the following primers: VDR (forward 5′- AGCCCTGTCTTCATCCCTTC-3′; reverse 5′- TTCTGCAATCCCATTCCACT-3′), CYP27A1 (forward 5′- CTTCATCGACAAAGGAGAC-3′; reverse 5′- CCAAGGCAAGGTGGTAGAGA-3′), CYP27B1 (forward 5′- TAGAGGCTGAAGTCCCTCCT-3′; reverse 5′- CATCGACACCTCCTCTC-3′). Data were normalized relative to GAPDH (forward 5′-CTGCGACTTACCCACTC-3′; reverse 5′-GAGTTGGGATAGGCCTCCTC-3′). Amplification reactions were performed in a 20 µL final volume which includes 4 µL of cDNA (1:4), 10 µL 2XPower SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL (10 µM) of each primer (forward and reverse) and 4 µL of H2O. Amplification was carried out with an initial denaturation step at 95° for 10’, followed by 40 cycles of 95° for 15”, 60° for 1’ using thermocycler StepOne Plus (Thermo Fisher Scientific Inc, Carlsbad, CA, USA). The expression ratios relative to GAPDH were then determined by calculating ∆∆CT, and results are expressed as fold changes compared with the endogenous control not stimulated with vitamin D. All samples were quantified in triplicate and positive and negative controls were included in all the reactions. Real-time data were collected by the StepOne Plus 2.1.

Subjects
This was a single centre prospective study conducted at the unit of Endocrinology, Andrology and Reproductive Medicine at the University of Padova. The investigation was conformed to the principles of the Declaration of Helsinki and all subjects gave informed consent to the study, which has been approved by the local ethical committee (protocol AOP0973).

We recruited 67 consecutive subjects with obesity (BMI> 30 kg/m²) and 54 age-matched normal weight controls (BMI< 25 kg/m²) between October 2013 and March 2014. All subjects were of Caucasian ethnicity and Italian origin according to self-report. Our inclusion criteria were age 18-60, vitamin D deficiency (serum 25(OH)D < 50 nmol/l), testosterone (T) >10.4 nmol/l and no intestinal or endocrine diseases. Our exclusion criteria were intestinal malabsorption, current intake of vitamin D in any form, renal or hepatic insufficiency, and no use of drugs that could interfere with calcium or vitamin D metabolism (steroids, vitamin K, ...
calcitonin, and biphosphonates). Subjects’ evaluation included physical examination (weight, height, body mass index (BMI), waist circumference), fasting glucose, hemochrome and serum insulin by standard biochemical methods. Serum levels of total T, estradiol, LH, FSH, PTH, and 25(OH)D were measured in all subjects by standard chemiluminescence methods. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting insulin (µIU/ml) × fasting glucose (mmol/l)/22.5. Vitamin D sufficiency was defined as serum 25(OH)D levels >50 nmol/l (28).

The treatment protocol included supplementation with either 25-hydroxyvitamin-D3 or Vitamin D3 for 1 year. Patients were randomly assigned to either supplementation group. Random assignment of subjects was blinded and computer generated. Recent estimates of the conversion factor between vitamin D3 and 25-hydroxyvitamin-D3 in humans in terms of elevating serum 25(OH)D is approximately three(29,30), therefore we used a starting dose for 25-hydroxyvitamin-D3 three times lower than Vitamin D3. Participants were given a starting dose of 50 µg/week oral supplementation of 25-hydroxyvitamin-D3 (Didrogyl, Bruno Farmaceutici, Rome, Italy) or 150 µg/week of Vitamin D3 (Dibase, Abiogen Pharma S.p.A., Pisa, Italy) in an oily solution on a slice of bread during lunch. The dosage was adjusted by determining the 25(OH)D levels at 6 months and increasing the starting dose by 50% unless vitamin D sufficiency (25(OH)D > 50 nmol/l) was achieved, then maintaining the dose till the end of the study. Compliance was assessed by self-report.

Statistical analysis
Statistical analysis of the data were conducted with SPSS 21.0 for Windows (SPSS, Chicago, IL). The results are expressed as means ± standard deviation (SD). The Kolmogorov–Smirnov test was used to check for normality of distribution. Variables not showing normal distribution were log transformed. Baseline characteristics of normal weight and obese subjects and data from in vitro experiments were compared by using unpaired Student’s t tests with Bonferroni-Holm correction for multiple comparisons. Differential release of D3 and 25(OH)D were tested with the interaction between adipocyte phenotype and adrenaline in a repeated-measures ANOVA. Repeated-measures ANOVA was performed to test for differences in serum 25(OH)D and PTH levels during the study at three time points (baseline, 6 months visit, endpoint). Main effects included treatment (25-hydroxyvitamin-D3 or Vitamin D3), BMI status (normal weight or obese) and their interaction; age was included as a covariate and post-hoc analysis with Bonferroni-Holm correction for multiple comparisons was performed to test differences between time-points. The Levene’s test was used to test the homogeneity of variance among groups. If homogeneity of variance assumption was violated, Welch test was performed and the respective p value was reported. Using multiple linear backward stepwise regression, we examined the relationship of the 25(OH)D increment with age, weight, BMI status, and average weekly dose for each treatment group. In the 25-hydroxyvitamin-D3 group, only BMI status and mean dose were significantly related (R² = 0.217, p=0.004) and were included in the final model. In the vitamin D3 group, only weight and BMI status were included in the final model (R² = 0.349, p<0.001). The proportion of vitamin D sufficient subjects during the study was compared with non-parametric Cochran’s test. Post-hoc pairwise comparisons were performed with McNemar’s test, with Bonferroni-Holm adjustments for multiple comparisons. p values <0.05 were considered as statistically significant.

Results
Vitamin D trafficking in cultured adipocytes
Cultured 3T3-L1 adipocytes exposed to dexamethasone and chronic insulin became insulin resistant within several days, as assessed by the ability of insulin to stimulate glucose uptake(22,23) (Fig. 1A). Assessment of adipocyte lipolysis were performed by measuring glycerol release. Basal (non-hormone stimulated) lipolysis was not different between controls. Effects on hormone-stimulated lipolysis were determined by incubating the cells with 10⁻⁶ M
adrenaline, a β₂-adrenoceptor agonist. Adrenaline induced a lower lipolytic response (Fig. 1B). By western-blotting, we found a significantly reduced expression of β₂-receptor in IR fat cells (Fig. 1C).

After overnight loading with D3, control and IR 3T3-L1 cells were incubated with or without adrenaline 10⁻⁶ M. We quantified both D3 and 25(OH)D by HPLC-MS (Supplemental Fig. 1). In the absence of D3 in the medium, both were undetectable (data not shown). In the absence of adrenaline, IR adipocytes showed a significantly higher uptake of D3 (p = 0.038), but a lower content of 25(OH)D (p = 0.004) compared with control adipocytes (Fig 1D). In adrenaline-stimulated adipocytes, both D3 and 25(OH)D concentrations decreased (all p < 0.05, Fig. 1D), but the lipolytic effect of adrenaline was significantly lower in IR cells for both D3 (F₁₁ = 5.159, p = 0.045) and 25(OH)D content (F₁₁ = 11.796, p = 0.009). In 3T3-L1 cells loaded with 25(OH)D, D3 was not detectable (data not shown). In the absence of adrenaline, IR adipocytes showed an increased concentration of 25(OH)D (p = 0.031; Fig. 1E). After adrenaline stimulation, although 25(OH)D significantly decreased in both IR and control adipocytes (p = 0.008 and p = 0.003; Fig. 1E), the rate of decrement did not differ between control and IR cells (F₁₁ = 0.180, p = 0.682).

We evaluated the gene expression pattern of vitamin D metabolizing enzymes before and after 4 hours stimulation with either D3 and 25(OH)D. In control adipocytes, both CYP27A1 and CYP27B1, but not VDR, were significantly upregulated after D3 stimulation (p = 0.030, p = 0.018 and p = 0.100 respectively), whereas in IR adipocytes CYP27A1 expression, but not CYP27B1 and VDR, significantly increased compared with basal conditions (p = 0.040, p = 1.000 and p = 1.000), but to a lower extent than control adipocytes (p = 0.043; Fig. 2A). In adipocytes stimulated with 25(OH)D, CYP27B1 and VDR, but not CYP27A1, were significantly upregulated from baseline in both control (p = 0.006, p = 0.015 and p = 0.897 respectively) and IR adipocytes (p = 0.046, p = 0.015 and p = 0.728), but the increment in CYP27B1 expression was much higher in control adipocytes than in IR adipocytes (p = 0.010; Fig. 2B).

**Vitamin D content and release from ex vivo human adipose tissue**

We assessed adrenaline-mediated lipolysis in SAT of lean and obese subjects by measuring glycerol release. Basal (non-hormone stimulated) lipolysis was not different between controls and adrenaline induced a lower lipolytic response in obese SAT (Fig. 3A). By western-blotting, we found a significantly reduced expression of β₂-receptor in SAT from obese males (Fig. 3B).

We quantified D3 and 25(OH)D content by LC-MS in ex vivo SAT samples from lean and obese subjects (mean BMI = 21.61 ± 2.12 and 35.56 ± 3.98 respectively, p < 0.001), before and after adrenaline stimulation. Lean and obese subjects did not differ for age (45.00 ± 13.76 respectively, p = 0.773) and serum 25(OH)D levels (58.32 ± 14.10 and 51.70 ± 11.91 respectively, p = 0.446). In obese SAT we found an higher concentration of both D3 and 25(OH)D, although not statistically significant (p = 0.023 and p = 0.693 respectively; Fig. 3C-D), with D3 being much more represented than 25(OH)D in SAT of both obese and lean subjects (p < 0.001 for both; Fig. 3C-D). After adrenaline stimulation, D3 concentration decreased in SAT from both lean and obese subjects (p < 0.001 and p = 0.006 respectively; Fig. 3C), but the decrease was significantly more pronounced in lean SAT (F₁,₈ = 6.157, p = 0.028). A similar pattern was observed for 25(OH)D (p < 0.001 and p = 0.005 respectively, Fig. 3D), indicating a blunted lipolytic-driven decrement of 25(OH)D in obese SAT (F₁,₈ = 7.157, p = 0.038).

**Effect of 25-hydroxyvitamin-D3 and Vitamin D3 supplementation in normal weight and obese subjects**

Baseline characteristics of normal weight and obese subjects are reported on Table 1. Of the 121 subjects recruited in the study, 97 completed the treatment or had a compliance >90%. No differences in baseline parameters have emerged within groups between subjects allocated to 25-hydroxyvitamin-D3 and vitamin D3 treatment groups (all p > 0.05). In a repeated-measures ANOVA corrected for age, serum 25(OH)D levels during the study significantly incremented within each group (Table 2), with a significant effect of treatment (F₁,₉₂ = 14.43, p < 0.001), BMI status (F₁,₉₂ = 55.69, p < 0.001) and their interaction (F₁,₉₂ = 7.75, p = 0.006). PTH concentration
were significantly reduced by both treatments (Table 2). Within BMI groups, ANOVA by treatment groups adjusted for age showed that there was no significant difference in the mean serum PTH concentration between the two treatment at any time point in both normal weight and obese subjects (Table 2).

The increments of serum 25(OH)D achieved at the end of the study were significantly different between treatment groups and BMI status, but it was more pronounced in the Obese group (Table 2; group-by-treatment interaction: $F_{1,93}^{} = 4.94$, $p = 0.029$). Using a multiple linear backward stepwise regression, in the 25-hydroxyvitamin-D3 group, mean dosage, but not BMI status, was a significant predictor of 25(OH)D increment ($B = 0.334$, 95% CI= 0.133 to 0.536, $p = 0.002$; $B = -5.04$, 95% CI= -10.521 to 0.44, $p = 0.071$, respectively), whereas in the vitamin D3 group, BMI status and weight were significant predictors ($B = -20.696$, 95% CI= -31.583 to -9.809, $p < 0.001$; $B = 0.30$, 95% CI= -0.007 to 0.607, $p = 0.050$, respectively). The mean dosage of 25-hydroxyvitamin-D3 during the study to achieve vitamin D-sufficiency did not differ between normal weight and obese subjects (Table 2, $p = 0.760$), whereas in the vitamin D3 group, the mean dosage was significantly higher in obese patients compared with normal weight subjects ($p < 0.001$). Based on these findings, we estimated that obese subjects supplemented with vitamin D3 would require a 1.82-fold increase in weekly dosage, on average, to increase serum 25(OH)D levels by 1 nmol/l, compared with normal weight subjects (mean weekly dosage for 1 nmol/l increment= 16.99±3.15 and 9.35±3.15 µg/week, respectively; $p< 0.001$), whereas in the group supplemented with 25-hydroxyvitamin-D3, obese subjects required only a 1.11-fold increase in weekly dose compared with normal weight subjects (3.02±0.88 and 2.71±0.62 µg/week, respectively; $p=0.174$). The proportion of vitamin D-sufficient patients increased over time within all groups (all $p< .0001$) and it did not differ at any time point between normal weight subjects treated with either 25-hydroxyvitamin-D3 or vitamin D3 (all $p > 0.05$, Fig. 4A), whereas the proportion of obese patients with 25(OH)D> 50 nmol/l was significantly higher after 6 months of treatment with 25-hydroxyvitamin-D3 compared with vitamin D3, but not at the end of the study ($p = 0.003$ and $p=0.125$ respectively; Fig. 4B).

**Discussion**

In this study, we analyzed a range of biological processes associated with vitamin D metabolism in either 3T3-L1 adipocytes rendered insulin-resistant or fat explants from obese male subjects. We observed a considerable reduction in adrenaline-stimulated lipolytic release of vitamin D3 and 25(OH)D in all models, which might be explained by a reduced expression of β2-adrenergic receptor in both human and 3T3-L1 models. However, the mechanisms controlling the deposition and release of vitamin D from AT are still unknown(17). It is likely that, since vitamin D is a fat soluble secosteroid, it is subjected to the same pathway of catecholamine-induced lipolysis observed for cholesterol(18).

Interestingly, we observed that adipocytes were capable of converting loaded D3 into 25(OH)D, but to a lesser extent in IR adipocytes, which was supported by reduced expression of 25-hydroxylating enzyme CYP27A1. Finally, supplementation with oral 25-hydroxyvitamin-D3 was more rapid in achieving vitamin D sufficiency in obese patients than vitamin D3. These findings demonstrate a clear alteration of vitamin D uptake and release in pathological AT.

The fat-solubility of vitamin D suggests a sequestration process in body fat depots, leading to lower bioavailability in the obese state(6), which is further promoted by catecholamine- and natriuretic peptide-lipolytic resistance in both obese patients(14) and insulin-resistant adipocytes(15), possibly leading to a reduced release of vitamin D from AT, given the fat soluble secosteroid nature of vitamin D.

We confirmed that IR 3T3-L1 cells had a reduced glucose uptake(22,23,31) and adrenaline-induced lipolytic response(32), compared with control adipocytes, in concordance with previous studies demonstrating abrogated catecholamine-induced lipolysis in adipocytes from obese subjects (reviewed by Arner and Langin(33)) and we showed for the first time reduced protein
expression of β2-adrenergic receptor expression in both 3T3-L1 cells with insulin resistance and SAT from obese subjects.

In vitro and in vivo data suggest that catecholamine resistance is restricted to the β2-adrenoceptor, which may be due to a decreased number and function of β2-adrenoceptors(34). We confirmed this hypothesis, showing reduced levels of β2-adrenoceptor in both IR 3T3-L1 and obese SAT, although the exact mechanism associated with this decrement needs to be further elucidated. Indeed it has been shown that impaired lipolysis in AT does not improve after weight loss(35), is a feature of childhood onset obesity(36) and affects adipocytes from first-degree relatives of obese subjects(37). These data indicate that catecholamine resistance may be an important factor causing obesity, rather than a consequence. In fact more evidence comes from polymorphisms in genes encoding key proteins of the lipolytic pathway (reviewed by Jocken and Blaak(32)). Based on the well-established reduction in catecholamine resistance and 25(OH)D levels in the obese state, we aimed to verify whether the AT is not only a passive storage, but also actively sequestrates vitamin D, due to its reduced lipolytic response. After incubation with D3, IR 3T3-L1 cells showed an increased intracellular content of D3 compared with control adipocytes, but we also showed a lower concentration of 25(OH)D in these cells, therefore the increased amount of D3 in IR cells could actually be the result of a greater conversion of D3 in 25(OH)D in control adipocytes, rather than a greater uptake in IR fat cells. Both D3 and 25(OH)D decreased after adrenaline-induced lipolysis, but the rate of decline was significantly lower in IR fat cells. On the other hand, in 3T3-L1 incubated with 25(OH)D, D3 was undetectable, as expected, and 25(OH)D content was higher in IR adipocytes. In the presence of adrenaline, in both control and IR cells 25(OH)D equally decreased, with 25(OH)D levels in IR adipocytes being higher due to the higher uptake of 25(OH)D, rather than a differential lipolytic response. However, quantification also of 1,25(OH)D would be necessary to show that the increased uptake of 25(OH)D is actually due to a reduced conversion to 1,25(OH)D in IR fat cells.

Notably, independently of the adipocyte phenotype, the absolute uptake of 25(OH)D was lower than the uptake of D3, despite the same concentration of both forms in the incubation medium. This could be explained by the different lipo-solubility of the two forms, where D3 has an higher partition coefficient (logK_{ow} = 10.2) than 25(OH)D (logK_{ow} = 8.43). This would explain also why in vivo D3 is mainly found in fat (approx. 75%) , whereas 25(OH)D is more equally distributed (approx. 35% in fat)(10,38), although in this case vitamin D binding protein affinity must be taken into consideration, since its lower for D3 than 25(OH)D(39), therefore more unbound D3 could freely diffuse into AT given its higher lipo-solubility.

As confirmed by our observation of significant amounts of 25(OH)D in adipocytes loaded with D3, 25-hydroxylation was already shown to be functional in AT due to the presence of CYP27A1, which is up-regulated by D3 treatment(40). In accordance, we found upregulation of CYP27A1 after D3 stimulation, but it was less pronounced in IR adipocytes, which could explain the reduced conversion of D3 into 25(OH)D in these cells. CYP27B1 was already shown to be functional in 3T3-L1 adipocytes(41), but at lower levels in SAT from obese(42). We found that its stimulation was reduced in IR adipocytes, this would imply a reduced capacity to synthesize 1,25(OH)D(7) and clearly requires further investigation. Notably, in both IR and control adipocytes VDR was upregulated by 25(OH)D. Recently, evidence was provided that 25(OH)D is an agonist for VDR and exhibited direct gene regulatory properties(43). Since it was shown that protein levels of CYP27B1 and VDR measured by western blotting were consistent with mRNA levels(44,45) these results can also be extended at a protein level, thus suggesting that autocrine/paracrine actions of vitamin D in AT could be altered(19).

Previous in vitro results were confirmed also in ex vivo explants of SAT from obese and lean subjects. 25(OH)D concentration was lower than D3, and both were comparable with previous reports from human AT measured by LC-MS(12,46). Adrenaline-stimulated SAT had lower content of both D3 and 25(OH)D than unstimulated SAT, but the reduction was significantly
more pronounced in SAT from lean subjects. The reduced bioavailability of vitamin D in obese individuals probably involves active sequestration in AT, due to a reduced catecholamine-induced release of both D3 and 25(OH)D, rather than an active uptake, and altered autocrine/paracrine actions due to altered activity of vitamin D-metabolizing enzymes.

Finally, in a cohort of vitamin D-deficient subjects randomly assigned to supplementation with either 25-hydroxyvitamin-D3 or vitamin D3, we found that normal weight subjects had comparable responses to either treatment. Conversely, the proportion of obese subjects achieving vitamin D sufficiency was significantly higher after 6 months of supplementation with 25-hydroxyvitamin-D3, suggesting a more rapid effect of this treatment. Since, at the 6 months follow-up, in patients that had not achieved serum 25(OH)D levels> 50 nmol/l the dosage was increased by 50%, we estimated that obese subjects supplemented with vitamin D3 would require an almost two-fold increase in weekly dosage to increase serum 25(OH)D levels by 1 nmol/l, which is comparable with previous reports(29,47). All these aspects must be taken into consideration when evaluating the most appropriate treatment in vitamin D-deficient subjects affected by obesity.

In conclusion, this is the first study to show 1) a reduced release of D3 and 25(OH)D in IR 3T3-L1 and SAT from obese subjects after adrenaline-induced lipolysis, concomitant with lower expression of β2-adrenoceptor; 2) a reduced response of vitamin D-metabolizing genes to D3 and 25(OH)D stimulation in IR 3T3-L1; 3) an increased effectiveness of 25-hydroxyvitamin-D3 supplementation in increasing vitamin D levels in obese patients, compared with vitamin D3, after intrinsic effectiveness was accounted for. Further studies are needed in order to elucidate the molecular mechanisms controlling the deposition and release of vitamin D from AT.

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Disclosure statement: The authors have nothing to disclose

CONFLICT OF INTEREST: The authors declare no conflict of interest


**Figure 1.** Insulin resistance characterization, lipolysis and uptake and release of D3 and 25(OH)D in 3T3-L1 cells. (A) Insulin resistant (IR) 3T3-L1 adipocytes treated with chronic insulin and dexamethasone were washed, serum-starved for 2 h, and then assessed for insulin-stimulated (Ins., grey bars) 2-deoxyglucose (2-DG) uptake. (B) Control and IR 3T3-L1 were assessed for inhibition of adrenaline-stimulated (Adr., black bars) glycerol release for lipolysis. (C) Control (empty bar) and IR 3T3-L1 cells (dotted bar) were immunoblotted with anti-β2-adrenergic receptor antibody. Protein levels are expressed as target band density on β-actin band density. (D) Control and IR 3T3-L1 were cultured in presence of D3 or (E) 25(OH)D for 16 hours and then incubated with or without 10^-6 M adrenaline and harvested for HPLC-MS quantification of D3 and 25(OH)D. D3 was undetectable in cells cultured with 25(OH)D and was therefore excluded from the analysis (data not shown). Data are expressed as means ± SD. *p<0.05 vs control; **p<0.001 vs control; †p<0.05 vs lean SAT.

**Figure 2.** Expression of vitamin D-related genes in normal and insulin resistant (IR) 3T3-L1 cells. Control (empty bars) and IR (grey bars) 3T3-L1 cells have been stimulated with either vitamin D3 (A) or 25(OH)D (B) for 4 hours. Gene expression of vitamin D-related genes (CYP27A1, CYP27B1 and VDR) were assessed by RT-PCR, relative to GAPDH. Results are expressed as fold changes compared with the endogenous control not stimulated with vitamin D (dotted line). Data are means ± SD. *p<0.05 vs control baseline; †p<0.05 vs normal3T3-L1 cells.
FIGURE 3. Subcutaneous adipose tissue (SAT) lipolysis, β₂-adrenergic receptor expression and content and lipolytic release of D₃ and 25(OH)D in lean and obese male subjects. (A) SAT from lean and obese subjects were assessed for inhibition of adrenaline-stimulated (Adr., black bars) glycerol release for lipolysis. (B) Lean (empty bar) and obese SAT (grey bar) were immunoblotted with anti-β₂-adrenergic receptor antibody. Protein levels are expressed as target band density on β-actin band density. (C) SAT samples from lean (empty bars, n =5) and obese (grey bars, n= 5) subjects were cultured in vitro and assessed for D₃ and (D) 25(OH)D content by HPLC-MS after incubation with or without 10⁻⁶ M adrenaline for 4 hours. Data are expressed as means ± SD. *p < 0.05 vs Basal; †p < 0.05 vs Lean SAT; #p < 0.05 vs control.

FIGURE 4. Proportion of vitamin D-sufficient subjects during the study. 25-hydroxyvitamin D₃ (straight line) or vitamin D₃ (dotted line) were orally-administrated to normal weight (A) and obese male subjects (B). Serum 25-hydroxyvitamin D (25(OH)D) levels were assessed at baseline, at 6 months follow-up visit and at the end of the study (12 months). Dosage was increased by 50% if vitamin D sufficiency (serum 25(OH)D> 50 nmol/l) was not achieved at follow-up visit, otherwise dosage was maintained. Starting dose was 50 µg/week for 25-hydroxyvitamin D₃ and 150 µg/week for vitamin D₃. *p < 0.05 vs vitamin D₃.

Table 1. Baseline clinical, metabolic, hormonal parameters and anthropometric measures of subjects.

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight (N=38)</th>
<th>Obese (N=59)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>42.28±6.59</td>
<td>42.48±6.19</td>
</tr>
<tr>
<td><strong>Height, cm</strong></td>
<td>170.34±6.12</td>
<td>171.08±8.02</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>65.21±8.26</td>
<td>98.09±10.10*</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>22.25±2.07</td>
<td>33.47±2.16*</td>
</tr>
<tr>
<td><strong>Waist, cm</strong></td>
<td>94.40±4.60</td>
<td>124.32±11.57*</td>
</tr>
<tr>
<td><strong>Testosterone, nmol/l</strong></td>
<td>16.32±2.47</td>
<td>15.47±2.40</td>
</tr>
<tr>
<td><strong>LH, IU/l</strong></td>
<td>4.29±2.22</td>
<td>3.72±2.09</td>
</tr>
<tr>
<td><strong>Estradiol, pmol/l</strong></td>
<td>94.59±20.28</td>
<td>111.76±37.42*</td>
</tr>
<tr>
<td><strong>Fasting glucose, mg/dl</strong></td>
<td>76.45±7.76</td>
<td>94.19±11.82*</td>
</tr>
<tr>
<td><strong>Insulin, mU/l</strong></td>
<td>4.34±3.29</td>
<td>9.56±5.58*</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>0.87±0.43</td>
<td>2.03±0.73*</td>
</tr>
<tr>
<td><strong>25(OH)D, nmol/l</strong></td>
<td>34.4±5.5</td>
<td>33.0±7.3</td>
</tr>
<tr>
<td><strong>PTH, ng/l</strong></td>
<td>83.20±8.00</td>
<td>86.28±7.04</td>
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<tr>
<td><strong>Calcium, nmol/l</strong></td>
<td>2.36±0.10</td>
<td>2.38±0.09</td>
</tr>
<tr>
<td><strong>Phosphorus, nmol/l</strong></td>
<td>1.08±0.19</td>
<td>1.06±0.19</td>
</tr>
</tbody>
</table>


*p < 0.05 vs normal weight subjects according to unpaired Student’s t-test with Bonferroni-Holm correction for multiple comparisons.
Table 2. Serum 25(OH)D and PTH levels at baseline, 6 months and endpoint, dosage and change in 25(OH)D levels in normal weight and obese subjects by treatment group.

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight</th>
<th>Obese</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum 25(OH)D, nmol/l</td>
<td></td>
<td>Serum 25(OH)D, nmol/l</td>
</tr>
<tr>
<td></td>
<td>25-hydroxyvitamin D3</td>
<td>Vitamin D3</td>
<td>25-hydroxyvitamin D3</td>
</tr>
<tr>
<td>Baseline</td>
<td>34.2±6.2</td>
<td>34.6±9.4</td>
<td>1.000</td>
</tr>
<tr>
<td>6 months</td>
<td>68.5±9.1</td>
<td>67.2±10.5</td>
<td>1.000</td>
</tr>
<tr>
<td>12 months</td>
<td>73.7±7.6</td>
<td>71.1±6.5</td>
<td>0.480</td>
</tr>
<tr>
<td></td>
<td>Within group</td>
<td>p</td>
<td>Serum PTH, ng/ml</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;0.001</td>
<td></td>
<td>82.8±7.97</td>
</tr>
<tr>
<td>6 months</td>
<td>71.47±12.62</td>
<td>77.24±9.69</td>
<td>0.174</td>
</tr>
<tr>
<td>12 months</td>
<td>61.21±6.15</td>
<td>63.04±7.50</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Within group</td>
<td>p</td>
<td>Mean dose, µg/week</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>50.6±11.5</td>
</tr>
<tr>
<td>6 months</td>
<td>153.7±11.5</td>
<td>&lt;0.001</td>
<td>134.5±11.1</td>
</tr>
<tr>
<td>12 months</td>
<td>172.50±18.69</td>
<td>&lt;0.001</td>
<td>172.50±18.69</td>
</tr>
</tbody>
</table>

Abbreviations: 25(OH)D: serum 25-hydroxyvitamin D; PTH: Parathyroid Hormone.

1 Repeated-measures ANOVA was used to test the treatment · BMI status interaction with pairwise comparisons (Bonferroni-Holm adjusted) at each time point with age as a covariate.

2 Significant differences during the study within each group were investigated by ANOVA for repeat measures with age as a covariate.

3 Significant differences between treatment groups were tested with ANOVA with age as a covariate; fixed factors: treatment and BMI status

4 Different superscript letters represent significant differences (p <0.05) between time points within groups.

Data are expressed as means ± SD.

* p < 0.001 vs vitamin D3 dose in normal weight subjects.