Obesity, ectopic lipids, and insulin resistance

*Tissue-specific defects in nutrient handling*

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Citation for published version (APA):


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CHAPTER 9

The vitamin D metabolites 25(OH)D and 1,25(OH)$_2$D are not related to either glucose metabolism or insulin action in obese women


Diabetes Metab 2016; 42: 416-23

* These authors contributed equally.
ABSTRACT

Aim
Vitamin D deficiency has been proposed to be involved in obesity-induced metabolic disease. However, data on the relationship between 25-hydroxycholecalciferol [25(OH)D] and insulin resistance have been inconsistent, and few studies have investigated the active vitamin D metabolite, 1,25-dihydroxycholecalciferol [1,25(OH)₂D]. This study aimed to determine the relationship between circulating levels of both 25(OH)D and 1,25(OH)₂D and direct measures of glucose metabolism and insulin action in obese women.

Methods
Serum levels of 25(OH)D and 1,25(OH)₂D, and glucose metabolism and tissue-specific insulin action, as assessed in the basal state and during a 2-step hyperinsulinemic-euglycemic clamp study with [6,6-²H₂]glucose infusion, were measured in 37 morbidly obese women [age: 43±10 years; body mass index (BMI): 44±6 kg/m²].

Results
Sixteen subjects had circulating 25(OH)D levels <50 nmol/L, consistent with vitamin D deficiency, and 21 had normal 25(OH)D levels. There were no differences in either baseline characteristics or parameters of glucose metabolism and insulin action between the groups. Serum 25(OH)D, but not 1,25(OH)₂D, was negatively correlated with both BMI (r=−0.42, p=0.01) and total body fat (r=−0.46, p<0.01). Neither 25(OH)D nor 1,25(OH)₂D levels were related to any measured metabolic parameters, including fasting glucose, fasting insulin, basal endogenous glucose production, and hepatic, adipose-tissue, and skeletal muscle insulin sensitivity.

Conclusions
Obesity was associated with lower levels of circulating 25(OH)D, but not with the hormonally active metabolite 1,25(OH)₂D. Neither 25(OH)D nor 1,25(OH)₂D were related to glucose metabolism and tissue-specific insulin sensitivity in obese women, suggesting that vitamin D does not play a major role in obesity-related insulin resistance.
INTRODUCTION

Vitamin D, a family of secosteroid hormones, has numerous effects beyond regulation of calcium and phosphorus balance [372]. Circulating 25(OH)D is considered a clinical indicator of vitamin D status [373], and vitamin D deficiency is diagnosed by measuring this metabolite. As several metabolic disorders, including obesity [374-377], the metabolic syndrome [378], and type 2 diabetes [379], are associated with low serum levels of 25(OH)D, vitamin D deficiency has been proposed to contribute to the development of metabolic disease [380].

Insulin resistance is closely linked to obesity and is a major contributor to its cardiometabolic complications [32]. Vitamin D metabolites have been suggested to exert some of their beneficial effects through improving insulin sensitivity [373,380]. Notably, the 25(OH) vitamin D metabolite is enzymatically hydroxylated to produce 1,25(OH)₂D. This hormonally active metabolite can directly stimulate expression of the insulin receptor [381] and/or have indirect insulin-sensitizing effects through regulation of extracellular calcium [380]. While production of 1,25(OH)₂D is tightly regulated to maintain calcium balance [382], more recently, it was suggested that 1,25(OH)₂D has independent effects on features of the metabolic syndrome [383]. Clinical data, however, have been scarce and inconsistent: some studies reported an association between reduced 25(OH)D and insulin resistance in various populations [373,376,379], whereas others reported no such associations [384,385]. To date, no studies have investigated the relationship between circulating concentrations of the active 1,25(OH)₂D metabolite and direct measures of glucose metabolism and insulin action in humans. Interpretation of these apparently conflicting results is further complicated by i) the confounding association between insulin resistance and adiposity, where obesity can contribute to low circulating vitamin D levels due to volumetric dilution and deposition in body fat compartments [374,375], ii) the use of indirect measures of insulin resistance in most studies [373], and iii) the use of inaccurate methods for measuring 25(OH)D and 1,25(OH)₂D levels [386,387].

Therefore, in the present study, the aim was to determine the relationship between circulating levels of both 25(OH)D and 1,25(OH)₂D, as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and direct measures of glucose metabolism and insulin action, using detailed metabolic tracer methods, in a cohort of obese adult women.

MATERIAL AND METHODS

Subjects
Obese women were recruited from the outpatients clinics of 3 bariatric surgery centers in The Netherlands. All had participated in different metabolic studies, the results of which have been partially described elsewhere [130]. Subjects were eligible to participate in the present study if they were aged >18 years, met the criteria for bariatric surgery according to national guidelines [18], were scheduled to undergo Roux-en-Y gastric bypass surgery, and had stable weight (<5% weight change) for at least 3 months prior
to examination. Those with insulin-dependent diabetes were excluded. Other exclusion criteria were the use of alcohol (>2 units/day) or recreational drugs, the use of antipsychotic or antidepressant medications, or any somatic disorder except for obesity-related conditions (such as secondary dyslipidaemia, secondary hypertension, or impaired glucose tolerance). All subjects had completed a medical evaluation, including history, physical examination and blood tests, prior to the study date. All procedures were approved by the Academic Medical Center medical ethics committee, and all subjects provided their written informed consent in accordance with the Declaration of Helsinki.

**Experimental protocol**

Body composition was determined by bioelectrical impedance analysis (Maltron BF-906; Maltron International, Rayleigh, Essex, UK). Indirect calorimetry was performed using a ventilated hood system (Vmax Encore 29N; CareFusion, San Diego, CA, USA). Liver fat content was assessed using double-voxel proton magnetic resonance spectroscopy on a 1.0-T Panorama scanner (Philips Healthcare, Best, The Netherlands) [135].

The basal rate of endogenous glucose production (EGP), insulin-mediated suppression of EGP (hepatic insulin sensitivity), insulin-mediated suppression of circulating free fatty acids (FFA) (adipose-tissue insulin sensitivity), and insulin-stimulated rate of disappearance (Rd) of glucose (peripheral/muscle insulin sensitivity) were assessed during a 2-step hyperinsulinemic-euglycemic clamp study, described in detail elsewhere [184]. In brief, subjects were admitted to the metabolic unit after an overnight fast. A primed continuous infusion of the stable isotope-labeled tracer $[6,6^{-2}\text{H}_2]$glucose (>99% enriched; Cambridge Isotope Laboratories, Tewksbury, MA, USA) was started. Basal EGP was determined after 2 h of tracer equilibration. Next, suppression of basal EGP and circulating FFA by insulin was assessed after 2 h of low-dose insulin infusion (Actrapid 20 mU·m$^{-2}$·min$^{-1}$; Novo Nordisk Nederland, Alphen aan den Rijn, The Netherlands). Finally, glucose Rd was assessed after an additional 2 h of high-dose insulin infusion (60 mU·m$^{-2}$·min$^{-1}$). During hyperinsulinemia, plasma glucose was maintained constant at 5.0 mmol/l by frequent bedside monitoring of glucose levels and variable infusions of exogenous glucose (enriched with 1% $[6,6^{-2}\text{H}_2]$glucose). Arterialized venous blood samples were drawn regularly for determination of tracer enrichment and hormones.

**Laboratory analyses**

Plasma glucose concentration was determined with the glucose oxidase method using a Biosen C-line glucose analyzer (EKF-diagnostic GmbH, Barleben near Magdeburg, Germany). Levels of glucoregulatory hormones and FFA were determined as previously described [130]. Plasma enrichment of $[6,6^{-2}\text{H}_2]$glucose (tracer-to-tracee ratio) was determined by gas chromatography–mass spectroscopy as previously described [136].

Serum concentrations of 25(OH)D were measured by isotope dilution LC-MS/MS as described elsewhere [387], but with minor adjustments. Briefly, a deuterated internal standard [25(OH)D-d6] was added to the samples, and 25(OH)D was released from its binding proteins with acetonitrile. Samples were extracted and analyzed by solid-phase extraction (Symbiosis™ Pharma Online SPE systems, Spark Holland, Emmen, The Neth-
erlands) coupled with MS/MS (Quattro Premier XE, Waters Corp., Milford, MA, USA). Serum concentrations of 1,25(OH)₂D were determined by isotope dilution LC-MS/MS as previously described [386].

Calculations
EGP and Rd were calculated using modified versions of Steele’s equation for steady state (basal EGP) or non-steady state (during insulin infusion) [40], and expressed as μmol·[kg fat-free mass (FFM)]⁻¹·min⁻¹ and μmol·(kg body weight)⁻¹·min⁻¹, respectively. Basal insulin secretion was estimated by an updated computer-based homoeostasis model assessment of beta cell function (HOMA2-%B) [388].

Statistical analyses
Data are presented as means ± standard deviation (SD) or medians [interquartile range (IQR)] unless otherwise stated. The study groups (sufficient vs deficient vitamin D status) were compared by 2-tailed χ², independent samples t or Mann–Whitney U tests, depending on the type and distribution of variables. Pearson’s coefficient was used to assess correlations between 2 normally distributed continuous variables. Multiple linear regression analysis was performed to assess the independent associations between vitamin D metabolites and measurements of glucose metabolism and insulin action. Findings were considered significant if the p value was <0.05. Analyses were performed using IBM SPSS Statistics v22 (Armonk, NY, USA).

![Figure 9.1. Serum concentrations of 25(OH)D did not correlate with serum concentrations of its hormonally active metabolite, 1,25(OH)₂D, in obese women (n=37).](image)

RESULTS

Participants
A total of 37 obese women were divided into 2 groups according to vitamin D status: 16 subjects (43%) had circulating 25(OH)D levels <50 nmol/l (<20 ng/ml), consistent with (subclinical) vitamin D deficiency [385], and 21 subjects (57%) had normal/sufficient serum 25(OH)D. Apart from the clear difference in circulating levels of 25(OH)D,
the groups were well-matched for most baseline clinical and biochemical characteristics (Table 9.1). Two women, 1 in each group, had (non-insulin-dependent) type 2 diabetes, and were being treated with oral hypoglycaemic agents. One woman had previously received oral cholecalciferol supplementation and had normal vitamin D status.

Table 9.1. Characteristics of obese women according to clinical vitamin D status (n=37).

<table>
<thead>
<tr>
<th></th>
<th>Deficiency</th>
<th>Sufficiency</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44±9</td>
<td>42±11</td>
<td>0.691</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>46±7</td>
<td>43±5</td>
<td>0.102</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>55±5</td>
<td>52±4</td>
<td>0.061</td>
</tr>
<tr>
<td>Liver fat content (%)</td>
<td>10.6 (2.3–23.3)</td>
<td>13.1 (2.8–19.0)</td>
<td>0.794</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.5±0.8</td>
<td>5.4±1.0</td>
<td>0.815</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>85±46</td>
<td>107±30</td>
<td>0.087</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.1 (0.8–1.7)</td>
<td>0.9 (0.7–1.1)</td>
<td>0.130</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8±1.1</td>
<td>4.5±0.7</td>
<td>0.336</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.0±0.2</td>
<td>1.2±0.3</td>
<td>0.031</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3.1±1.0</td>
<td>2.9±0.7</td>
<td>0.404</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
<td>0.575</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>9 (4–15)</td>
<td>9 (4–15)</td>
<td>0.765</td>
</tr>
<tr>
<td>25(OH)D (nmol/l)</td>
<td>24 (18–35)</td>
<td>64 (58–80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1,25(OH)₂D (pmol/l)</td>
<td>101±46</td>
<td>108±34</td>
<td>0.622</td>
</tr>
<tr>
<td>Antihypertensive drug use (%)</td>
<td>5 (31)</td>
<td>7 (33)</td>
<td>0.893</td>
</tr>
<tr>
<td>Proton pump inhibitor use (%)</td>
<td>3 (19)</td>
<td>4 (19)</td>
<td>0.982</td>
</tr>
<tr>
<td>Oral hypoglycemic agent use (%)</td>
<td>1 (6)</td>
<td>1 (5)</td>
<td>0.843</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>0.376</td>
</tr>
<tr>
<td>Vitamin D supplementation (%)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>0.376</td>
</tr>
<tr>
<td>Physical activity (h/week)</td>
<td>0 (0–1.5)</td>
<td>0 (0–0.8)</td>
<td>0.941</td>
</tr>
<tr>
<td>REE (kcal/day)</td>
<td>1899±256</td>
<td>1799±181</td>
<td>0.252</td>
</tr>
</tbody>
</table>

Data are count (%), mean ± SD, or medians (IQR). ¹ Circulating 25(OH)D levels <50 nmol/l.

**Serum levels of vitamin D metabolites were not mutually related**

Subjects with 25(OH)D deficiency did not have lower 1,25(OH)₂D levels than those with sufficient 25(OH)D (Table 9.1). Consistent with the hormonal regulation of the active vitamin D metabolite, circulating levels of 25(OH)D and 1,25(OH)₂D were not correlated (Figure 9.1).
**Figure 9.2.** Serum concentrations of 25(OH)D correlated negatively with (A) BMI and (B) body fat content in obese women (n=36-37). Serum 1,25(OH)₂D levels did not correlate with either (C) BMI or (D) body fat content in obese women (n=36-37).

**Serum 25(OH)D, but not 1,25(OH)₂D, was negatively associated with obesity**

In all subjects, circulating levels of 25(OH)D were negatively correlated with both BMI and total body fat content (Figures 9.2A-B), indicating that more severe obesity and/or adiposity is associated with lower levels of 25(OH)D. Notably, 6 out of 8 women with BMI scores >50 kg/m² had 25(OH)D levels <50 nmol/L, consistent with vitamin D deficiency. In contrast, 1,25(OH)₂D was not associated with either BMI or total body fat content (Figures 9.2C-D).

**Vitamin D metabolites were not related to glucose metabolism and insulin action**

Vitamin D deficiency was not associated with differences in either fasting plasma glucose or insulin (Table 9.1). In all obese women, neither 25(OH)D nor 1,25(OH)₂D metabolites were correlated with fasting plasma glucose or insulin (Supplemental Figure S1).
To assess whether vitamin D status relates to basal glucose metabolism and insulin action, these metabolic fluxes were measured using the glucose clamp technique and infusion of a stable isotope-labeled glucose tracer. Vitamin D deficiency was not associated with differences in basal EGP rates (Figure 9.3A), nor with insulin sensitivity in the liver (Figure 9.3B), adipose-tissue (Figure 9.3C) or peripheral tissues/muscle (Figure 9.3D). Moreover, in the entire cohort of morbidly obese women, who were characterized by moderate-to-severe insulin resistance compared with healthy non-obese subjects [184], clamp-derived measurements of glucose metabolism and insulin action did not correlate with either circulating 25(OH)D or 1,25(OH)₂D metabolites (Figures 9.4A–H). In fact, none of the measured metabolic parameters [including plasma lipids, liver fat content (Supplemental Figure S2), HOMA2-%B (Supplemental Figure S3), C-reactive protein (CRP) and resting energy expenditure (REE)] correlated with serum levels of vitamin D metabolites (not shown), indicating that low levels of vitamin D, whether prehormone or active, are not associated with metabolic disturbances in obese women.

Multiple regression analysis, including age, total body fat content and CRP as covariates, confirmed that neither vitamin D metabolite was associated with clamp-derived measurements of insulin sensitivity after correction for possible confounders (Supplemental Tables S1 to S4).

**DISCUSSION**

Data from the present study demonstrate that i) BMI is associated with lower serum levels of 25(OH)D, but not with the hormonally active metabolite 1,25(OH)₂D, ii) vitamin D deficiency is not associated with impaired glucose metabolism or insulin sensitivity, iii) neither 25(OH)D nor 1,25(OH)₂D are related to (direct measurements of) glucose metabolism and tissue-specific insulin action across the full range of serum concentrations in morbidly obese women.
Vitamin D and insulin sensitivity

Figure 9.4. Serum concentrations of neither (A–D) 25(OH) vitamin D nor (E–H) 1,25(OH)₂ vitamin D correlated with clamp-derived measurements of glucose metabolism and insulin action in obese women (n=32-36).
In line with previous findings [389,390], BMI and total body fat content, as reflections of obesity and adiposity, were negatively correlated with circulating levels of 25(OH)D, indicating that obesity is associated with reduced 25(OH)D levels. The association between obesity and vitamin D deficiency raises the question of causality, and several lines of evidence now suggest that obesity can lead to low vitamin D levels. Firstly, volumetric dilution of vitamin D in the large adipose-tissue mass results in decreased circulating levels [374,375]. Secondly, physical activity, dietary calcium, vitamin D intake and sunlight exposure are lower in obesity and may contribute to vitamin D deficiency [391,392]. Thirdly, a large bidirectional genetic analysis using Mendelian randomization recently found that a higher BMI leads to lower 25(OH)D, whereas the effects of lower 25(OH)D on increasing BMI are likely limited [377]. Importantly, as obesity can contribute to lower 25(OH)D levels and insulin resistance [32], it is undoubtedly a major confounder when assessing the relationship between vitamin D and insulin resistance/metabolic health. When comparing metabolic outcomes in individuals with normal and deficient vitamin D status, groups should be well-matched for BMI, adiposity and physical activity (Table 9.1). Our findings show that vitamin D deficiency is not associated with either direct measurements of glucose metabolism or insulin resistance in our well-matched obese groups.

In addition, active vitamin D [1,25(OH)2D] was associated with neither levels of 25(OH)D nor BMI, indicating that hydroxylation of 25(OH)D to produce 1,25(OH)2D is regulated independently of vitamin D status and obesity. To our knowledge, this is the first study in humans to investigate the relationship between 1,25(OH)2D and direct (gold standard) measurements of glucose metabolism and insulin action. This has allowed the proper determination of the relationship between active vitamin D and insulin resistance, with our data indicating that circulating levels of 1,25(OH)2D are unrelated to various measured parameters of metabolic health.

These findings are clinically relevant, given the current interest in the role of vitamin D in metabolic disease and widespread promotion of vitamin D supplementation. Although several epidemiological studies have found an association between vitamin D deficiency and indirect markers of insulin resistance (reviewed in [373]), the available evidence from most [376,384,385], but not all [393], gold standard hyperinsulinemic-euglycemic clamp studies supports our main finding: circulating levels of vitamin D metabolites are not associated with insulin resistance. More importantly, clinical trials investigating the effects of vitamin D supplementation on indirect markers of insulin resistance have been inconclusive [373,394,395] and, in 2 randomized controlled trials assessing insulin resistance by clamp technique, supplementation with cholecalciferol and with 1,25(OH)2D had no benefits over placebo [396,397]. Taken altogether, these observations suggest that vitamin D does not play a major role in the development of obesity-related disruption of glucose homoeostasis, and that normalization of vitamin D in obese individuals is not likely to lead to improved glucose metabolism and/or insulin sensitivity.

The reference range for 1,25(OH)2D in our laboratory is 59–159 pmol/l [386]. Only 3 (8%) of our present study subjects had 1,25(OH)2D concentrations below this range. Thus, the present study failed to address the question of whether low 1,25(OH)2D levels might con-
Vitamin D and insulin sensitivity

contribute to poor metabolic health. Previously, intravenous administration of \(1,25(\text{OH})_2\text{D}\) to uremic hemodialysis patients, who are characterized by severe \(1,25(\text{OH})_2\text{D}\) deficiency and hyperparathyroidism, improved insulin resistance and secretion \[398\], suggesting that this metabolite may play a role in the development of metabolic disease in patients with severe \(1,25(\text{OH})_2\text{D}\) deficiency. In addition, \(1,25(\text{OH})_2\text{D}\) may have beneficial effects on metabolic parameters not measured in the present study. The nuclear vitamin D receptor and \(1\alpha\)-hydroxylase, responsible for hydroxylation of \(25(\text{OH})\text{D}\) to \(1,25(\text{OH})_2\text{D}\), are present in pancreatic \(\beta\) cells \[399\], and \(1,25(\text{OH})_2\text{D}\) has been suggested to improve insulin secretion \[380,398\]. Although \(1,25(\text{OH})_2\text{D}\) levels were not correlated with HOMA2-%B-estimated insulin secretion in our cohort (Supplemental Figure S3), this indirect marker of insulin secretion may not fully reflect direct measurements of insulin secretion. Furthermore, the vitamin D receptor and \(1\alpha\)-hydroxylase are expressed in many rodent and human brain areas involved in energy metabolism \[400-403\], and vitamin D metabolites can be detected in human cerebrospinal fluid \[404\], suggesting the intriguing hypothesis that vitamin D influences central regulation of metabolism and energy balance. The effects of vitamin D on brain function, and the role of the central nervous system in obesity and insulin resistance, are active interests of ongoing research.

Several factors may limit the strength and generalizability of our present results. Firstly, the cross-sectional design of our study does not allow any conclusions on causality. Secondly, the labor-intensive nature of metabolic tracer studies limits the number of participants in studies using such gold standard measurement methods for glucose metabolism. Thirdly, there were no data on dietary vitamin D (or calcium) intake, and circulating levels of calcium and parathyroid hormone were also not determined. Thus, it is not possible to rule out that (variations in) these factors may have influenced our results.

Finally, in line with previous findings in obese subjects \[385\], the prevalence of vitamin D deficiency in our cohort was high (43%), and none of those with \(25(\text{OH})\text{D}\) deficiency were receiving vitamin D supplementation at the time of investigation, suggesting that vitamin D deficiency, irrespective of its role in glucose homeostasis, is underrecognized in obese patients, and additional clinical attention may be required. However, no differences were observed in our results when all analyses were repeated, but excluding the subject who received oral cholecalciferol supplementation.

CONCLUSIONS

Based on our present study results, it is concluded that i) morbid obesity is associated with lower levels of circulating \(25(\text{OH})\text{D}\), but not with the hormonally active metabolite \(1,25(\text{OH})_2\text{D}\), ii) a vitamin D-deficient status is not associated with metabolic disturbances compared with well-matched but vitamin D-sufficient controls, and iii) the vitamin D metabolites \(25(\text{OH})\text{D}\) and \(1,25(\text{OH})_2\text{D}\) are not related to (direct measurements of) glucose metabolism and tissue-specific insulin action in obese women. These results suggest that vitamin D does not play a major role in obesity-related insulin resistance.

Supplemental information to this chapter is available online (https://www.journals.elsevier.com/diabetes-and-metabolism).