Effects of vitamin D supplementation on metabolic and endocrine parameters in PCOS: a randomized-controlled trial

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Supplemental Materials and Methods

Procedures

Anthropometric measurements (height, weight, waist and hip circumference, blood pressure) were obtained from all subjects at each study visit. Blood pressure was measured after a resting period of at least five minutes. Body-mass index (BMI) was calculated as body weight [kg] divided by the height in meters squared. To evaluate the presence of clinical hyperandrogenism for the diagnosis of PCOS, hirsutism was classified according to the modified Ferriman-Gallwey score [1].

Mass spectrometry (MS) measurements

Measurements of 25(OH)D and TT by ID-LC-MS/MS were performed at the Endocrine Laboratory of the VU University Medical Center, Amsterdam, the Netherlands, as previously described [2,3].

For 25(OH)D, the internal standards $^{13}C_5$–25(OH)D_3 and $^{2}H_6$–25(OH)D were added to the samples and 25(OH)D was released from its binding proteins with acetonitrile. After a liquid-liquid extraction by hexane, the samples were analyzed by LC-MS/MS (Acquity UPLC coupled to a Quattro Premier XE MS/MS, Waters Corp., Milford, MA, USA). For concentrations between 17 and 160

nmol/L, intraassay and interassay coefficients of variation (CVs) were <4.5% and <5.5%, respectively. The limit of quantification (LOQ) was 4.0 nmol/L.

For TT, the internal standard $^{13}C_3$-testosterone was added to the samples and testosterone was released from its binding proteins with acetonitrile. After a liquid-liquid extraction by hexane, the samples were analyzed by LC-MS/MS (Acquity 2D-UPLC coupled to a Xevo TQ-S tandem mass spectrometer, Waters Corp., Milford, MA, USA). Interassay variation at 0.1 nmol/L was 10.6% and <6% between 0.9 and 14 nmol/L. The LOQ was 0.10 nmol/L.

Both methods to measure either 25(OH)D and TT were well-standardized [2,4].

Biochemical analyses

To evaluate inclusion criteria, 25(OH)D was initially measured by a commercially available enzyme immunoassay (IDS, Boldon, UK) with intra- and interassay CVs of 5.6 and 6.4%, respectively. To establish the diagnosis of PCOS, TT was initially measured by luminescence immunoassay (Siemens, Erlangen, Germany) with intra- and interassay CVs of <10%. Insulin was measured by luminescence immunoassay (Siemens, Erlangen, Germany) with intra- and interassay CVs of 4% and 2.6%, respectively. PTH was measured by ElectroChemiLuminescence immunoassay (ECLIA; Roche Diagnostics, Mannheim, Germany) with intra- and interassay CVs of 1.5-2.7% and 3.0-6.5%, respectively. Sex hormone-binding globulin (SHBG; for calculation of FT and FAI) was measured by luminescence immunoassay (Cobas, Roche, Basel, Switzerland) with intra- and interassay CVs of 1.3% and 2.1%, respectively. Androstendione and dehydroepiandrostendione-sulfate (DHEAS) were measured by ELISA (DiaMetra, BioVendor, Brno, Czech Republic and LDN Labor Diagnostika Nord GmbH, Nordhorn, Germany) with intra- and interassay CVs of <10%. Estradiol was measured by chemiluminescence immunoassay (Siemens, Erlangen, Germany) with intra- and interassay CVs of 6.3-15% and 6.4-16%, respectively. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by enzyme immunoassay (DiaMetra S.r.l., Segrate (MI), Italy) with intra- and interassay CVs of <10%. $1,25(OH)_2D$ was measured by chemiluminescence immunoassay (IDS, Boldon, UK) with intra- and interassay CVs of 6.4-12.1% and 6.6-9.6%, respectively.

All other parameters [plasma glucose, HbA1c, triglycerides, TC, HDL-cholesterol, LDL-cholesterol, C-reactive protein (CRP), plasma calcium] were determined by routine laboratory diagnostics. To avoid glycolysis and therefore incorrect measurements, plasma glucose was measured from tubes containing sodium fluoride.

Supplemental Table 1: Normal ranges of baseline biochemical and anthropometric parameters (where available) and participants within these ranges

Parameter	Normal Range	Participants [%]
Body-mass index (kg/m²)	<18.5 underweight 18.5-25.0 normal weight 25.0-30.0 overweight >30.0 obese	0.8 49.2 19.7 30.3
Waist circumference (cm) [5]	<80	32.1
WHR (cm/cm) [6]	<0.85	54.2
Systolic BP (mmHg) [7]	<130	79.2
Diastolic BP (mmHg) [7]	<85	70.8
Fasting glucose (mg/dL) [8]	<100	98.4
OGTT glucose 120 min (mg/dL) [8]	<140 (for prediabetes) <200 (for diabetes)	95.1 100
Fasting insulin (mU/L)	3.0-25.0	85.4
HbA1c (mmol/mol) [8]	<39 (for prediabetes) <48 (for diabetes)	91.9 100
Triglycerides (mg/dL)	<150	91.7
Total cholesterol (mg/dL)	<200	78.5
HDL-cholesterol (mg/dL)	>40	92.5
CRP (mg/L)	<5	84.9
25(OH)D (nmol/L)*	75-150	0
PTH (pg/mL)	15.0-65.0	89.4
Plasma calcium (mmol/L)	2.20-2.65	95.1
Total testosterone (nmol/L)$^{+}$ [9]	0.30-1.69	60.2
Androstendione (ng/mL)	0.75-3.20	47.2
DHEAS (µg/mL)	0.46-2.75	73.2

*Normal range of the commercially available immunoassays used to evaluate study eligibility. $^{+}$Normal range of the ID-LC-MS/MS method used to measure total testosterone; for recruitment and to establish the clinical diagnosis of polycystic ovary syndrome, a commercially available immunoassay measurement was used

25(OH)D = 25-hydroxyvitamin D; BP = blood pressure; CRP = C-reactive protein; DHEAS = dehydroepiandrostendione-sulfate; HbA1c = glycated hemoglobin; HDL-cholesterol = high density lipoprotein-cholesterol; LDL-cholesterol = low density lipoprotein-cholesterol; OGTT glucose 120 min = plasma glucose at 120 minutes during 75g oral glucose tolerance test; PTH = parathyroid hormone; WHR = waist-to-hip ratio

Supplemental Table 2: Primary and secondary outcome variables at baseline and final follow-up after 12 weeks in study participants with available values at both study visits

	Baseline	Follow-Up (12 weeks)	Treatment Effect (95% CI)	p-value
AUCgluc				
Vitamin D (n=92)	228.05±46.45	210.59±46.35	-12.89 (-24.70 to -1.08)	0.033
Placebo (n=45)	210.04±40.39	209.77±47.54		
Fasting glucose [mg/dL]				
Vitamin D (n=94)	84±8	83±9	-0.5 (-2.9 to 1.9)	0.681
Placebo (n=46)	84±8	84±7		
OGTT glucose 30 min [mg/dL]				
Vitamin D (n=91)	133±25	131±29	-3.0 (-11.0 to 4.9)	0.452
Placebo (n=45)	127±24	130±23		
OGTT glucose 60 min [mg/dL]				
Vitamin D (n=91)	122±40	108±35	-10.8 (-20.0 to -1.6)	0.022
Placebo (n=45)	107±31	108±39		
OGTT glucose 120 min [mg/dL]				
Vitamin D (n=92)	99±24	87±21	-2.8 (-9.9 to 4.3)	0.430
Placebo (n=45)	91±24	86±25		
*HbA1c [mmol/mol]**				
Vitamin D (n=42)	33 (31-35)	34 (32-35)	0.4 (-0.2 to 1.0)	0.166
Placebo (n=85)	34 (32-35)	33 (32-35)		
*HOMA-IR**				
Vitamin D (n=94)	1.92 (1.09-3.49)	2.32 (1.34-3.62)	0.52 (-0.96 to 1.99)	0.134
Placebo (n=46)	2.15 (1.28-3.00)	2.02 (1.06-3.23)		
*QUICKI**				
Vitamin D (n=94)	0.346 (0.318-0.378)	0.336 (0.316-0.366)	-0.009 (-0.022 to 0.003)	0.129
Placebo (n=46)	0.340 (0.324-0.369)	0.343 (0.321-0.380)		
*Triglycerides [mg/dL]**				
Vitamin D (n=91)	63 (49-90)	69 (53-94)	5 (-4 to 15)	0.455
Placebo (n=45)	74 (51-114)	76 (58-95)		
*Total cholesterol [mg/dL]**				
Vitamin D (n=91)	172 (156-190)	179 (151-194)	0.3 (-7 to 7)	0.780
Placebo (n=45)	175 (142-203)	172 (148-197)		
*Total testosterone [nmol/L]**				
Vitamin D (n=92)	1.55 (1.13-2.18)	1.50 (1.10-2.00)	-0.14 (-0.34 to 0.06)	0.164
Placebo (n=44)	1.40 (1.20-1.80)	1.60 (1.13-1.88)		
*Free testosterone [nmol/L]**				
Vitamin D (n=91)	0.021 (0.016-0.032)	0.021 (0.014-0.029)	-0.001 (-0.004 to 0.002)	0.271
Placebo (n=44)	0.019 (0.015-0.035)	0.022 (0.015-0.031)		

Data are shown as means with standard deviation or medians and interquartile range, as appropriate. Treatment effects with 95% confidence interval and p-values were calculated by ANCOVA for group differences at follow-up with adjustment for baseline values.

*Skewed variables for which logarithmic transformed values were used in ANCOVA, but untransformed values are shown in the table.

AUCgluc = plasma glucose area under the curve; HbA1c = glycated hemoglobin; HOMA-IR = homeostatic model assessment-insulin resistance; OGTT glucose 30 min = plasma glucose at 30 minutes during 75g oral glucose tolerance test; OGTT glucose 60 min = plasma glucose at 60 minutes during 75g oral glucose tolerance test; OGTT glucose 120 min = plasma glucose at 120 minutes during 75g oral glucose tolerance test; QUICKI = quantitative insulin sensitivity check index

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