Vitamin D₃ increases in abdominal subcutaneous fat tissue after supplementation with vitamin D₃

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Abstract

Objective: The objective was to assess the amount of vitamin D₃ stored in adipose tissue after long-term supplementation with high dose vitamin D₃.

Design: A cross-sectional study on 29 subjects with impaired glucose tolerance who had participated in a randomized controlled trial with vitamin D₃ 20 000 IU (500 µg) per week vs placebo for 3–5 years.

Methods: Abdominal subcutaneous fat tissue was obtained by needle biopsy for the measurements of vitamin D₃ and 25-hydroxyvitamin D₃ (25(OH)D₃). Body fat was measured with dual-energy X-ray absorptiometry, and serum 25(OH)D₃ level was quantified.

Results: In the subjects given vitamin D₃, the median concentrations of serum 25(OH)D₃, fat vitamin D₃, and fat 25(OH)D₃ were 99 nmol/l, 209 ng/g, and 3.8 ng/g, respectively; and correspondingly in the placebo group 62 nmol/l, 32 ng/g, and 2.5 ng/g. If assuming an equal amount of vitamin D₃ stored in all adipose tissue in the body, the median body store was 6.6 mg vitamin D₃ and 0.12 mg 25(OH)D₃ in those given vitamin D₃.

Conclusions: Subcutaneous adipose tissue may store large amounts of vitamin D₃. The clinical importance of this storage needs to be determined.

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Introduction

Vitamin D is essential for the development and maintenance of a healthy skeleton, and severe lack of vitamin D leads to rickets in children and osteomalacia in adults. Since there is a wide distribution of the vitamin D receptor (VDR) in the body, vitamin D probably also has a multitude of other functions (1).

Vitamin D is produced in the skin by sun exposure and is also available from food, in particular fatty fish, or as vitamin supplements. In Northern Norway at 70° latitude, there is no effective UVB radiation and no cutaneous vitamin D synthesis from October till March (2), and the daily average intake of vitamin D is only 300 IU (3, 4). Given that 25-hydroxyvitamin D₃ (25(OH)D₃), the

metabolite used to evaluate a subject's vitamin D status, has a half-life of 10–40 days (5), one would expect the serum 25(OH)D₃ levels by the end of the UVB winter to be very low. However, data from Tromsø show only a 25% decline during this period (6). Accordingly, there must be some regulatory mechanisms or vitamin D sources that we are not aware of, or we are using vitamin D stores accumulated during the summer. Thus, it is well known that vitamin D is present to some degree in adipose tissue (7), but the extent and clinical importance are not known. In Tromsø, we are presently performing a 5-year intervention study with vitamin D₃ at a dose of 20 000 IU (500 µg) per week vs placebo in subjects with

impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) (8). Those given vitamin D₃ after 5 years should have a considerable amount stored in their adipose tissue, and to evaluate that, we invited subjects who came to their last visit in the intervention study to undergo a subcutaneous fat biopsy.

Subjects and methods

Subjects

The subjects included in this study were participants in an ongoing 5-year study administrated with vitamin D₃ (20 000 IU/week, Dekristol Mibe, Jena, Germany) vs identical-looking placebo capsules containing arachis oil (Hasco-lek, Wroclaw, Poland) for the prevention of type 2 diabetes (T2DM), and the trial was carried out at the Clinical Research Unit, University Hospital of North Norway (8). Those included in this sub-study were also included in the main study between March 2008 and February 2010. At inclusion, all subjects had IGT and/or IFG (9) and were followed by annual oral glucose tolerance tests (OGTT). The subjects who developed T2DM (fasting blood glucose >6.9 mmol/l or the 2-h value >11.0 mmol/l at OGTT, or HbA1c >6.4% (the latter criterion included after May 2013)) were excluded from the intervention study. During the study, the subjects were asked not take vitamin D supplementation (including cod liver oil) exceeding 400 IU/day. Study medication was supplied every 6 months.

Subjects who came to the final 5 years visit or who were excluded due to development of T2DM were invited to a 1-year follow-up study that included an optional fat biopsy. These biopsies were taken immediately after the end of the supplementation. At this visit, the subjects were also asked regarding the intake of vitamin D supplementation. Height and weight were measured while the subjects wore light clothing and no shoes. BMI was calculated as weight (kg) divided by squared height (m²).

Total body dual-energy X-ray absorptiometry (DEXA) was performed for the determination of the total amount of body fat (10). The fat samples were taken from the abdominal subcutaneous adipose tissue with a needle biopsy as described by Mutch *et al.* (11). The levels of serum calcium, parathyroid hormone (PTH), creatinine and HbA1c, and 25(OH)D₃ were analyzed as described previously (8).

More specifically for the analyses of 25(OH)D, 80 µl serum was used. Following liquid–liquid extraction method, separation was performed using a Waters Acquity

HSS PFP Column (2.1×100 mm, 100 Å, 1.8 µm) maintained at 50 °C and a mobile phase of 0.1% aqueous formic acid:methanol (23:77). For quantification, the internal standard 26,26,26,27,27,27-[D6]25(OH)D₃ was used. The limit of quantification was <4 nmol/l. Within-day variation was <2% based on analyses of two serum samples (34.3 and 58.6 nM) analyzed six times on the same day, and between-day variation (*n*=3 days) was <9%. The accuracy for 25(OH)D₃ was found to be 105% based on the mean concentration of MassCheck (*n*=3 days).

Vitamin D₃ and 25(OH)D₃ levels in the fat biopsies were determined by a sensitive liquid chromatography with tandem mass spectrometry method (12). Briefly, 0.2–1 g fat samples were saponified, liquid–liquid extracted, and furthermore cleaned-up by a normal-phase solid-phase extraction method. The analytes were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione to improve the ionization efficiency by electrospray ionization positive mode. Separation was performed on a reversed column (Ascentis Express C18, 2.1 mm×10 cm, 2.7 µm) maintained at 50 °C, and a gradient mobile phase of Milli-Q water, 5 mM methylamine and 0.1% formic acid up to 60% methanol, 5 mM methylamine and 0.1% formic acid at a flow rate of 0.5 ml/min. The deuterated standards 26,26,26,27,27,27-[D6]vitamin D₃ and 26,26,26,27,27,27-[D6]25(OH)D₃ were used as internal standard. The limit of quantification was <0.1 ng/g. In this study, the analyses of variation were assessed by using house-reference pork fat materials (*n*=6) 8.2% for vitamin D₃ (5.8 ng/g) and 8.5% for 25(OH)D₃ (2.4 ng/g). The accuracy was 84–96% for vitamin D₃ and 113–114% for 25(OH)D₃ assessed on spiking (*n*=9).

All data files were sent directly from the laboratories to the Hospital's Randomization Unit and returned to the investigators (R Jorde and A Didriksen) with the randomization code but without person identification. Accordingly, all the researchers and the staff at the Clinical Research Unit were kept blinded.

Statistical analyses

Most of the data were not normally distributed and the data are therefore presented as median and range, unless otherwise specified. Mann–Whitney *U* tests were used for comparisons between groups and Spearman's ρ for correlations. A formal power calculation was not performed for the present sub-study. A *P*<0.05 was considered to be statistically significant.

Ethics

All subjects gave written informed consent and the study was approved by the Regional Committee for Medical Research Ethics. The trial is registered at [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01729013) (NCT01729013).

Results

A total of 511 subjects, of whom 256 were randomized to vitamin D₃, were included in the original prevention of T2DM study (8). During the time period, November 2012 till January 2014, 92 subjects were invited to participate in a 1-year follow-up study that included an optional fat biopsy. Seventy-six subjects accepted to participate in the follow-up study, of whom 29 agreed to have a fat biopsy taken. Eighteen of these subjects (three females) had been randomized to vitamin D₃ and 11 (two females) to placebo. In the vitamin D group, four subjects were taking vitamin D supplementation at a dose of 100–400 IU/day, and in the placebo group three subjects were taking 200–400 IU/day. In both groups, three subjects had participated for 3 years, three subjects for 4 years, and the rest for 5 years.

In the vitamin D group, the median serum 25(OH)D₃ level increased from baseline (start of the main 5 years study) 61 to 99 nmol/l at time of biopsy (after 3–5 years) ($P<0.001$), whereas there was a nonsignificant change in the placebo group from 54 to 62 nmol/l. Other characteristics of the subjects at time of the fat biopsy are given in Table 1.

The baseline blood samples and the fat biopsies (and accordingly the blood samples at time of biopsy) were all taken at the same time of the year in each individual. In the vitamin D group, this was done during the winter months (October–February) in 14 subjects and during the spring/summer months (April–June) in four subjects; correspondingly in the placebo eight and three subjects. In the vitamin D group at time of biopsy, the median serum 25(OH)D₃ concentration in the winter samples was 99 nmol/l and in the summer samples 105 nmol/l; corresponding values in the placebo group were 63 and 53 nmol/l. In neither group did the summer and winter values differ significantly, nor did adjusting for season affect the results given.

The fat biopsies were successfully performed in all subjects with no serious complications except for a slight bruising. The weight of the biopsy samples used in the vitamin D analyses varied between 0.2 and 1.1 g. The median concentration of vitamin D₃ in subcutaneous adipose tissue in the vitamin D group was 209 ng/g and in the placebo group 32 ng/g ($P<0.001$). The corresponding values for 25(OH)D₃ were 3.8 and 2.5 ng/g ($P<0.001$). Assuming equal concentration of vitamin D in all adipose tissue, the median total amounts of vitamin D₃ stored were 6.6 mg (0.26 mill IU) in the vitamin D group and 0.95 mg (0.04 mill IU) in the placebo group; and the part stored as 25(OH)D₃ 0.12 mg in the vitamin D group and 0.08 mg in the placebo group.

In both groups, there were highly significant correlations between baseline and at biopsy serum 25(OH)D₃ levels, whereas the statistical significance of

Table 1 Characteristics of the subjects in the vitamin D and placebo groups at time of inclusion in the main study and at time of biopsy after 3–5 years. The values are median (range).

	At inclusion in the main study		At time of biopsy after 3–5 years	
	Vitamin D group (n=18)	Placebo group (n=11)	Vitamin D group (n=18)	Placebo group (n=11)
Age (years)	63 (39–73)	62 (47–77)	68 (44–78)	67 (52–82)
BMI (kg/m ²)	30.7 (25.2–38.6)	29.1 (23.8–41.0)	30.9 (25.0–41.5)	28.7 (24.7–42.2)
Serum calcium (mmol/l)	2.32 (2.20–2.42)	2.30 (2.24–2.38)	2.35 (2.20–2.50)	2.38 (2.26–2.55)
Serum PTH (pmol/l)	5.0 (3.5–19.3)	6.7 (2.8–12.0)	4.7 (2.7–13.4)	5.7 (2.9–8.3)
Serum creatinine (μmol/l)	70 (48–104)	79 (51–95)	75 (52–108)	76 (59–105)
HbA1c (%)	5.9 (5.4–6.5)	6.0 (5.7–6.4)	6.1 (5.6–6.8)	6.1 (5.8–7.0)
Serum 25(OH)D ₃ (nmol/l)	60.6 (23.6–93.3)	54.2 (34.4–94.6)	99 (70–142)	62 (36–93)*
Fat tissue vitamin D ₃ (ng/g)			209 (89–510)	32 (3.6–118)*
Fat tissue 25(OH)D ₃ (ng/g)			3.8 (2.4–5.9)	2.5 (1.5–3.5)*
Total body fat (kg)			31.7 (20.6–58.3)	30.3 (13.3–58.8)
Vitamin D ₃ in total body fat (mg)			6.6 (3.7–14.5)	0.95 (0.11–2.9)*
Vitamin D ₃ in total body fat (mill IU)			0.26 (0.14–0.58)	0.04 (0.004–0.12)*
25(OH)D ₃ in total body fat (μg)			120 (94–192)	80 (36–152)*

* $P<0.001$, Mann–Whitney *U* test.

Table 2 Correlations (Spearman's ρ) between serum 25(OH)D₃ at inclusion of the 5-year intervention study, serum 25(OH)D₃ at time of biopsy (after 3–5 years), vitamin D₃ and 25(OH)D₃ level in fat tissue biopsies, and BMI and total body fat at time of biopsy in the vitamin D group ($n=18$) and the placebo group ($n=11$).

	Serum 25(OH)D ₃ at time of biopsy	Vitamin D ₃ in fat tissue biopsies	25(OH)D ₃ in fat tissue biopsies
Vitamin D group			
Serum 25(OH)D ₃ at inclusion	0.65 [†]	0.42	0.61 [†]
Serum 25(OH)D ₃ at time of biopsy		0.70 [†]	0.85 [†]
Vitamin D ₃ in fat tissue biopsies			0.68 [†]
BMI at time of biopsy	−0.48*	−0.44	−0.46
Total body fat at time of biopsy	−0.54*	−0.60*	−0.53*
Placebo group			
Serum 25(OH)D ₃ at inclusion	0.72*	0.66*	0.72*
Serum 25(OH)D ₃ at time of biopsy		0.47	0.86 [†]
Vitamin D ₃ in fat tissue biopsies			0.45
BMI at time of biopsy	−0.34	−0.38	−0.32
Total body fat at time of biopsy	−0.18	−0.43	−0.13

* $P<0.05$; [†] $P<0.01$.

the correlations between serum 25(OH)D₃ at biopsy, fat vitamin D₃ and fat 25(OH)D₃, and BMI and total body fat depended on intervention group (Table 2, Figs 1 and 2).

Discussion

In this study, we have found considerable storage of vitamin D₃ in adipose tissue, and almost six times higher levels in subjects who took vitamin D₃ 20 000 IU/week for 3–5 years compared with the placebo group.

Our results regarding the placebo group with a median subcutaneous vitamin D₃ concentration of 32 ng/g is quite similar to that reported by other groups (7, 13, 14, 15) (Table 3). In all of these studies, there was a great inter-individual variability in the fat vitamin D₃ concentration as exemplified in our study by the lowest concentration of 3.6 ng/g and the highest concentration of 118 ng/g in the placebo group, and of 89 and 510 ng/g in the vitamin D group. This could be due to several factors, the most obvious being differences in intake (and for the vitamin D group compliance) and skin production of vitamin D. Furthermore, as vitamin D in the circulation is bound to the vitamin D-binding protein (DBP) and albumin, it is likely that what diffuses into the fat cells comes from the free fraction of vitamin D. Accordingly, differences in DBP serum concentrations as well as the differences in binding coefficients for the various DBP phenotypes may be important (16). Adiposity may also play a role, as there were negative correlations between both BMI and total body fat and fat vitamin D₃. However, our study was also too small to evaluate the effect of other potential determinants such as age and gender.

Although the storage of vitamin D in adipose tissue is well known, the importance of this is uncertain. It is also uncertain if the storage is similar in all types of adipose tissue. Thus, in the study by Pramyothin *et al.* (15), four subjects with BMI > 40 kg/m² had biopsies taken not only from subcutaneous fat but also from omental and mesenteric fat with very little correlation between

Figure 1

Relation between serum 25(OH)D₃ at time of biopsy and vitamin D₃ in fat tissue from biopsies in the vitamin D group subjects (black diamonds, $n=18$; upper correlation line ($\rho=0.70$, $P<0.01$)) and the placebo group (open squares, $n=11$; lower correlation line ($\rho=0.47$, P NS)). A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0870>.

Figure 2

Relation between serum 25(OH)D₃ at the time of biopsy and 25(OH)D₃ in fat tissue from biopsies in the vitamin D group (black diamonds, $n=18$; upper correlation line ($\rho=0.85$, $P<0.01$)) and the placebo group (open squares, $n=11$; lower correlation line ($\rho=0.86$, $P<0.01$)). A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0870>.

vitamin D₃ concentrations at these sites. As an example, one subject had vitamin D₃ concentrations of 4, 187, and 57 ng/g in omental, mesenteric, and subcutaneous tissue, whereas another subject had corresponding concentrations of 143, 6, and 7 ng/g. However, if assuming an even concentration in all adipose tissue, the subjects in the placebo group in our study had a median amount of 0.95 mg or 0.04 mill IU fat-stored vitamin D. If this could

be mobilized and used at least as efficiently as vitamin D from supplementation, it is equivalent to 90 daily doses of 400 IU. If the estimate that serum 25(OH)D₃ levels increase by 2.5 nmol/l for every 100 IU vitamin D₃ given is correct (17), and the vitamin D stored is released gradually as could be expected during the winter decline in 25(OH)D₃, then this supply from adipose tissue in the placebo group would account for 10 nmol/l of the serum 25(OH)D₃ level during 3 months; in the subjects in the vitamin D group who had a median amount of 6.6 mg or 0.26 mill IU of vitamin D in adipose tissue, this would be equivalent to 328 daily doses of 800 IU or account for 20 nmol/l for almost a year. In addition, a small amount of 25(OH)D₃ was also stored in fat tissue and could contribute to maintaining the serum 25(OH)D₃ levels.

Similarly, when adipose tissue is lost after bariatric or bypass surgery for obesity, and the stored vitamin D presumably released, one would expect a sustained increase in serum 25(OH)D level. However, so far no or only small increases have been reported. Thus, in the study by Pramyothin *et al.* (15), where 17 subjects underwent a Roux-en-Y gastric bypass (RYGP) and had a fat mass loss of ~40 kg the first year, there was no increase in serum 25(OH)D₃ level after 3, 6, 9, or 12 months; in the study by Lin *et al.* (18) in 20 patients who also underwent RYGP and had a weight loss of ~30 kg in the first 6 months, there was only a transient 14 nmol/l increase in serum 25(OH)D level after 1 month; on the other hand, in the study by Aasheim *et al.* (19) in 59 subjects with a mean BMI of 55 kg/m² of whom 31 had RYGP and 28 bilio-pancreatic diversion with duodenal switch and a BMI loss of 16.3 and 22.8 kg/m², respectively, in the RYGP group there was an increase in serum

Table 3 Vitamin D in fat tissue in previously published studies.

Reference	Type of fat	n	BMI (kg/m ²)	Serum 25(OH)D (nmol/l)	Vitamin D ₃ in fat tissue (ng/g)
(7)	Subcutaneous adipose tissue, obtained at gastric bypass surgery	17	50.5±6.4	43.3±15.4	38.6±16.8
(13)	Renal adipose tissue obtained at autopsy for sudden death	15			45.3±22.2
	Pericardial adipose tissue obtained at autopsy for sudden death	4			62.1±30.8
	Axillary fat obtained at autopsy for sudden death	6			115.6±52.4
	Cervical fat obtained at autopsy for sudden death	2			82.2±100.2
(14)	Subcutaneous adipose tissue at baseline	9			76.1±15.9
	Subcutaneous adipose tissue, increase from baseline after 1250 µg vitamin D weekly for 12 weeks	7			104±77.2
(15)	Subcutaneous adipose tissue, obtained at gastric bypass surgery	10 ^a		52.8±24.8	77.9±106.5

^aOne outlier excluded.

25(OH)D level that peaked after 6 months with 15 nmol/l higher levels than before surgery, whereas a slight decrease was seen in the other group.

These surgical methods induce malabsorption of nutrients including vitamin D as seen in the study by Pramyothin *et al.* (15), where seven of the subjects were post-operatively given high dose vitamin D₂ supplementation (50 000 IU (1250 µg) per week) with no effect on the serum 25(OH)D levels, which were similar to those not given supplementation. It is likely that vitamin D from other sources was similarly poorly absorbed, and if assuming an unaltered production of vitamin D in the skin, one should therefore have expected at least a modest fall in serum 25(OH)D levels, which was not seen. A similar line of reasoning can be applied to the studies by Lin *et al.* (18) and Aasheim *et al.* (19) and one obvious explanation for the lack of decline in serum 25(OH)D levels in these three studies would be mobilization of vitamin D in adipose tissue.

What regulates the storage of vitamin D in adipose tissues is not clear. It has been suggested by Drincic *et al.* (20) that there is a simple passive diffusion with an equilibrium between vitamin D in serum and in fat globules, which could explain the lower serum 25(OH)D level seen in obese subjects (21). However, in theory it is also possible that active mechanisms are involved, for which longitudinal studies with measurements of serum and fat vitamin D as well as 25(OH)D levels are needed.

Although not a primary objective of the present sub-study, the high correlation between serum 25(OH)D₃ at baseline and after 3–5 years in the placebo group is noteworthy and a confirmation of the high degree of tracking for serum 25(OH)D₃ level (3).

This study has several short-comings. We did not have repeated biopsies in individuals and compared subjects in the two randomized groups, we did not have measurements of serum vitamin D, and we had only one level of supplementation. We could therefore not answer many of the important questions regarding storage of vitamin D in adipose tissue, such as dose–response effects, storage capacity, active and regulated storage or simple passive diffusion, and moreover, whether this has clinical importance. Also, if storage in adipose tissue is a regulated process, it is possible that this could shed light on what is our basic need for vitamin D intake or production, which again could be important in the consideration of what are sufficient or optimal serum 25(OH)D levels.

Our study also has some strengths; it is the largest so far with determination of vitamin D₃ and 25(OH)D₃ level in adipose tissue in humans, and we had the opportunity

to include subjects who for 3–5 years had been on a high vitamin D₃ supplementation dose.

In conclusion, a considerable amount of vitamin D₃ is stored in subcutaneous adipose tissue. The importance of this is uncertain, and larger studies with repeated biopsies in subjects given graded doses of vitamin D are warranted.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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