CLINICAL STUDY

Vitamin D-associated polymorphisms are related to insulin resistance and vitamin D deficiency in polycystic ovary syndrome

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Abstract

Introduction: Women with polycystic ovary syndrome (PCOS) frequently suffer from metabolic disturbances including insulin resistance (IR), which might be related to vitamin D metabolism. We aimed to investigate the association of polymorphisms in the vitamin D receptor (VDR) gene as well as vitamin D level-associated genes with metabolic and endocrine parameters in PCOS women. Moreover, we examined whether there are associations with PCOS susceptibility.

Methods: Metabolic, endocrine, and anthropometric measurements and oral glucose tolerance tests were performed in 545 PCOS and 145 control women. Genotyping of VDR (*Cdx2*, *Bsm-I*, *Fok-I*, *Apa-I*, and *Taq-I*), *GC*, *DHCR7*, and *CYP2R1* polymorphisms was performed.

Results: 25-Hydroxyvitamin D (25(OH)D) levels showed significant negative correlation with IR and positive correlation with insulin sensitivity ($P < 0.05$ for all) in PCOS women. In PCOS women, the VDR *Cdx2* 'AA' genotype was associated with lower fasting insulin ($P = 0.039$) and homeostatic model assessment-IR ($P = 0.041$) and higher quantitative insulin-sensitivity check index ($P = 0.012$) and MATSUDA index ($P = 0.003$). The VDR *Apa-I* 'AA' genotype was associated with lower testosterone ($P = 0.028$) levels. In PCOS women, 170 women (31.2%) presented with 25(OH)D levels < 20 ng/ml. PCOS women carrying the *GC* 'GG' genotype and the *DHCR7* 'GG' genotype had a significantly higher risk for 25(OH)D levels < 20 ng/ml (OR 2.53 (1.27–5.06), $P = 0.009$, and OR 2.66 (1.08–6.55), $P = 0.033$ respectively) compared with PCOS women carrying the *GC* 'TT' genotype and *DHCR* 'TT' genotype in multivariate analyses. We observed no association of genetic variations and PCOS susceptibility.

Conclusion: VDR and vitamin D level-related variants are associated with metabolic and endocrine parameters including 25(OH)D levels in PCOS women.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common female endocrine disorder with a prevalence of ~5–10% in women of reproductive age (1–3); the incidence, however, might increase due to nutritional changes. PCOS is characterized by increased ovarian and adrenal androgen secretion; hyperandrogenic symptoms such as hirsutism, acne, and/or alopecia; menstrual irregularity; and polycystic ovaries. In addition, insulin resistance (IR) is common in PCOS women (4), who are therefore at an increased risk of type 2 diabetes (5).

Accumulating evidence suggests that vitamin D deficiency might be a causal factor in the pathogenesis of IR and the metabolic syndrome in PCOS (6, 7). The results from our study in PCOS women investigating the effect of vitamin D supplementation indicate a favorable

effect of vitamin D supplementation on glucose metabolism (8). This notion is supported by the fact that the vitamin D receptor (VDR) regulates more than 3% of the human genome, including genes that are crucial for glucose metabolism (9, 10). However, data on the role of gene variants involved in vitamin D metabolism in PCOS are sparse. PCOS status has been shown to be associated with the VDR *Apa-I* and *Fok-I* polymorphism was associated with IR in a cohort including 162 PCOS women (11). By contrast, a small study including 56 PCOS women reported an association of VDR *Bsm-I* variants with sex hormone binding globulin (SHBG) levels in PCOS women, whereas no association was found between VDR polymorphisms and metabolic parameters (12). Recently, the results from a genome-wide association study of 25-hydroxyvitamin D (25(OH)D) levels in 33 996 individuals of

European descent from 15 cohorts established a role for common genetic variants in the regulation of 25(OH)D levels (13). Variants near genes involved in cholesterol synthesis (*DHCR7*), hydroxylation (*CYP2R1*), and vitamin D transport (*GC*) affect vitamin D status. So far, the role of those polymorphisms in PCOS has not been investigated.

The aim of this study was to investigate the association of *VDR* variants including *Cdx2*, *Bsm-I*, *Fok-I*, *Apa-I*, and *Taq-I* as well as *GC*, *DHCR7*, and *CYP2R1* variants with metabolic and endocrine parameters including 25(OH)D levels in a cohort of PCOS women. Moreover, we examined whether there are associations with PCOS susceptibility.

Methods

Subjects

The study cohort consisted of 545 women with PCOS aged 16–45 years, who were routinely referred to our outpatient clinic for PCOS evaluation from 2006 to 2010. Totally, 145 body mass index (BMI)-matched women within the same age range, who were referred for routine thyroid evaluation to our outpatient clinic from 2009 to 2010, were invited to participate in the trial as a control group. All the control women had normal thyroid function, regular menstrual cycles, normal serum androgens, and no clinical signs of hyperandrogenism.

PCOS women

PCOS was diagnosed using the Rotterdam criteria (14). Of the following three criteria, two are required to confirm the diagnosis: clinical and/or biochemical signs of hyperandrogenism, oligo- and/or anovulation, and polycystic ovaries (by ultrasound). Hyperandrogenism was defined by the clinical presence of hirsutism (Ferriman–Gallwey score ≥ 6), acne, or alopecia and/or elevated androgen levels (testosterone normal range <0.77 ng/ml and free testosterone normal range <3.18 pg/ml). Oligo- and/or anovulation were defined by the presence of oligomenorrhea or amenorrhea. Polycystic ovarian morphology was examined by ultrasound (14). Hyperprolactinemia, Cushing's syndrome, congenital adrenal hyperplasia, and androgen secreting tumors were excluded by specific laboratory analysis (cortisol, ACTH, 17α (OH)-progesterone, and DHEAS). PCOS women and control women did not take any medication known to affect endocrine parameters, carbohydrate metabolism, or serum lipid profile for at least 3 months before entering the study. The study protocol was approved by the local ethics committee, and written informed consent was obtained from each patient.

Procedures

Standard anthropometric data (height, weight, waist circumference (WC), hip circumference, and blood pressure) were obtained from each subject. WC was measured in a standing position midway between the lower costal margin and the iliac crest. Hip circumference was measured in a standing position at the maximum circumference over the buttocks. The BMI was calculated as the weight in kilograms divided by the square of height in meters. Hirsutism was quantified with the modified Ferriman–Gallwey score. Moreover, basal blood samples for hormonal (total testosterone, free testosterone, SHBG, androstenedione, DHEAS, free tri-iodothyronine, free thyroxine, TSH, 17α (OH)-progesterone, and cortisol) and metabolic (glucose, insulin, total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein cholesterol, and triglycerides (TG)) determinations were collected at 0800–0900 h after overnight fast. All participants underwent a fasting 75 g oral glucose tolerance test. Blood samples were drawn after 30, 60, and 120 min for glucose and insulin determination. IR was estimated using the homeostatic model assessment-IR (HOMA-IR). HOMA-IR was calculated as the product of the fasting plasma insulin value (μ U/ml) and the fasting plasma glucose value (mg/dl) divided by 405 (15). Quantitative insulin-sensitivity check index (QUICKI) was used to estimate insulin sensitivity. QUICKI was calculated as $1/\log$ fasting insulin (μ U/ml) + $\log$ fasting glucose (mg/dl) (16). To assess β -cell function, HOMA- β was calculated as $(20 \times \text{fasting insulin } (\mu\text{U/ml})) / (\text{fasting glucose (mmol/l)} - 3.5)$. MATSUDA index was calculated as $10000 / \sqrt{(\text{fasting glucose} \times \text{fasting insulin}) \times (\text{mean glucose}_{\text{OGTT}} \times \text{mean insulin}_{\text{OGTT}})}$ (17). The free androgen index (FAI) was calculated as testosterone (nmol/l)/SHBG (nmol/l) $\times 100$.

Genotyping

Blood samples were collected in tubes containing EDTA as anticoagulant. DNA was extracted using the NucleoSpin Blood method. Genotyping of *VDR* *Cdx2* (rs11568820), *Bsm-I* (rs1544410), *Fok-I* (rs2228570), *Apa-I* (rs7975232), and *Taq-I* (rs731236) as well as *GC* (rs2282679), *DHCR7* (rs12785878), and *CYP2R1* (rs10741657) were performed by predesigned SNP Genotyping Assay (Applied Biosystems, Carlsbad, CA, USA).

Biochemical analysis

25(OH)D was measured using a commercially available enzyme immunoassay (IDS, Boldon, UK) with intra- and interassay coefficients of variation (CV) of 5.6 and 6.4% respectively. Insulin was measured by ELISA (Siemens, Erlangen, Germany) with intra- and interassay CV of 4.0 and 2.6 and 5.1 and 8.4% respectively. Free testosterone was determined using an RIA (DSL, Webster,

TX, USA). SHBG, PTH (Roche), and total testosterone (Siemens, Erlangen, Germany) were measured by luminescence immunoassay. Androstenedione was determined using ELISA (Biotec, Suffolk, UK).

Statistical analysis

Data are presented as median (interquartile range) unless stated. The Kolmogorov–Smirnov test and descriptive statistics were used to evaluate the distribution of data. All continuous parameters following a non-normal distribution were logarithmically transformed when parametric tests were performed. After transformation, all parameters showed a normal distribution. Hardy–Weinberg equilibrium was tested by a χ^2 goodness-of-fit test. Within the group of PCOS women, AN(C)OVA with *post hoc* analyses (Bonferroni) was used to compare continuous parameters between genotypes. We performed adjustment for age, BMI, 25(OH)D, month of blood sampling, and calcium levels, as appropriate. Nominal variables were analyzed using the χ^2 and the Fisher exact tests. Binary logistic regression analyses were performed to examine the associations of hypovitaminosis D (dependent variable) with genotype, age, BMI, and month of blood sampling. We calculated two binary logistic regression analyses using two different thresholds for hypovitaminosis D: 25(OH)D levels <20 ng/ml (vitamin D deficiency) and 25(OH)D levels <30 ng/ml (combined vitamin D insufficiency and deficiency).

A *P* value <0.05 was considered significant. Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Table 1 shows clinical and biochemical characteristics of PCOS and control women. PCOS women were significantly younger, had higher fasting insulin levels, HOMA-IR, HOMA- β , testosterone, and androstenedione levels, and significantly lower 25(OH)D, QUICKI, MATSUDA, and SHBG levels.

Median Ferriman–Gallwey score of PCOS women was 7 (2–11), and 52% of PCOS women were hirsute. Oligo- or amenorrhea was present in 475 out of 545 PCOS women (87%), and polycystic ovaries were found in 264 women (48%).

In PCOS women, 170 women (31.2%) presented with vitamin D deficiency (<20 ng/ml), 189 women (34.7%) had vitamin D insufficiency (20–29.9 ng/ml), and 186 women (34.1%) had a sufficient vitamin D status. In control women, vitamin D deficiency and vitamin D insufficiency were less common (*P*<0.001). Among the control women, 25 women (17.2%) presented with vitamin D deficiency (<20 ng/ml), 38 women (26.2%) had vitamin D insufficiency (20–29.9 ng/ml), and 82 women (56.6%) had a sufficient vitamin D status.

Genotyping

There was no significant difference in genotype frequencies between PCOS and control women. In detail, distribution of the *VDR Cdx2* genotype was 64% (*n*=346) GG, 32% (*n*=176) GA, and 4% (*n*=23) AA in PCOS women and 66% (*n*=96) GG, 32% (*n*=46) GA, and 2% (*n*=3) AA in control women (*P*=0.420).

Table 1 Baseline characteristics (median (interquartile range)) of PCOS and control women. To convert 25(OH)D levels into nM/l, multiply by 2.496.

	PCOS women (<i>n</i> =545)	Control women (<i>n</i> =145)	<i>P</i> value
Age (years)	27 (23–31)	29 (26–36)	<0.001
BMI (kg/m ²)	24.2 (21.2–29.0)	24.4 (20.9–29.2)	0.845
WHR	0.79 (0.74–0.86)	0.81 (0.76–0.86)	0.234
Fasting glucose (mg/dl)	86 (80–92)	88 (82–91)	0.753
Glucose 2 h (mg/dl)	98 (84–119)	93 (78–112)	0.121
AUCgluc	176.8 (154.8–202.0)	167.5 (146.5–194.8)	0.090
HbA1c (%)	5.1 (5.0–5.3)	5.2 (5.0–5.4)	0.794
Fasting insulin (μU/ml)	6.3 (4.0–10.5)	4.9 (3.0–7.8)	<0.001
Insulin 2 h (μU/ml)	34.0 (20.4–58.0)	32.5 (17.8–57.2)	0.133
AUCins	61.3 (37.9–90.6)	57.7 (37.3–87.9)	0.338
HOMA-IR	1.35 (0.82–2.3)	1.16 (0.64–1.74)	0.009
HOMA- β	105.6 (68.7–174.6)	72.2 (45.9–119.8)	0.003
QUICKI	0.37 (0.33–0.40)	0.37 (0.35–0.41)	0.005
MATSUDA	7.88 (4.8–12.5)	8.03 (4.64–13.67)	0.006
25(OH)D (ng/ml)	25.7 (18.3–34.8)	32.0 (22.9–40.5)	0.002
PTH (pg/ml)	35.3 (28.0–45.8)	34.1 (26.5–42.8)	0.632
Testosterone (ng/ml)	0.63 (0.48–0.80)	0.44 (0.32–0.57)	0.001
SHBG (nmol/l)	44.8 (29.8–61.5)	76.4 (51.6–200)	0.003
Free testosterone (pg/ml)	2.60 (1.98–3.47)	1.71 (1.18–2.03)	<0.001
Androstenedione (ng/ml)	2.8 (2.0–4.2)	1.5 (1.0–2.5)	<0.001

The allelic frequency of *VDR Fok-I* genotype was 15% ($n=82$) AA, 45% ($n=241$) AG, and 40% ($n=215$) GG in PCOS women and 16% ($n=22$) AA, 45% ($n=60$) AG, and 39% ($n=53$) GG in control women ($P=0.954$). The distribution of the *VDR Bsm-I* genotype was 40% ($n=216$) GG, 45% ($n=244$) GA, and 15% ($n=77$) AA in PCOS women and 36% ($n=49$) GG, 48% ($n=66$) GA, and 16% ($n=22$) AA in control women ($P=0.622$). The allelic frequency of *VDR Apa-I* genotype was 26% ($n=142$) AA, 51% ($n=274$) AC, and 23% ($n=127$) CC in PCOS women and 33% ($n=48$) AA, 41% ($n=60$) AC, and 26% ($n=37$) CC in control women ($P=0.155$). The distribution of the *VDR Taq-I* genotype was 42% ($n=226$) TT, 44% ($n=238$) TC, and 14% ($n=72$) CC in PCOS women and 36% ($n=49$) TT, 47% ($n=65$) TC, and 17% ($n=23$) CC in control women ($P=0.335$). The allelic frequency of GC genotype was 50% ($n=221$) TT, 38% ($n=172$) TG, and 12% ($n=55$) GG in PCOS women and 46% ($n=67$) TT, 42% ($n=61$) TG, and 12% ($n=17$) GG in control

women ($P=0.836$). The distribution of the *DHCR7* genotype was 51% ($n=227$) TT, 41% ($n=185$) TG, and 8% ($n=35$) GG in PCOS women and 53% ($n=77$) TT, 36% ($n=52$) TG, and 11% ($n=16$) GG in control women ($P=0.440$). The distribution of the *CYP2R1* genotype was 38% ($n=172$) GG, 47% ($n=212$) GA, and 15% ($n=66$) AA in PCOS women and 44% ($n=64$) GG, 36% ($n=52$) GA, and 20% ($n=29$) AA in control women ($P=0.121$). None of the genotype frequency distributions deviated significantly from the Hardy–Weinberg equilibrium ($P>0.05$).

VDR Cdx2

PCOS women with the AA genotype had significantly lower fasting insulin, HOMA-IR, and significantly higher QUICKI and MATSUDA levels than women with the GG or GA genotype. Moreover, there was a trend toward lower AUCins and HOMA- β levels in PCOS women carrying the AA genotype (Table 2).

Table 2 Metabolic parameters (median (interquartile range)) according to *VDR Cdx-2*, *GC* and *DHCR7* genotypes. To convert 25(OH)D levels into nM/l, multiply by 2.496.

		Values		P value	P value ^a
VDR Cdx-2					
Genotypes	GG	GA	AA		
Fasting insulin (μ U/ml)	6.4 (4.0–11.1)	6.3 (3.3–9.5)	5.2 (2.9–7.7)	0.039 ^b	0.045 ^b
AUCins	64.7 (40.8–88.8)	57.4 (36.8–95.6)	38.8 (28.9–72.2)	0.061	0.251
HOMA-IR	1.37 (0.86–2.55)	1.27 (0.65–2.1)	1.09 (0.55–1.75)	0.041 ^b	0.052
HOMA- β	108.3 (64.5–174.6)	100.6 (73.8–173.8)	70.1 (57.0–120.3)	0.063	0.061
QUICKI	0.36 (0.33–0.39)	0.37 (0.34–0.41)	0.38 (0.35–0.43)	0.014 ^b	0.031 ^b
MATSUDA	7.79 (4.71–11.0)	7.85 (4.9–14.1)	10.59 (6.24–19.7)	0.003 ^b	0.015 ^b
GC					
Genotypes	TT	TG	GG		
Weight (kg)	65 (57–78)	70 (60–83)	68 (58–81)	0.008 ^c	0.023 ^c
Height (cm)	165 (162–170)	168 (163–171)	165 (161–170)	0.027 ^c	0.021 ^c
BMI (kg/m^2)	23.3 (20.7–28.0)	25.3 (21.6–29.9)	24.6 (20.8–30.9)	0.049 ^c	0.056 ^d
WC (cm)	79 (72–90)	81 (73–96)	83 (74–101)	0.047 ^e	0.030 ^{d,e}
HC (cm)	103 (95–111)	105 (99–114)	106 (96–115)	0.094	NA
WHR	0.77 (0.74–0.83)	0.78 (0.73–0.87)	0.82 (0.74–0.90)	0.065	NA
TC (mg/dl)	182 (158–204)	174 (155–197)	171 (152–152)	0.008 ^e	0.008 ^e
HDL (mg/dl)	67 (56–79)	60 (51–76)	68 (62–76)	0.005 ^{c,f}	0.030 ^{c,f}
QChol/HDL	2.70 (2.10–3.50)	2.80 (2.35–3.4)	2.50 (2.10–3.00)	0.017 ^f	0.011 ^f
AUCins	56.8 (35.9–88.5)	65.3 (47.9–100.6)	48.6 (36.9–36.9)	0.048 ^f	0.380 ^f
DHCR7					
Genotypes	TT	TG	GG		
BMI (kg/m^2)	23.80 (20.55–27.7)	24.77 (21.88–30.11)	23.71 (20.96–28.2)	0.073	NA
HC (cm)	102 (96–110)	105 (99–116)	103 (96–112)	0.025 ^c	0.071
Fasting glucose (mg/dl)	85 (79–90)	88 (82–94)	87 (81–90)	0.036 ^c	0.439
Glucose 2 h (mg/dl)	94 (81–108)	103 (88–122)	100 (86–123)	0.010 ^c	0.057
Fasting insulin (μ U/ml)	6.0 (3.4–9.3)	7.0 (4.6–12.5)	6.0 (5.0–10.7)	0.027 ^c	0.305
Insulin 2 h (μ U/ml)	28.5 (19.0–56.6)	38.8 (24.0–65.4)	47.0 (29.3–67.1)	0.006 ^c	0.049 ^c
HOMA-IR	1.23 (0.73–1.95)	1.62 (0.93–2.81)	1.46 (1.05–2.38)	0.020 ^c	0.195
MATSUDA	9.09 (5.80–13.77)	6.91 (4.00–10.38)	6.71 (4.43–12.3)	0.006 ^c	0.241
QUICKI	0.37 (0.35–0.40)	0.36 (0.33–0.39)	0.36 (0.34–0.38)	0.039 ^c	0.234
Phosphate (mmol/l)	1.09 (0.94–1.19)	1.03 (0.89–1.14)	1.05 (0.96–1.17)	0.022 ^c	0.085
TC (mg/dl)	178 (159–202)	177 (154–199)	174 (155–201)	0.029 ^e	0.018 ^e
TG (mg/dl)	76 (55–96)	82 (58–121)	63 (56–100)	0.024 ^c	0.109

^aAdjusted for age, BMI, 25(OH)D, month of blood sampling, serum calcium.

^bSignificant for GG vs AA.

^cTT vs TG.

^dAdjusted for age, 25(OH)D, month of blood sampling, serum calcium.

^eTT vs GG.

^fTG vs GG.

In multivariate-adjusted analyses, the results remained materially unchanged for fasting insulin, QUICKI, and MATSUDA but were attenuated for HOMA-IR.

We did not observe an association of *Cdx2* polymorphism with endocrine parameters or 25(OH)D levels.

VDR *Apa-I*

In PCOS women, the androgen levels were different according to VDR *Apa-I* genotypes. In detail, the testosterone ($P=0.028$) and androstenedione ($P=0.062$) levels were higher in women carrying the AA allele compared with PCOS women with the CC genotype (0.69 (0.51–0.82) ng/ml versus 0.59 (0.46–0.75) ng/ml and 3.0 (2.2–4.6) ng/ml versus 2.4 (1.7–3.5) ng/ml for testosterone and androstenedione respectively). In age and BMI-adjusted analyses, the results were attenuated ($P=0.052$ and $P=0.087$ for testosterone and androstenedione respectively). After further adjustment for 25(OH)D, calcium, and month of blood sampling, the P values were 0.128 and 0.150 for testosterone and androstenedione respectively.

We did not observe an association of *Apa-I* polymorphism with metabolic parameters or 25(OH)D levels.

VDR *Bsm-I*, *Fok-I*, and *Taq-I*

There was no association of VDR *Bsm-I*, *Fok-I*, and *Taq-I* polymorphisms with anthropometric, endocrine, or metabolic parameters.

GC

We observed a significant association of GC polymorphism with 25(OH)D levels in crude (Fig. 1a) as well as in multivariate-adjusted (age and month of blood sampling; $P=0.049$) analyses. PCOS women carrying the GG genotype had significantly higher risk for vitamin D deficiency than women carrying the TT genotype in crude (OR 2.98 (1.54–5.77), $P=0.001$) as well as in multivariate-adjusted analyses (Fig. 1c). In binary logistic regression analyses calculating ORs for combined vitamin D insufficiency and deficiency (25(OH)D levels <30 ng/ml), we found a significant higher risk in PCOS women carrying the GG allele compared with TT carriers in the crude model (OR 2.47 (1.12–5.47); $P=0.026$) as well as in the multivariate analysis adjusting for age, BMI, and month of blood sampling (OR 2.36 (1.03–5.39); $P=0.042$).

Moreover, the weight, height, BMI, WC, TC, HDL, QChol/HDL, and AUCins levels were significantly different within GC genotypes (Table 2). The association of GC polymorphism with weight, height, WC, TC, HDL, and QChol/HDL remained significant in multivariate-adjusted analyses (age, BMI, 25(OH)D, month of blood sampling, and serum calcium).

Figure 1 25(OH)D levels and ORs for vitamin D deficiency (<20 ng/ml) according to GC and DHCR7 genotypes in PCOS women. (a) 25(OH)D levels in PCOS women according to GC polymorphism (27.6 (20.0–35.7) ng/ml in TT, 25.9 (17.1–34.8) ng/ml in TG, and 20.1 (16.1–25.6) ng/ml in GG carriers; $P=0.027$ for TT versus GG). To convert 25(OH)D levels into nM/l, multiply by 2.496. (b) 25(OH)D levels in PCOS women according to DHCR7 polymorphism (26.5 (19.2–36.5) ng/ml in TT, 25.7 (17.5–33.3) ng/ml in TG, and 17.1 (13.3–30.8) ng/ml in GG carriers; $P=0.006$ for TT versus GG). To convert 25(OH)D levels into nM/l, multiply by 2.496. (c) ORs for vitamin D deficiency (<20 ng/ml) according to GC genotype (TT reference). OR 2.53 (1.27–5.06), $P=0.009$, adjusted for age, BMI, and month of blood sampling. (d) ORs for vitamin D deficiency (<20 ng/ml) according to DHCR7 genotype (TT reference). OR 2.66 (1.08–6.55), $P=0.033$, adjusted for age, BMI, and month of blood sampling.

DHCR7

We found a significant association of *DHCR7* variants with 25(OH)D levels in crude (Fig. 1b) as well as in multivariate-adjusted analyses (adjusted for age, BMI, and month of blood sampling, $P=0.035$). In binary logistic regression analyses, PCOS women carrying the GG genotype had significantly higher risk for vitamin D deficiency than women carrying the TT genotype in crude (OR 3.27 (1.45–7.39), $P=0.004$) as well as in multivariate-adjusted analyses (Fig. 1d). In binary logistic regression analyses calculating ORs for combined vitamin D insufficiency and deficiency (25(OH)D levels < 30 ng/ml), we found no significant association with *DHCR7* genotype.

Moreover, the hip circumference, fasting glucose, glucose 2 h, fasting insulin, insulin 2 h, TC, TG, HOMA-IR, MATSUDA, QUICKI, and serum phosphate levels were significantly different within *DHCR7* genotypes (Table 2).

In multivariate-adjusted analyses (age, BMI, month of blood sampling, serum calcium, and 25(OH)D), the association of *DHCR7* with 2 h insulin and TC remained significant, whereas the other association lost significance.

CYP2R1

The 25(OH)D levels were similar within the genotypes (28.1 (20.0–39.4) ng/ml, 25.2 (18.3–32.2) ng/ml, and 25.2 (17.4–34.6) for AA, AG, and GG respectively, $P=0.412$). In binary logistic regression analyses, we observed a trend toward an increased risk for vitamin D deficiency in PCOS women carrying the GG genotype compared with PCOS women carrying the AA genotype in multivariate-adjusted analyses (OR 2.18 (0.97–4.92), $P=0.059$). In binary logistic regression analyses calculating ORs for combined vitamin D insufficiency and deficiency (25(OH)D levels < 30 ng/ml), we found no significant association with *CYP2R1* genotype.

We found a significant association of the *CYP2R1* polymorphism with 30 min glucose and AUC glucose levels ($P<0.005$ for both).

Combined analyses

We performed additional analyses to assess the combined effect of the three variants influencing the risk for vitamin D deficiency (GC, *DHCR7*, and *CYP2R1*). As only one woman presented with all the three risk genotypes, we combined PCOS women with two (8.3% of all women) and three risk genotypes. ORs for vitamin D deficiency were 1.68 (1.01–2.81) ($P=0.047$) and 2.66 (1.12–6.32) ($P=0.026$) for PCOS women carrying 1 or ≥ 2 risk genotypes respectively.

Discussion

We present evidence that *VDR* and vitamin D level-related genetic variants are associated with metabolic and endocrine parameters including 25(OH)D levels in PCOS women. In detail, variants in the *VDR Cdx2* and *DHCR7* genes are associated with IR and insulin sensitivity and *VDR Apa-I* variants are associated with testosterone levels in PCOS women. We did not observe any association of vitamin D-related polymorphisms and PCOS susceptibility. However, we found an independent association of GC and *DHCR7* polymorphisms with vitamin D deficiency in PCOS women.

Polymorphisms of the *VDR* gene might be associated with PCOS and biochemical markers related to PCOS (11, 12). These studies involved the analyses of variants that are located at the 3'-end of the *VDR* gene such as the *VDR Bsm-I*, *Apa-I*, and *Taq-I*. However, those variants are not likely to influence the function of the *VDR* itself because they are in an intron or do not change amino acid sequence (18). In contrast, *CDX2* acts as a transcription factor of *VDR* (19) and *VDR* regulates the transcription of about 3% of the human genome (9). Yamamoto *et al.* (20) first described a functional binding site for the intestinal-specific transcription factor *CDX2* in the 1a promoter region of the *VDR* gene. The G to A substitution, which was investigated in our study, was first described by Arai *et al.* (19) and was found to modulate the intestine-specific transcription of the *VDR* gene. The *CDX2* protein binds more specifically to the A-allele and leads to increased transcription of the *VDR* gene. Of note, the *VDR* itself is a transcription factor and regulates the transcription of other downstream genes in many tissues including genes that are crucial for glucose metabolism (9, 10).

Whereas the *VDR Cdx2* polymorphism has been shown to be associated with BMD and fracture risk (18), the association with metabolic parameters has not been investigated so far. It can be hypothesized that *VDR Cdx2* A-allele carriers have higher intestinal calcium absorption, because of the elevated expression of intestinal calcium channel proteins (21). Calcium fluxes and regulation of intracellular calcium stores are essential in the regulation of insulin secretion by the β -cells. This makes vitamin D, the central hormone in calcium regulation, as well *VDR* genes a candidate for influencing β -cell function. This notion is further supported by the fact that low vitamin D levels are associated with IR and type 2 diabetes in PCOS (6, 7) as well as in other cohorts (10). The exact mechanisms underlying the association of vitamin D and IR are not fully understood. First, vitamin D may have a beneficial effect on insulin action by stimulating the expression of insulin receptor and thereby enhancing insulin responsiveness for glucose transport (10). The vitamin D-responsive element is present in the promoter of the human insulin gene (22) and the transcription of the

human insulin gene is activated by 1,25(OH)₂D (23). Secondly, vitamin D regulates extracellular and intracellular calcium that is essential for insulin-mediated intracellular processes in insulin-responsive tissues such as skeletal muscle and adipose tissue (10). Moreover, alterations in calcium flux can have adverse effects on insulin secretion, which is a calcium-dependent process (24). Finally, as vitamin D has a modulating effect on the immune system (25), hypovitaminosis D might induce a higher inflammatory response, which is again associated with IR (26).

Interestingly, Mahmoudi *et al.* (11) report an association of *VDR Apa-I* polymorphism with PCOS status in a small cohort. In our cohort, no association was found with PCOS susceptibility. In particular, we observed an association of *VDR Apa-I* with androgen levels in crude analyses. However, these association lost significance in multivariate-adjusted analyses including 25(OH)D. Previously, we demonstrated a BMI-independent association of 25(OH)D levels with hirsutism score (7) and others (6) showed an association of 25(OH)D and FAI levels in PCOS. Of note, 25(OH)D levels are correlated with androgen levels in men and display a similar seasonal variation (27). Thus, one might speculate on an association of vitamin D with androgens. The underlying mechanisms, however, remain to be explored.

Low 25(OH)D levels have been linked to increased risk for cancer (28), autoimmune disease, diabetes (9), cardiovascular disease (29), and increased mortality (30), indicating the importance of sufficient 25(OH)D levels. Although there is no consensus on optimal levels of 25(OH)D, a level of 20 ng/ml has been suggested to cover the requirements of at least 97.5% of the population according to the latest public health report (31). Others (32), however, consider a 25(OH)D level of 30–44 ng/ml sufficient. In our study, only 34.1% of PCOS women present with 25(OH)D levels >30 ng/ml and 68.8% with 25(OH)D levels >20 ng/ml. In line with the genome-wide association study (13), we observed a strong independent association of GC 'GG' and *DHCR7* 'GG' genotype with risk for vitamin D deficiency. The detection of those alleles might assist in the identification of a subgroup of patients who are at an especially high risk for vitamin D deficiency. Of note, interindividual differences have been reported in response to treatment with identical doses of vitamin D (33). Thus, further studies investigating whether genetic predisposition modifies the response to sun exposure or dietary supplementation are warranted especially in these high-risk PCOS women, who are frequently affected by obesity and metabolic disturbances. Identification of genetic association patterns might pave the way to a more personalized approach to therapy.

The GC gene encodes a multifunctional plasma protein GC, also known as a group-specific component, or vitamin D binding protein (VDBP), a protein that is

synthesized in the liver that binds and transports vitamin D and its metabolites. Apart from its specific sterol binding capacity, VDBP exerts several other important biological functions such as actin scavenging, fatty acid transport, macrophage activation, and chemotaxis (34). We observed an independent association of GC polymorphism with lipids in our PCOS cohort. Of note, a major function of VDBP is the binding of mainly monounsaturated and saturated fatty acids (34). Moreover, we observed an association of GC polymorphism with anthropometric measures that was independent of 25(OH)D levels. The link of obesity with vitamin D has been described in PCOS (7) as well as in other cohorts (35), the relationship with VDBP is, however, less clear. Taes *et al.* (2006) (36) described a positive relationship between VDBP concentrations and BMI in a cohort of elderly men. Overall, the underlying mechanisms of this association are not known so far but deserve further investigation.

DHCR7 encodes the enzyme 7-dehydrocholesterol (7-DHC) reductase, which converts 7-DHC to cholesterol. 7-DHC reductase removes 7-DHC, which is a precursor of 25(OH)D, from the synthetic pathway of vitamin D₃. Rare mutations in *DHCR7* lead to Smith–Lemli–Opitz syndrome. People affected by the syndrome present with reduced activity of 7-DHC reductase, accumulation of 7-DHC, low cholesterol, and many congenital abnormalities (37). In our study, we found an association of *DHCR7* polymorphism with 25(OH)D levels as well as metabolic parameters including glucose, insulin, and lipids. Most of these associations were attenuated in multivariate analyses, which indicates that they are most likely mediated via 25(OH)D levels underlining again the important association of vitamin D with IR and action. Nevertheless, those polymorphisms might be important in risk prediction or risk calculation in PCOS women. One might also speculate that vitamin D supplementation is of especially high importance in certain vitamin D-deficient women with the GG genotype of *DHCR7*.

CYP2R1 encodes a hepatic microsomal enzyme that might be the enzyme responsible for the 25-hydroxylation in the liver. However, many other enzymes with 25-hydroxylase activity have been described (38). Whereas the results from the large genome-wide association study (13) demonstrated a strong association of *CYP2R1* with 25(OH)D levels, we observed a trend without a significant association.

One limitation of our study is the relatively small sample size of our control group, which might limit the power to detect differences in genotype distribution of PCOS and control women. Moreover, we did not measure serum levels of VDBP. Thus, our analyses are restricted to the GC polymorphism and cannot be extended to circulating VDBP concentrations, which might have allowed further insights into the association of GC polymorphism with anthropometric and biochemical parameters. The strength of this study is the

number and precise metabolic characterization of PCOS women by oral glucose tolerance tests.

In summary, variants in the *VDR Cdx2* and *DHCR7* genes are associated with IR and insulin sensitivity and *VDR Apa-I* variants are associated with testosterone levels in PCOS women. Moreover, we confirmed the results from previous genome-wide association studies showing an association of *GC* and *DHCR7* polymorphisms with an increased risk for vitamin D deficiency. Further studies investigating whether genetic predisposition modifies response to vitamin D supplementation 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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