Dissertation

The Role of Vitamin D in Polycystic Ovary Syndrome-

Metabolic, Endocrine and Genetic Aspects

eingereicht von

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zur Erlangung des akademischen Grades

Doktorin der medizinischen Wissenschaft

(Dr. scient. med.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Innere Medizin

unter der Anleitung von

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Graz, am 19.01.2011 _____

Danksagung

Als erster Stelle bedanke ich mich bei meiner Betreuerin und Mentorin Frau Univ.-Prof. Dr. Barbara Obermayer-Pietsch, die bereits meine Diplomarbeit betreut hat und mich sowohl in beruflicher als auch privater Hinsicht immer wunderbar unterstützt hat.

Mein besonderer Dank gebührt auch meinem Velobten David, der mir nicht nur bei dieser Arbeit sondern während meines gesamten Studiums in vielerlei Hinsicht eine Stütze war.

Weiters möchte ich meiner Mutter danken, die mir durch ihre Unterstützung mein Studium und somit auch diese Arbeit ermöglicht hat.

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1 Abstract

Introduction: Women with polycystic ovary syndrome (PCOS) frequently suffer from metabolic disturbances, in particular from insulin resistance. Accumulating evidence suggests that vitamin D deficiency may contribute to the development of insulin resistance. The association of vitamin D with endocrine, metabolic, and genetic aspects in PCOS is largely unknown.

Methods: We performed a cross-sectional study including 206 PCOS women to investigate the association of serum 25-hydroxyvitamin D (25[OH]D) levels with metabolic and endocrine parameters. Moreover, we carried out an intervention study including 57 PCOS women, who received 20.000 IU cholecalciferol weekly for 24 weeks. Further, genotyping of vitamin D receptor (VDR) (*Cdx-2, Bsm-I, Fok-I, Apa-I*, and *Taq-I*), GC, DHCR7, and CYP2R1 polymorphisms was performed in 545 PCOS and 145 control women

Results: *Cross-sectional analyses:* We found a significantly negative correlation between 25(OH)D levels and BMI, weight, waist circumference, hip circumference, WHR, blood pressure, fasting and stimulated glucose, AUCglucose, fasting insulin, homeostatic model assessment-insulin resistance (HOMA-IR), HOMA-β, triglycerides, and QChol/HDL and a positive correlation with Quantitative Insulin-sensitivity Check Index (QUCKI) and HDL ($p<0.05$ for all). In multivariate regression analysis, 25(OH)D and BMI were independent predictors of HOMA-IR and QUICKI ($p<0.05$ for all). PCOS women with impaired glucose tolerance and metabolic syndrome had lower 25(OH)D levels than PCOS women without these features ($p<0.05$ for all). In logistic regression analysis, 25(OH)D (OR 0.86, $p=0.021$) and BMI (OR 1.3, $p<0.001$) were independent predictors of metabolic syndrome in PCOS women. There were no significant associations of 25(OH)D with endocrine parameters in PCOS women.

Intervention study: 46 PCOS women finished the study. 25(OH))D levels significantly increased from 28.0±11.0 ng/ml at baseline to 51.3±17.3 and 52.4±21.5 after 12 and 24 weeks, respectively ($p<0.001$). We observed a significant decrease of fasting and stimulated glucose (24 weeks, $p<0.05$) and C-peptide levels (12 and 24 weeks, $p<0.001$) after vitamin D treatment. Moreover, triglyceride and estradiol levels significantly decreased after 24 weeks

(p=0.001) and 12 weeks (p=0.022), respectively, whereas total cholesterol (12 weeks, p=0.008) and LDL cholesterol levels (12 weeks, p=0.005; 24 weeks, p=0.026) significantly increased after vitamin D treatment. There were no changes in androgens. After 12 weeks, 14 out of 46 PCOS women previously affected by menstrual disturbances (30.4%) reported improvement of menstrual frequency; after 24 weeks, 23 out of 46 women (50.0%), who were oligo- or amenorrhoeic at baseline reported improvement.

Genetics: In PCOS women, the VDR *Cdx-2* "AA" genotype was associated with lower fasting insulin (p=0.039) and HOMA-IR (p=0.041) and higher QUICKI (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) when compared to 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: We demonstrate that low 25(OH)D levels are associated with obesity, insulin resistance, impaired β cell function, impaired glucose tolerance, and metabolic syndrome in PCOS women. Further, our results suggest that vitamin D treatment might improve glucose metabolism and menstrual frequency in PCOS women. Further randomized controlled trails are warranted to confirm our findings. Finally, VDR and vitamin D level associated variants influence metabolic and endocrine parameters including 25(OH)D levels in PCOS women.

2 Zusammenfassung

Einleitung: Frauen mit polycystischem Ovar-Syndrom (PCOS) sind häufig von metabolischen Problemen, vor allem von Insulinresistenz, betroffen. Man vermutet eine Verbindung zwischen Vitamin D-Mangel und der Entwicklung von Insulinresistenz. Der Zusammenhang zwischen Vitamin D und endokrinen, metabolischen und genetischen Aspekten beim PCOS ist hingegen weitgehend unklar.

Methoden: Wir führten eine Querschnittsstudie bei 206 Frauen mit PCOS durch, um den Zusammenhang zwischen 25-Hydroxyvitamin D (25[OH]D)-Spiegeln und metabolischen und endokrinen Parametern zu untersuchen. Weiters führten wir eine Interventionsstudie bei 57 Frauen mit PCOS durch, die über 24 Wochen 20.000 IE Cholecalciferol wöchentlich erhielten. Außerdem erfolgte eine Genotypisierung von Vitamin D Rezeptor (VDR) (*Cdx-2, Bsm-I, Fok-I, Apa-I*, and *Taq-I*), GC, DHCR7, und CYP2R1 Polymorphismen bei 545 PCOS-Frauen und 145 Frauen ohne PCOS.

Ergebnisse: *Querschnittsstudie:* Es bestand eine signifikant negative Korrelation zwischen 25(OH)D-Spiegeln und BMI, Gewicht, Bauchumfang, Hüftumfang, WHR, Blutdruck, nüchtern und stimulierten Glucosewerten, AUCgluc, nüchtern Insulin, homeostatic model assessment (HOMA)-Insulinresistenz, HOMA-β, Triglyceride, und QChol/HDL und eine signifikant positive Korrelation mit dem Quantitative Insulin-sensitivity Check Index (QUCKI) und HDL ($p<0.05$ für alle Parameter). In einer multivariaten Regressionsanalyse waren 25(OH)D und der BMI unabhängige Prädiktoren von HOMA-IR und QUICKI. PCOS Frauen mit Glucosetoleranzstörung und metabolischem Syndrom hatten niedrigere 25(OH)D-Spiegel als Frauen ohne diesen Problemen ($p<0.05$ für beide). In einer logistischen Regressionsanalyse waren sowohl 25(OH)D (OR 0.86, $p=0.021$) als auch der BMI (OR 1.3, $p<0.001$) unabhängige Prädiktoren für das metabolische Syndrom. Es gab keinen signifikanten Zusammenhang zwischen 25(OH)D und endokrinen Parametern.

Interventionsstudie: 46 Frauen mit PCOS beendeten die Studie. 25(OH)D-Spiegel stiegen signifikant von 28.0±11.0 ng/ml zu Beginn der Studie auf 51.3±17.3 ng/ml nach 12 Wochen und 52.4±21.5 ng/ml nach 24 Wochen, ($p<0.001$) an. Nach Vitamin D-Supplementierung kam es zu einem signifikanten Abfall der nüchtern und stimulierten Glucosewerte (24 Wochen,

p<0.05) und C-Peptid-Werte (12 und 24 Wochen, p<0.001). Weiters sanken Triglyceride (24 Wochen, p=0.001) und Östradiol-Spiegel (12 Wochen, p=0.022), wohingegen das Gesamtcholesterin (12 Wochen, p=0.008) und LDL Cholesterin (12 Wochen, p=0.005; 24 Wochen, p=0.026) nach Vitamin D-Gabe anstiegen. Die Androgenspiegel blieben unverändert. Nach 12 wöchiger Vitamin D-Gabe berichteten 14 von 46 anfangs oligo- oder amenorrhoischen Frauen (30.4%) von einer regelmäßigeren Menses, nach 24 wöchiger Vitamin D-Gabe 23 von 46 (50%).

Genetik: Der VDR *Cdx-2* "AA" Genotyp war mit signifikant niedrigeren nüchtern Insulin (p=0.039) und HOMA-IR (p=0.041) und höherem QUICKI (p=0.012) und MATSUDA-index (p=0.003) bei Frauen mit PCOS assoziiert. Der VDR *Apa-I* "AA" Genotyp war mit signifikant niedrigeren Testosteronspiegeln (p=0.028) assoziiert. Von 545 Frauen mit PCOS hatten 170 (31.2%) 25(OH)D-Spiegel <20 ng/ml. In einer multivariaten Anlayse hatten PCOS-Frauen mit dem GC "GG" Genotyp und dem DHCR7 "GG" Genotyp ein signifikant höheres Risiko für 25(OH)D-Spiegel <20 ng/ml (OR 2.53 [1.27-5.06], p=0.009, und OR 2.66 [1.08-6.55], p=0.033) im Vergleich zu PCOS-Frauen mit dem GC "TT" Genotyp und dem DHCR "TT" Genotyp. Wir fanden keinen Zusammenhang zwischen genetischen Polymorphismen und dem Auftreten des PCOS.

Schlussfolgerung: Bei Frauen mit PCOS gibt es einen Zusammenhang zwischen 25(OH)D-Spiegeln und Adipositas, Insulinresistenz, verminderter β-Zellfunktion, gestörter Glucosetoleranz und dem metabolischen Syndrom. Weiters konnten wir zeigen, dass eine Vitamin D-Supplementierung einen positiven Effekt sowohl auf den Glucosestoffwechsel als auch auf endokrine Parameter bei Frauen mit PCOS hat. Darüberhinaus besteht ein Einfluss von VDR und Vitamin D assoziierten Polymorphismen auf metabolische und endokrine Parameter einschließlich 25(OH)D-Spiegel.

3 Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D
7-DHC	7-dehydrocholestererol
ACTH	adrenocorticotropic hormone
AES	Androgen Excess Society
AHA	American Heart Association
ANCOVA	analysis of covariance
AUC	area under the curve
BMI	body mass index
CRP	C-reactive protein
CVD	cardiovascular disease
DHEAS	dehydroepiandrosterone sulphate
GnRH	gonadotropin-releasing hormone
GWAS	genome-wide association study
HDL	high-density lipoprotein
FAI	free androgen index
FSH	follicle-stimulating hormone
fT3	free triiodothyronine
fT4	free thyroxin
HGH	human growth factor

HOMA-IR	homeostatic model assessment-insulin resistance
IGF-1	insulin-like growth factor-1
IL-6	interleukin 6
IU	international unit
IVF	in-vitro fertilization
LDL	low-density lipoprotein
LH	luteinizing hormone
oGTT	oral glucose tolerance test
OR	odds ratio
PCOS	polycystic ovary syndrome
PTH	parathyroid hormone
QChol/HDL	quotient total cholesterol/HDL
QUICKI	Quantitative Insulin-sensitivity Check Index
SD	standard deviation
SHBG	sex hormone–binding globulin
TSH	thyroid stimulation hormone
VDBP	vitamin D-binding protein
VDR	vitamin D receptor
WHR	waist-to-hip ratio

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6 Introduction

6.1 PCOS

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of women of reproductive age affecting about 5-10% of all women (1-3). The disorder that possibly would be known as PCOS was initially characterized by an Italian physician named Antonio Vallisnerie in 1721 (4). He described the presence of polycystic ovaries in overweight women affected by infertility. In 1921, Achard and Thiers described the association of type 2 diabetes with hyperandrogenism (5). The first systematic characterization of the syndrome was done in 1935. Stein and Leventhal published a manuscript reporting on 7 women affected by amenorrhea, hirsutism, obesity and polycystic ovaries (4). Thus, PCOS was long known as „Stein-Leventhal-Syndrome“referring to the first authors who decribed the syndrome.

6.1.1 Definition PCOS

There is little disagreement that PCOS should be considered a syndrome; the precise definition of PCOS is, however, less clear. A clear and contemporaneous definition of the syndrome has important clinical as well as scientific implications.

Clinically, diagnosing a woman as having PCOS implies an increased risk for infertility, dysfunctional bleeding, endometrial carcinoma, obesity, type 2 diabetes mellitus, dyslipidemia, hypertension, and possibly cardiovascular disease (CVD). Furthermore, it has important familial implications, principally, but not exclusively, for her sisters and daughters.

Finally, a diagnosis of PCOS may mandate life-long treatments (e.g., the use of insulin sensitizers). Consequently, the diagnosis of PCOS should not be assigned lightly, and diagnostic criteria should be based on robust data (6).

The most recent definition, that is the Androgen Excess Society (AES) criteria, is described below (see also figure 1 and 2).

6.1.1.1 AES criteria

The AES reviewed all available data and recommend a definition for PCOS based on published peer-reviewed data to guide clinical diagnosis and future research (6). The Task Force stated the following conclusions and recommendations regarding the phenotype of PCOS:

a) PCOS is a hyperandrogenic disorder; the Task Force felt that PCOS was above all a disorder of androgen biosynthesis, utilization, and/or metabolism in women. As such, with currently available evidence the diagnosis of PCOS should not be established without evidence of either clinical or biochemical hyperandrogenism. Although the exact measures for these may vary, the most reliable indices of this feature include hirsutism and free testosterone levels.

b) The ovarian morphology should be considered when establishing the diagnosis, as polycystic ovaries are found in the majority, although not all, women with PCOS: 70% to 90% of women with PCOS demonstrate a polycystic ovarian morphology on ultrasound, although the false positive rate is high with up to one-quarter of unselected reproductive aged women demonstrating this ovarian morphology. The

diagnosis of polycystic ovaries requires the use of clear and strict criteria. Consistent with recommendation a) above, in women with polycystic ovaries, but no evidence of clinical or biochemical hyperandrogenism, the diagnosis of PCOS is less certain, regardless of the presence of concomitant ovulatory dysfunction.

c) Ovulatory dysfunction is a prominent, but not universal feature, of PCOS: some patients with PCOS may demonstrate regular ovulation at the time of their evaluation, the so-called ‘‘ovulatory PCOS’’. However, the patients with ‘‘ovulatory PCOS’’ constitute a minority of the PCOS population, and have less severe androgenic and metabolic features than anovulatory women with PCOS. There exists little data regarding the long-term maintenance of ovulation in women with ovulatory PCOS. Ovulatory function in PCOS often improves as patients near the perimenopause.

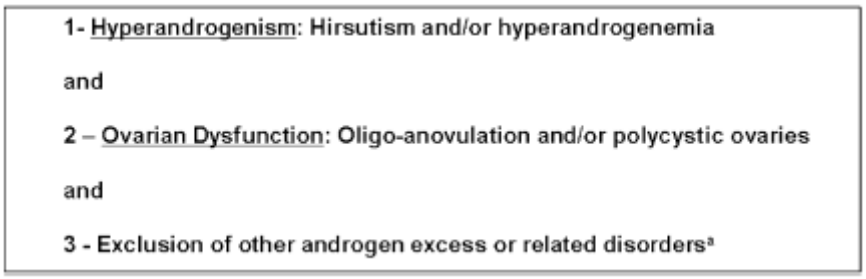
d) Eumenorrhea in the presence of dermatologic features suggestive of hyperandrogenism (e.g. hirsutism) can not reliably be used to establish the presence of normal ovulation: in patients with no clinical signs of hyperandrogenism a history of regular predictable vaginal bleeding could be used as strong evidence of normal ovulation. Alternatively, a history of ‘‘regular’’menstrual cycles in patients who demonstrated hyperandrogenic features (e.g., hirsutism) could not be relied upon as evidence of normal ovulation, with up to 40% of these women having oligo-anovulation. In these patients, confirmation of ovulatory function by more objective means is required.

e) Other well-defined disorders that could result in ovulatory dysfunction, polycystic ovaries, or clinical or biochemical hyperandrogenism have to be excluded: the initial

screening for androgen secreting neoplams and Cushing's syndrome is primarily clinical, and the prevalence of thyroid dysfunction, hyperprolactinemia, or premature ovarian failure among patients with frank hyperandrogenism, or of 21-hydroxylase-deficient non classical congenital adrenal hyperplasia in certain ethnic groups (e.g., those of Anglo-Saxon descent) is relatively low. Consequently, the prevalence of these disorders in the population being studied should be considered and might potentially limit the disorders excluded.

f) Recognition of associated abnormalities: the presence of obesity, insulin resistance, and hyperinsulinism, and increased luteinizing hormone (LH) levels or an LH/follicle-stimulating homone (FSH) ratio, while observed in a significant fraction of patients, should not be used as part of the definition of PCOS

Figure 1: AES criteria for defining PCOS (6)



However, when this study was started (2008) the current definition was the Rotterdam criteria (7) and thus, these criteria were used to select the study population (see also figure 2).

6.1.1.2 Rotterdam Criteria

Two out of three of the following are required to confirm the diagnosis of PCOS: oligo – and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries (by ultrasound). Disorders with a similar clinical presentation, such as congenital adrenal hyperplasia, Cushing's syndrome, and androgen-secreting tumours, must be excluded. Oligo- and/or anovulation are defined by presence of oligomenorrhea or amenorrhea. Hyperandrogenism is defined by the clinical presence of hirsutism (Ferriman Gallwey-Score ≥6), acne or alopecia and/or elevated androgen levels. Polycystic ovarian morphology was examined by ultrasound. Polycystic ovaries were defined as the presence of 12 or more follicles in each ovary measuring 2 to 9 mm in diameter and/or increased ovarian volume (>10 mL; calculated using the formula 0.5 x length x width x thickness). Hyperprolactinemia, Cushing's syndrome, congenital adrenal hyperplasia and androgen secreting tumors were excluded by specific laboratory analysis (cortisol, adrenocorticotropic hormone [ACTH], 17αOH-progesterone, dehydroepiandrosterone sulfate [DHEAS]).

However, clinical features may not be constant even in a single patient affected by PCOS and can be modified by changes in body weight and lifestyle choices. Moreover, there may be a number of women who have features suggestive of PCOS, but who do not fulfill the criteria; clearly, these women and their symptoms should be treated accordingly, regardless of whether a diagnosis of PCOS is established or not.

Figure 2: All possible phenotypes of PCOS based on the presence or absence of oligo- or anovulation, hyperandrogenemia, hirsutism and polycystic ovaries (6)

	Potential Phenotypes															
Features	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Hyperandrogenemia	+	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−
Hirsutism	+	+	−	−	+	+	+	+	−	−	+	−	−	+	−	−
Oligo- oder anovulation	+	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−
Polycystic ovaries	+	−	+	−	+	−	+	+	+	+	−	+	−	−	−	−
NIH 1990 criteria	√	√	√	√	√	√										
Rotterdam 2003 criteria	√	√	√	√	√	√	√	√	√	√						
AE-PCOS 2006 criteria	√	√	√	√	√	√	√	√	√							

6.1.2 Pathogenesis

No single etiologic factor fully accounts for the spectrum of abnormalities in PCOS. But there is a complex interaction of various factors contributing to the pathogenesis of PCOS.

6.1.2.1 Gonadotropins

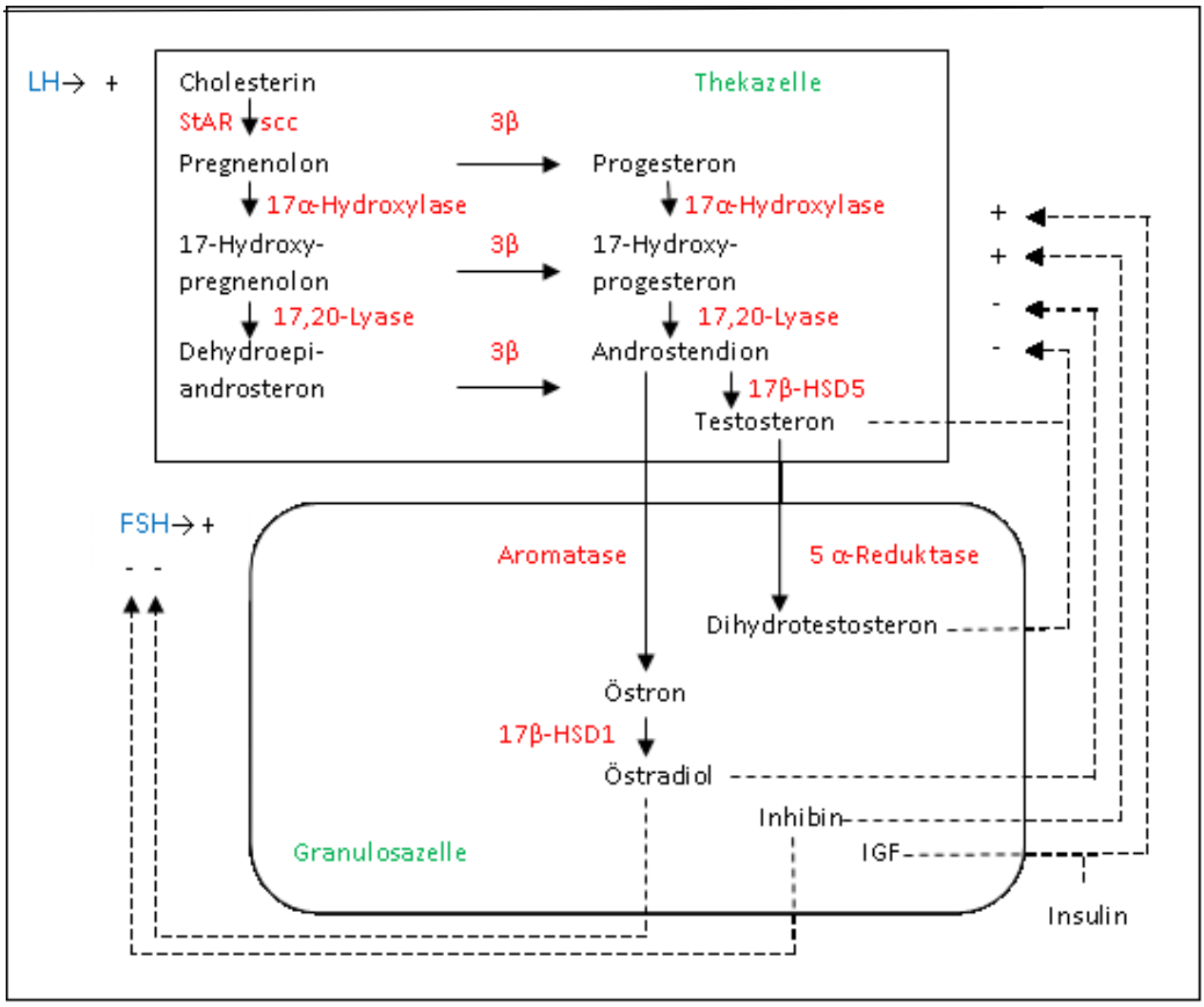
The pathogenesis of PCOS is multifactorial and depends on the biosynthesis of steroid hormones. Triggered by the LH from the pituitary gland, ovarian theca cells produce androgens (figure 3): cytochrome P-450c17 (an enzyme with 17α-hydroxylase and 17,20-lyase-activity) induces the production of androstenedione. Androstenedione is metabolized into testosterone by 17β-hydroxysteroid dehydrogenase or into estron by aromatase. In PCOS patients, this metabolic step is in favor of testosterone production. The FSH from the pituitary gland regulates aromatase-activity in granulosa cells and thereby the production of estrogens.

Increased LH levels, compared to the FSH, result in increased androgen production in the ovaries. The Gonadotropin-releasing hormone (GnRH) dominates LH/FSH secretion in the pituitary. High GnRH expression increases LH and low GnRH increases FSH synthesis. In PCOS patients the increased LH/FSH ratio relates to a high GnRH excretion, but it is still uncertain if an intrinsic malfunction of the GnRH pulse generator or relatively low levels of progesterone, as a result of anovulatory menstrual cycles, are the underlying cause (figure 3) (4). High GnRH expression could also be caused by low pituitary progesterone sensitivity, due to low circulating androgens. This is supported by the fact that progesterone sensitivity can be increased by the therapeutic use of flutamide, an androgen receptor antagonist (8).

6.1.2.2 Insulin

Insulin plays both a direct as well as an indirect role in the pathogenesis of hyperandrogenemia in PCOS (figure 3). Insulin acts synergistically with LH to enhance the androgen production of ovarian theca cells. Moreover, insulin inhibits hepatic synthesis of sex hormone–binding globulin (SHBG), the key circulating protein that binds to testosterone, and thus increases the proportion of testosterone that circulates in the unbound, biologically available state. Because women with PCOS typically have hyperinsulinemia, the concentration of free testosterone is often elevated with total testosterone concentration being at the upper range of normal or only modestly elevated (4).

Figure 3: Pathogenesis of PCOS (modified, 9)



6.1.3 Genetics

Several lines of evidence suggest that PCOS is heritable. Its inherited basis was established by studies demonstrating an increased prevalence of PCOS and hyperandrogenemia, insulin resistance, and disordered insulin secretion in relatives of women with PCOS (10). Current understanding of the pathogenesis of the syndrome suggests, however, that it is a complex

multigenic disorder. Candidate genes chosen from logical pathways that may regulate the hypothalamic–pituitary–ovarian axis, as well as those responsible for insulin resistance and its sequelae, have been the principal focus of linkage and case–control studies. Whereas several positive results have been reported, no genes are universally accepted as important in PCOS pathogenesis, largely due to lack of replication of positive results. This is caused, in part, by various factors, most importantly the lack of a universally accepted diagnostic scheme for PCOS, the ability to assign PCOS diagnosis only in women of reproductive age, inadequate coverage of genes by the analysis of only one or two variants, and of small case-control cohorts in most studies. Moreover, candidate gene selection has been limited by our incomplete knowledge of the pathophysiology of PCOS. In Microarray analyses of target tissues in the polycystic ovary syndrome have been used to identify novel candidate genes involved in the condition, and a number of them appear to contribute modestly to the phenotype. In the future, strict and uniform diagnostic criteria, improved application of the candidate gene approach using haplotype-based analyses, intermediate phenotypes, replication of positive results in large cohorts, more family-based studies, gene selection from expression studies, and whole-genome approaches will enhance gene discovery in PCOS (10). Recently, a genome-wide association study (GWAS) of PCOS was conducted in Han Chinese women (11). The discovery set included 744 PCOS cases and 895 controls; subsequent replications involved two independent cohorts (2,840 PCOS cases and 5,012 controls from northern Han Chinese; 498 cases and 780 controls from southern and central Han Chinese). The authors found evidence of associations between PCOS and three loci. Despite this promising approach, a lot of work remains to do to further elucidate the underlying genetic causes of this highly prevalent disorder.

6.1.4 Metabolic disturbances

6.1.4.1 Impaired glucose tolerance

Insulin resistance occurs in approximately 60–80% of women with PCOS and in 95% of obese women with PCOS (12). In most classic cases of PCOS, insulin-mediated glucose uptake is impaired, but the precise underlying mechanism remains elusive. These women with PCOS have insulin resistance independent and additive with that of obesity, with PCOS and obesity acting synergistically to impair insulin sensitivity. Consequently, impaired glucose tolerance and metabolic syndrome, as predictors of type 2 diabetes and premature CVD mortality, are common findings in PCOS women with an odds ratio of approximately 4:1. Alarmingly, impaired glucose tolerance and type 2 diabetes are highly prevalent among PCOS adolescents, and up to 40% of women with classic PCOS develop impaired glucose tolerance or type 2 diabetes by the fourth decade of life, with age and weight gain worsening glycemic control. In one study, classic PCOS patients had a 5-fold risk of developing type 2 diabetes over 8 yr vs. age- and weight-matched controls, although only 12% of PCOS patients without obesity developed glucose abnormalities (12).

6.1.4.2 Dyslipidemia

Dyslipidemia is very common in PCOS patients (up to 70% in the United States) and may present with different patterns, including low levels of high-density lipoprotein (HDL)-cholesterol, increased values of triglycerides and total and low-density lipoprotein (LDL)-cholesterol, as well as altered LDL quality. These different patterns may be related to the

associated effects ofinsulin resistance and hyperandrogenism that combine with environmental (diet, physical exercise) and genetic factors.

The most common pattern is probably the so-called atherogenic lipoprotein phenotype that is characterized by hypertriglyceridemia, increased small dense LDL levels, and decreased HDL levels. This lipid pattern is similar to that found in type 2 diabetes, and it is mainly the consequence of insulin reistance that impairs the ability of insulin to suppress lipolysis, thereby increasing mobilization of free fatty acids from adipose stores. Consequently, increased hepatic delivery of free fatty acids impairs insulin inhibition of hepatic very low-density lipoprotein 1 synthesis, causing altered catabolism of very low-density lipoprotein. Because excessive adipose tissue increases insulin resistance, this pattern is more common in obese patients with PCOS. It occurs in the United States in about 70% of women with classic PCOS but is less common in other countries where mean bodyweight is lower (12).

6.1.5 Depression, anxiety, and reduced quality of life

There is growing evidence that mood disturbances, mostly severe depression, are independent CVD risk factors (18) and prevalent in women with PCOS. The presence of clinically significant eating disorders and a 7-fold increase in the suicide rate have been reported in women with PCOS (13). A recent meta-analyses investigating the association of PCOS with depression revealed that the odds ratio for abnormal depression scores was 4.03 in women with PCOS compared with those in the control groups (14). A subanalysis showed that the odds ratio for abnormal depression scores was independent of body mass index (BMI). Several studies show increased depression and anxiety in PCOS patients, in whom impaired

quality of life from body image concerns cause fatigue, sleep disturbances, phobia, appetite changes, and binge eating. As a result, depressed women with PCOS have higher BMI and greater insulin resistance as CVD risk factors than nondepressed women with PCOS without differences in androgen excess, whereas weight loss through an energy-restricted diet improves their depression and quality of life. It remains to be determined how mood disturbances as CVD risk factors are linked with altered stress reactivity in women with PCOS, as evidenced by exaggerated ACTH and cortisol stress responses, impaired IL-6 up-regulation after stress, and heightened sympathetic nerve activity (12).

6.1.6 Assessment of CVD risk in PCOS

Because lifetime risk for CVD in all women is high and mostly preventable, screening all women for CVD risk factors is recommended. Evidence-based guidelines for women provided by the American Heart Association (AHA) classify women for CVD risk as: 1) optimal risk; 2) at risk; or 3) at high risk.

Recognizing that PCOS women with increased obesity are at greater risk for type 2 diabetes, stroke, and CVD, the AES recommends that PCOS-related CVD risk be categorized as „at risk“ and „at high risk“ (see table 1):

Table 1: Classification of PCOS-related CVD risk

At risk—PCOS women with:
• Obesity (especially increased abdominal adiposity)
• Cigarette smoking
• Hypertension
• Dyslipidemia (increased LDL-C and/or non-HDL-C)
• Subclinical vascular disease
• Impaired glucose tolerance
• Family history of premature CVD (<55 yr of age in male relative, <65 yr of age in female relative)
At high risk—PCOS women with:
• Metabolic syndrome
• Type 2 diabetes
• Overt vascular or renal disease

Given the high CVD risk of PCOS women, all women with PCOS should be assessed for CVD risk including anthropometric (BMI, waist circumference, blood pressure) and biochemical measures (lipids, oral glucose tolerance test):

1. Waist circumference/BMI

   Waist circumference and BMI should be determined at every visit. A waist circumference of at least 88 cm in Caucasian/African-American women or at least 80

cm in Hispanic, Native American, Asian (East and South), and European women is the easiest way to establish the presence of abdominal obesity.

2. Lipids

   A complete lipid profile (total cholesterol, LDL, non-HDL cholesterol, HDL, and triglycerides) should be determined. Based upon AHA guidelines, if the fasting serum lipid profile is normal, it should be reassessed every 2 years or sooner if weight gain occurs. The primary target goal is LDL, with non-HDL cholesterol estimating numbers of circulating small and large LDL particles as the secondary target for lifestyle and medical therapy. In women with PCOS without additional CVD risk factors, LDL levels should be less than 130 mg/dl. Those with the metabolic syndrome or type 2 diabetes/overt vascular/renal disease should have serum LDL levels less than 70–100 mg/dl or 70 mg/dl, respectively. Optimal (target) serum non-HDL cholesterol levels should be 30 mg/dl higher than the designated LDL goal; serum triglyceride levels, as an independent CVD risk factor in women, should be less than 150 mg/dl. Routine use of LDL subfractions appears premature because it is unclear whether there is added benefit to traditional risk factor assessment. Routine apolipoprotein B testing is not recommended until it becomes universally standardized, although apolipoprotein B levels may bemore accurate than non-HDL as a CVD risk predictor.

3. Oral glucose tolerance test

   A 2-h post 75-g oral glucose challenge should be performed in PCOS women with a BMI greater than 30 kg/m^2,or alternatively in lean PCOS women with advanced age (>40 years), personal history of gestational diabetes, or family history of type 2

diabetes. This outpatient test appears to be the best screening tool to detect impaired glucose tolerance or type 2 diabetes in women with PCOS, who often have a normal fasting glucose. Patients with normal glucose tolerance should be rescreened every 2 years or sooner if additional risk factors are identified. Those with impaired glucose tolerance should be screened annually for developing type 2 diabetes, acknowledging efficacy of treating impaired glucose tolerance, but not necessarily impaired fasting glucose, to prevent type 2 diabetes. HemoglobinA1c above 6.5% has been proposed as the defining criterion for diabetes. The AES endorses this criterion for risk assessment, but further studies will be needed to determine whether this criterion is useful in implementing lifestyle interventions and medical management for CVD prevention.

4. Blood pressure

   Blood pressure should be routinely checked at each visit. Ideal blood pressure is 120 mm Hg systolic and 80 mm Hg diastolic or lower, and prehypertension should be detected and treated. Blood pressure control has the largest benefit for reducing CVD.

6.1.7 Treatment

Lifestyle intervention, insulin sensitizers, and oral contraceptives are the most common therapeutic approaches for the management of PCOS. Their application depends on the individual therapeutic aim as well as on side effects.

6.1.7.1 Lifestyle modification

Lifestyle modification is the first-line therapy for safety, public health benefit, and avoidance of risks and side effects of drug use. For overweight/obese women with PCOS, lifestyle modification, including diet, exercise, smoking cessation, and behavioral techniques, can be used to reduce CVD risk. It has been shown that short-term weight-loss intervention in PCOS women decreases abdominal fat and reduces androgenicity and insulin resistance; it also improves dyslipidemia, depression, and quality of life, although long-term weight loss is unlikely. In two large intervention studies, almost 60% relative risk reduction in conversion to diabetes mellitus occurred in obese individuals (BMI >30 kg/m^2) with impaired glucose tolerance randomized to intensive lifestyle modification to reduce body weight by 5–7%; less dramatic improvement occurred in less obese individuals (BMI, 25–30 kg/m^2). A hypocaloric, low saturated fat, increased mono- and polyunsaturated fat diet (500–1000 kcal/d reduction;<30% calories from fat, <10% calories from saturated fat; increased consumption of fiber, whole-grain breads, cereals, fruits, and vegetables) is recommended, along with at least 30 min of moderate-intensity physical activity daily to maintain weight. Together, both reduce BMI and improve insulin resistance and cardiopulmonary function in overweight PCOS women and provide greater reductions in fat mass in PCOS women.

Overweight/obese PCOS women should initially attempt 5–10% weight loss to reduce obesity-related CVD risk factors, with long-term goals of achieving and maintaining reduced weight of 10 to 20% and a waist circumference of less than 88–80 cm, depending upon ethnicity.

6.1.7.2 Insulin sensitizers

Insulin lowering agents allow increased SHBG production and decrease ovarian testosterone production. Data regarding the effects of metformin on primary prevention of CVD are not consistent. Metformin has a small effect on body weight (less than 2–3% of BMI) and may improve atherogenic dyslipidemia, increasing HDL and decreasing triglycerides. However, in some studies, no changes in HDL or triglycerides were observed. Metformin does not improve LDL or non-HDL and should not be used when these lipid parameters are elevated. Metformin decreases circulating C-reactive protein (CRP) and may improve subclinical atherosclerosis, reduce carotid intima-media-thickness and improve endothelial function. The addition of metformin to lifestyle modification may be considered in those women who have metabolic syndrome or subclinical atherosclerotic CVD, although more studies are needed to confirm this benefit. Medical treatment is not recommended for women with PCOS who have isolated hypertriglyceridemia and/or decreased HDL but do not have metabolic syndrome or for those who are not considered to be at risk or at high risk (see table 1). Metformin should only be used in women with PCOS who are already undergoing lifestyle treatment and do not have improvement in impaired glucose tolerance and in those women with impaired glucose tolerance who are of normal weight, where weight loss in not appropriate. Although combined metformin/lifestyle modification to prevent type 2 diabetes in PCOS has not been established prospectively, small studies show that almost 30% of reproductive-aged women with PCOS have impaired glucose tolerance, of whom one half revert to normal glucose tolerance with metformin. Improved glucose tolerance also has been shown in nonobese women with PCOS with impaired glucose tolerance receiving thiazolidinediones, although increased bone fracture risk and exacerbation of preexisting congestive heart failure limit thiazolidinedion use

for primary CVD prevention. Whether metformin should be used in PCOS to prevent conversion of normal glucose tolerance to impaired glucose tolerance is debatable.

6.1.7.3 Oral contraceptives

Estrogen–gestagen combinations are preferably used in the treatment of hirsutism and acne. Estrogens lower LH levels resulting in decreased androgen production. Moreover, estrogens increase hepatic SHBG production resulting in decreased levels of free testosterone. Of note, oral contraceptives containing an anti-androgenic gestagens should be used in PCOS treatment, as some gestagens may be crucial, as they may feature androgenetic activity.

While oral contraceptives exerts beneficial effects with respect to hirsutism, acne and amenorrhoea, there is evidence showing an adverse effect of these drugs on insulin resistance, glucose tolerance, as well as vascular function (12).

6.1.7.4 Vitamin D

As vitamin D deficiency is related to metabolic disturbances including insulin resistance and type 2 diabetes, it has been hypothesized that vitamin D supplementation might be an interesting, cheap and safe therapeutic approach in PCOS. However, evidence regarding vitamin D treatment in PCOS is sparse.

6.2 Vitamin D

6.2.1 Vitamin D metabolism

Vitamin D is a steroid hormone. The vitamin D precursor, 7-dehydrocholesterol is a normal intermediary in the cholesterol pathway and is present in the skin (15). UV-B radiation induces conversion of 7-dehydrocholesterol to provitamin D3, which spontaneously isomerizes to vitamin D3 (cholecalciferol) (15). Vitamin D3 is released into circulation and transported by the vitamin D-binding protein (VDBP). Approximately 80-90% derives from sunlight-induced (solar ultraviolet B radiation; wavelength, 290 to 315 nm) production in the skin. There is also a small amount of the body's total vitamin D derived from diet and or supplements. This may derive from plants or fungi containing vitamin D2 (ergolcalciferol) or fatty fish or cod-liver oil containing vitamin D3 (cholecalciferol) (15). Vitamin D from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D (25[OH]D), which is used to determine a patient`s vitamin D status into vitamin D sufficient (25[OH]D ≥30 ng/ml), vitamin D insufficient (25[OH]D 20-29 ng/ml), and vitamin D deficient (25[OH]D <20 ng/ml] (15). 25(OH)D is metabolized in the kidneys by the enzyme 1α-hydroxylase to its active form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The enzyme 1α-hydroxylase is also found in many other tissues allowing the local conversion of 25(OH)D to the active $1,25(OH)_2D_3$.

Biological actions of vitamin D are mediated through the vitamin D receptor (VDR) that is distributed across various tissues including skeleton, parathyroid glands, as well as reproductive tissues (15, 16). Vitamin D binds to the nuclear VDR, which then heterodimerizes with the retinoid X receptor. This in turn binds to the vitamin D responsive element located in the promoter regions of the target genes (17). The VDR interacts with

other transcription factors such as co-activator proteins and transcription integrators such as calcium-binding proteins (18). This genomic pathway leading to changes in gene transcription takes hours to days (19). Another pathway is the interaction with a cell surface receptor and second messengers, leading to a more rapid response taking seconds to minutes (15, 19). Catabolization of $1,25(OH)_2D_3$ and 25(OH)D to biologically inactive calcitroic acid is catalyzed by 24-hydroxylase (15).

6.2.2 Vitamin D and insulin resistance

The growing incidence of prediabetes and clinical type 2 diabetes, in part characterised by insulin resistance, is a critical health problem with consequent devastating personal and health-care costs. Vitamin D status, assessed by serum 25(OH)D levels, is inversely associated with diabetes in epidemiological studies. Several clinical intervention studies also support that vitamin D, or its active metabolite $1,25(OH)_2D_3$, improves insulin sensitivity, even in subjects with normal glucose tolerance. The mechanisms proposed which may underlie this effect include potential relationships with improvements in lean mass, regulation of insulin release, altered insulin receptor expression and specific effects on insulin action. These actions may be mediated by systemic or local production of $1,25(OH)_2D_3$ or by suppression of parathyroid hormone, which may function to negatively affect insulin sensitivity. Thus, substantial evidence supports a relationship between vitamin D status and insulin sensitivity; however, the underlying mechanisms require further exploration (20).

6.2.3 Vitamin D and fertility

There is accumulating evidence, that in addition to sex steroid hormones, the classic regulators of reproduction, vitamin D also modulates reproductive processes in women; its nuclear receptor has been identified in the uterus, oviduct, ovary, placenta, and fetal membranes (21). It has been shown that vitamin D deficiency reduces mating success and fertility in female rats (22); female rats fed a vitamin D deficient diet are capable of reproduction, but overall fertility is decreased by 75%, and litter size is reduced by 10% (22). Both VDR and 1α-hydroxylase knockout female mice are infertile and present with uterine hypoplasia, impaired folliculogenesis, and anovulation (21, 23, 24). It is notable that male fertility is also reduced, as demonstrated by reduced sperm number and mobility, and histological changes in testicular morphology (16).

However, there might be various mechanisms explaining the role of vitamin D in human reproduction. First, a possible mechanism may be the direct stimulatory effect of $1,25(OH)_2D_3$on the aromatase gene expression in reproductive tissues, which has been demonstrated in female and male mice (16). Supplementation with estradiol corrected the reproductive phenotype of VDR null mice, whereas partial correction of calcium homeostasis by a high calcium diet was only partially effective, which indicates a direct effect of vitamin D on the reproductive system (16).

Second, it has been shown that HOXA10 expression is up-regulated by $1,25(OH)_2D_3$ in human endometrial stroma cells indicating that altered vitamin D signalling might impact HOXA10 expression and fertility (21). Hox genes were first recognized as an evolutionary conserved family of transcription factors critical to the control of early embryonic development. However, HOXA10 expression is important for the development of the uterus

and essential for endometrial development, allowing uterine receptivity to implantation (25). Aberrant HOXA10 expression in patients with infertility confirms its function in human implantation (26).

Third, vitamin D is the key regulating hormone in calcium homeostasis. It has been shown that calcium plays a role in oocyte activation and maturation resulting in the progression of follicular development (27). In this context, vitamin D and calcium repletion might lead to normalization of menstrual cycles and restoration of ovulation in PCOS women (28). More recently, it has been shown that women with a sufficient vitamin D level undergoing in vitro fertilization (IVF) are more likely to achieve clinical pregnancy than women with low vitamin D levels (29).

Serum levels of 25(OH)D show a seasonal variation with high levels in summer and autumn and lower levels in winter and spring. In northern countries, where a strong seasonal contrast in luminosity exists, the conception rate is decreased during the dark winter months, whereas a peak in conception rate during summer leading to a maximum in birth rate in spring has been observed (30). Moreover, ovulation rates and endometrial receptivity seem to be reduced during long dark winters in northern countries (31). There are several possible explanations for these findings including altered hypothalamic-pituitary axis, brain neurotransmitters such as serotonin, dopamine, and endogenous opioids. However, this fact might also be partly explained by the seasonal variation of vitamin D levels, which might influence several pathways including altered HOXA10 gene expression and altered oocyte development.

Moreover, it has been shown that VDR deficiency also influences fetal and postnatal development in VDR null mice. Fetuses from these animals are growth retarded and usually, but not always, have abnormal serum calcium levels and skeletal mineralization defects (19,

32). However, the effects of vitamin D deficiency on human reproduction and fetal development are poorly studied.

6.2.4 Vitamin D polymorphims

There is evidence that VDR polymorphisms are related to type 1 (33) as well as 2 diabetes (34). The aa genotype of VDR Apa-I is associated with defective insulin secretion in Bangladeshi Asians who are at an increased risk for type 2 diabetes (35). Moreover, the VDR Apa-I polymorphism is associated with glucose intolerance as well as metabolic syndrome in a community-based cohort (34). Moreover, the VDR Bsm-I polymorphism is associated with insulin resistance in the Rancho Bernardo study (34). In contrast, others were not able to detect an assoaition VDR polymorphism with type 2 diabetes (36).

However, there is little data on the association of vitamin related polymorphism with PCOS.

6.3 Aims

The aim of this work was 1.) to investigate the association of serum 25(OH)D levels with metabolic and endocrine parameters in a cohort of PCOS women. 2.) to examine the effect of vitamin D supplementation on metabolic and endocrine parameters in PCOS women; 3.) to investigate the association of VDR variants including Cdx-2, 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 of these variants with PCOS susceptibility.

7 Methods

7.1 Cross-sectional analyses

7.1.1 Subjects

We evaluated 206 women with PCOS aged 16-41 years, who were routinely referred to our outpatient clinic. The diagnosis was based on the Rotterdam criteria (7).

Metabolic syndrome was defined by the National Cholesterol Education Program and the Adult Treatment Panel-III in subjects presenting at least three of the following criteria: waist circumference >88cm, HDL cholesterol <50mg/dl, triglyceride level >150 mg/dl, raised blood pressure (systolic >130 mmHg, diastolic >85mmHg), and raised fasting glucose (>110mg/dl) or impaired glucose tolerance during oGTT (oral glucose tolerance test) (37). The study participants 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.

7.1.2 Procedures

Standard anthropometric data (height, weight, waist and hip circumference) were obtained from each subject. Blood pressure was measured after PCOS women have been seated for at least 5 minutes. The BMI was calculated as the weight in kilograms divided by the square of height in meters. Waist circumference 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. Hirsutism was quantified with the modified Ferriman-Gallwey-Score. Moreover, basal blood samples for hormonal (25(OH)D, PTH [parathyroid hormone], total testosterone, free testosterone, SHBG, androstendione, DHEAS, fT3 [free triiodothyronine], fT4 [free thyroxin], TSH (thyroid stimulation hormone], 17αOH-progesterone, cortisol) and metabolic (glucose, insulin, C-peptide, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) determinations were collected at 8.00-9.00 after overnight fast. All participants underwent a fasting 75g oGTT. Blood samples were drawn after 60 and 120 minutes for glucose, insulin and C-peptide determination. Insulin resistance was estimated using the HOMA-IR (homeostatic model assessment-insulin resistance). HOMA-IR was calculated as the product of the fasting plasma insulin value [μU/ml] and the fasting plasma glucose value [mg/dl], divided by 405 (38). QUICKI (Quantitative Insulin-sensitivity Check Index) was used to estimate insulin sensitivity. QUICKI was calculated as 1/log fasting insulin [μU/ml] + log fasting glucose [mg/dl] (39). To assess β cell function, HOMA-β was calculated as (20 x fasting insulin [μU/ml]) / (fasting glucose [mmol/l] -3.5). Hyperinsulinemia was assessed by calculating the area under the insulin response curve (AUCins). The free androgen index (FAI) was calculated as testosterone (nmol/l)/SHBG (nmol/l) x 100.

7.1.3 Biochemical analysis

25(OH)D [normal range 30-60 ng/ml] 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 [2.0-25.0 μU/ml] and C-peptide [0.5-3.2 ng/ml] were

measured by ELISA (Siemens Healthcare Diagnostics, Eschborn Germany) with intra- and interassay CV of 4.0 and 2.6, and 5.1 and 8.4%, respectively. Fasting glucose [≤115 mg/dl], triglycerides [≤150 mg/dl], total cholesterol [≤200 mg/dl], HDL cholesterol [>40 mg/dl], and CRP [≤8 mg/l] were determined using Modular Analytics SWA (Roche, Basel, Switzerland). Free testosterone [0.29-3.18 pg/ml] was determined using a radioimmunoassay (DSL, Webster, Texas, USA). SHBG [19-117 nmol/l], PTH [15-65 pg/ml] (Roche, Basel, Switzerland), ACTH [10-51 pg/ml], cortisol [43.0-220.0 ng/ml], human growth homone (HGH) [0.0-7.0 ng/ml] (Siemens DPC Bühlmann, Salzburg, Austria), and insulin-like growth factor-1 (IGF-1) [100-400 ng/ml], prolactin [2.8-29.2], total testosterone [0.14-0.77 ng/ml] and TSH [0.1-4.0 μU/ml] (Bayer, Leverkusen, Germany) were measured by luminescence immunoassay.

7.1.4 Statistical analysis

For the purpose of this study and according to widely used cut-offs, subjects were divided into groups: vitamin D sufficiency (25(OH)D ≥30 ng/ml), hypovitaminosis D (25(OH)D <30 ng/ml). Furthermore, we defined severe vitamin D deficiency (25(OH)D <10ng/ml), moderate vitamin D deficiency (25(OH)D 10-19.9ng/ml), and vitamin D insufficiency (25(OH)D 20-29,9ng/ml) (15, 40). Data are presented as means ± standard deviation (SD) unless otherwise stated. Kolmogorov-Smirnov test was used to examine for normal distribution and variables following a skewed distribution were logarithmically transformed before being used in correlation or regression analyses. Pearson correlations and partial correlation analyses were used to determine relationships between variables. Depending on the distribution of data the student's T-test for independent samples and the nonparametric Mann-Whitney-U-test for

independent samples were applied to test for differences between groups. CRP values within the normal range (≤8mg/l) were included in the analyses. To study seasonal variation, we subdivided the year into 4-month measurement periods: February-May (season 1), June-September (season 2), October-January (season 3) to address the seasonal changes in availability of sunlight (41). Multiple linear regression analyses were calculated with HOMA-IR and QUICKI as dependent variables and 25(OH)D, season, BMI, and age as independent variables. Binary logistic regression analyses were performed to examine associations of metabolic syndrome (dependent variable) with 25(OH)D, season, BMI, and age (independent variables). Statistical analyses were performed by SPSS version 16.0 (SPSS Inc., Chicago, IL). A p-value of <0.05 was considered statistically significant.

7.2 Intervention Study

7.2.1 Subjects

The criteria for eligibility were the diagnosis of PCOS, age 18-45 years, and serum calcium levels <2.65 mmol/l. We excluded patients with prevalent diabetes, pregnant or breast feeding women, patients with an intake of vitamin D supplements or any medication known to affect endocrine parameters, carbohydrate metabolism or serum lipid profile for at least 3 months before entering the study.

The diagnosis of PCOS was based on the Rotterdam criteria (7).

We evaluated 70 women with PCOS aged 18-41 years, who were routinely referred to the outpatient clinic of our department. The study was conducted from May 2009 to April 2010.

The study protocol was approved by the local ethics committee and written informed consent was obtained from each patient.

7.2.2 Study design

This was a prospective, outpatient clinical trial with one treatment group over 24 wk; the primary outcome was change in glucose metabolism. Prespecified secondary outcomes included changes in anthropometric measurements (BMI, waist circumference, and absolute body weight), blood pressure, serum 25(OH)D, insulin resistance, reproductive hormone levels, lipid profiles (total cholesterol, HDL, LDL, triglycerides, and lipoprotein a), CRP, and changes in menstrual frequency.

7.2.3 Treatment protocol

Subjects underwent the following clinical, metabolic, and laboratory evaluations before (visit 1), after 12 wk (visit 2), and after 24 wk (visit 3) of study treatment.

Standard anthropometric data (height, weight, waist and hip circumference) were obtained from each subject. Blood pressure was measured after PCOS women have been seated for at least 5 minutes. The BMI was calculated as the weight in kilograms divided by the square of height in meters. Waist circumference 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. Hirsutism was quantified with the modified Ferriman-Gallwey-Score. Vitamin D intake was assessed using questionnaires (15).

All patients were on an unrestricted diet. Basal blood samples for hormonal (25(OH)D, PTH, total testosterone, free testosterone, SHBG, fT3, fT4, TSH, 17αOH-progesterone, cortisol) and metabolic (glucose, insulin, C-peptide, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) determinations were collected at 07.30-9.00 after overnight fast at day 2-5 in women with menstrual bleeding or at random in amenorrhoeic women. All participants underwent a fasting 75g oGTT. Blood samples were drawn after 30, 60 and 120 minutes for glucose, insulin and C-peptide determination. Insulin resistance was estimated using the HOMA-IR. To assess β cell function, HOMA-β was calculated. Hyperinsulinemia was assessed by calculating the area under the insulin response curve. The FAI was calculated as testosterone (nmol/l)/SHBG (nmol/l) x 100.

All PCOS women received 20.000 international units (IU) orally administered cholecalciferol (Oleovit D3®, Fresenius Kabi, Austria) weekly for 24 weeks (equivalent to 2857 IU/day). It has been shown that supplementation with vitamin D can be achieved equally well with daily, weekly, or monthly dosing frequencies (42). To enhance compliance, the authors decided to administer vitamin D3 weekly. To ensure a replete vitamin D status in younger adults, a daily dose of more than 2000 IU is recommended (43); similar doses have been used previously in intervention trials (44). All laboratory testing and anthropometric measures described for the pretreatment evaluation was repeated at wk 12 and wk 24.

Subjects recorded the presence/absence of menstrual bleeding daily in a menstrual cycle diary distributed at the time medication was dispensed. The menstrual cycle diary was stored and a urine pregnancy test performed in all patients at wk 12 and wk 24.

7.2.4 Biochemical analysis

$1,25(OH)_2D_3$ was measured using a commercially available enzyme immunoassay (IDS, Boldon, UK) with intra- and interassay coefficients of variation (CV) of <10% and <15%, respectively. 25(OH)D, insulin, C-peptide, fasting glucose [≤115 mg/dl], triglycerides, total cholesterol, HDL cholesterol, total testosterone, free testosterone, SHBG, PTH, ACTH, cortisol, HGH, IGF-1, prolactin, and TSH were measured as described above.

7.2.5 Statistical analyses

To convert 25(OH)D levels in nmol/l, multiply by 2.5. Baseline characteristics are presented as means ±SD for continous variables. Kolmogorov-Smirnov test and descriptive statistics were used to examine for normal distribution and variables following a skewed distribution were logarithmically transformed for parametric statistical analyses. Paired student´s t-test was used to compare means in continuous variables at baseline to mean values at wk 12 and wk 24. Statistical analyses were performed by SPSS 17.0 (SPSS Inc, Chicago, USA) and a p-value below 0.05 was considered statistically significant.

7.3 Genetics

7.3.1 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. 145 BMI-matched women within the same age range, who were referred for routine thyroid evaluation to our outpatient clinic,

were invited to participate in the trial as a control group. All control women had normal thyroid function, regular menstrual cycles, normal serum androgens and no clinical signs of hyperandrogenism.

7.3.1.1 PCOS women

PCOS was diagnosed using the Rotterdam criteria (7).

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.

7.3.2 Procedures

Standard anthropometric data (height, weight, waist circumference [WC] and hip circumference, 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, fT3, fT4, TSH, 17α(OH)-progesterone, cortisol) and metabolic (glucose, insulin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) determinations were collected at 8.00-9.00 after overnight fast. All participants underwent a

fasting 75g oral glucose tolerance test. Blood samples were drawn after 30, 60 and 120 minutes for glucose and insulin determination. Insulin resistance was estimated using the HOMA-IR. QUICKI was used to estimate insulin sensitivity. To assess β cell function, HOMA-β was calculated. MATSUDA-index was calculated as 10000/√((fasting glucose*fasting insulin)*(mean $glucose_{OGTT}$*mean $insulin_{OGTT}$)) (45). The FAI was calculated as testosterone (nmol/l)/SHBG (nmol/l)x100.

7.3.3 Genotyping

Blood samples were collected in tubes containing EDTA as anticoagulant. DNA was extracted using the NucleoSpin® Blood method. Genotyping of VDR *Cdx-2* (rs11568820; assay-no: C___2880808_10), *Bsm-I* (rs1544410; assay-no: C___8716062_10), *Fok-I* (rs2228570; assay-no: C__12060045_20), *Apa-I* (rs7975232; assay-no: C__28977635_10), *Taq-I* (rs731236; assay-no: C___2404008_10) as well as GC (rs2282679; assay-no: C___26407519_10), DHCR7 (rs12785878; assay-no: C__32063037_10), and CYP2R1 (rs10741657; assay-no: C___2958430_10) were performed by predesigned SNP Genotyping Assay (Applied Biosystems).

7.3.4 Biochemical analysis

25(OH)D, insulin, free testosterone, SHBG, PTH, and total testosterone were measured as described above.

7.3.5 Statistical analysis

Data are presented as median (interquartile range) unless otherwise stated. Kolmogorov-Smirnov test and descriptive statistics were used to evaluate 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. Correlations of 25(OH)D levels with anthropometric, metabolic and endocrine parameters were analysed using Pearson correlation. 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 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 of <0.05 was considered significant. Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

8 Results

8.1 Cross-sectional analyses

8.1.1 Findings for entire cohort

Anthropometric and biochemical characteristics of PCOS women stratified by 25(OH)D levels are shown in table 2. Hypovitaminosis D was present in 150 out of 206 PCOS women (72.8%), 6 out of 206 PCOS women (2.9%) had 25(OH)D levels <10ng/ml, 74 out of 206 PCOS women (35.9%) had 25(OH)D levels between 10-19.9 ng/ml, 70 women (34.0%) presented with 25(OH)D levels between 20 and 29.9 ng/ml and 56 women (27.2%) showed sufficient 25(OH)D levels ≥30ng/ml.

Table 2: Clinical and biochemical characteristics of PCOS subjects based on 25(OH)D status.

	All PCOS n=206		**Hypovitaminosis D (<30ng/ml), n=150**		**Vitamin D sufficiency (≥30ng/ml), n=56**		
	Mean	SD	Mean	SD	Mean	SD	p
Clinical characteristics							
Age [years]	29	7	29	6	27	7	0.027
Weight [kg]	72.3	19.7	75.6	21.0	63.7	12.4	0.001
Height [cm]	166.1	6.4	166.0	6.4	166.2	6.5	0.980
BMI [kg/m^2]	26.2	6.9	27.4	7.4	23.0	4.1	0.001
Waist circumference [cm]	90	19	94	19	79	10	<0.001
Hip circumference [cm]	106	13	108	13	98	10	0.001
WHR	0.85	0.10	0.86	0.09	0.81	0.10	0.005
Systolic blood pressure [mmHg]	113	15	114	16	108	12	0.026
Diastolic Blood pressure [mmHg]	75	11	76	11	73	11	0.115
Metabolic biochemical characteristics							
Fasting glucose [mg/dl]	83	9	83	10	82	9	0.237
1h glucose [mg/dl]	115	40	120	43	103	28	0.003
2h glucose [mg/dl]	98	30	99	32	93	22	0.259
AUCgluc	102.5	27.8	105.5	29.9	94.6	18.9	0.046
HbA1c [%]	5.2	0.3	5.2	0.3	5.1	0.3	0.065
HOMA-IR	1.72	1.93	1.96	2.16	1.11	0.92	0.002
HOMA-β	160.2	122.7	173.3	131.1	126.7	90.9	0.014
QUICKI	0.39	0.09	0.38	0.10	0.40	0.05	0.002
Fasting insulin [µU/ml]	8.0	7.6	9.0	8.4	5.4	4.0	0.002
1h insulin [µU/ml]	58.0	44.1	61.7	45.0	48.9	40.6	0.049
2h insulin [µU/ml]	48.1	43.8	52.5	49.1	37.4	24.6	0.267
AUCins	42.8	31.5	45.9	33.3	35.2	25.3	0.074
Total cholesterol [mg/dl]	177.5	35.4	174.2	34.9	185.9	35.8	0.088
Triglycerides [mg/dl]	89.6	44.6	94.8	48.8	76.5	27.9	0.042
HDL [mg/dl]	64.1	16.2	61.7	15.7	70.0	16.3	0.002
QChol/HDL	2.9	0.8	3.0	0.8	2.8	0.7	0.106
LDL [mg/dl]	99.5	29.2	99	29	103.9	31.8	0.221
CRP [mg/l]	2.3	2.0	2.5	2.1	1.6	1.3	0.030

Endocrine biochemical characteristics							
Testosterone [ng/l]	0.66	0.28	0.65	0.28	0.69	0.28	0.243
Free testosterone [pg/ml]	2.79	1.19	2.80	1.25	2.76	0.99	0.788
SHBG [nmol/l]	51.6	27.8	50.0	29.1	55.7	23.9	0.038
FAI	5.8	4.3	6.1	4.7	5.2	3.2	0.549

Comparisons between PCOS women with hypovitaminosis D and sufficient 25(OH)D levels were performed by student's T-test or Mann-Whitney-U-test as appropriate.

25(OH)D levels were determined between October and January (season 3) in the majority of PCOS women (n=93, 45.1%). Blood samples of 76 women (36.9%) were collected between February and May (season 1), and 37 women (18%) were examined between June and September (season 2). 25(OH)D levels were significantly higher in season 2 when compared to season 1 and 3 ($p<0.05$ for all).

25(OH)D levels correlated significantly negative with BMI (figure 4), weight, waist circumference, hip circumference, WHR (waist-to-hip ratio), systolic and diastolic blood pressure, fasting and stimulated glucose, AUCgluc (figure 5), fasting and stimulated insulin, AUCins, PTH, triglycerides, QChol/HDL (quotient total cholesterol/HDL), and CRP (table 2). We found a significantly positive correlation between 25(OH)D levels and HDL cholesterol (table 3). These latter results did not materially change when controlling for season and age in partial correlation analyses. However, the associations of 25(OH)D with anthropometric parameters, glucose, insulin, and triglycerides were no longer significant after additional adjustment for BMI. The positive correlation of 25(OH)D with HDL remained significant after adjustment for BMI ($r=0.303$, $p=0.006$).

Table 3: Correlation of 25(OH)D levels with metabolic and endocrine parameters in PCOS women (n=206)

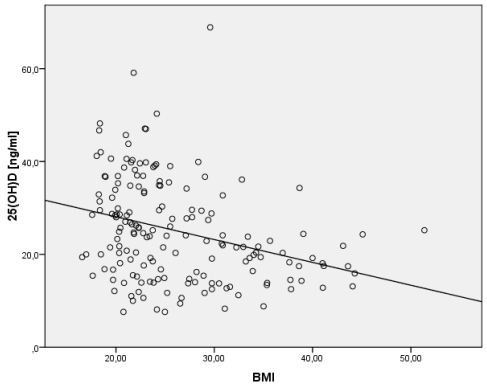
	25(OH)D	
Variable	r	p
Clinical characteristics		
Age [years]	-0.130	0.084
Weight [kg]	-0.318	<0.001
Height [cm]	0.021	0.789
Waist circumference [cm]	-0.409	<0.001
Hip circumference [cm]	-0.378	<0.001
WHR	-0.316	0.023
Systolic blood pressure [mmHg]	-0.197	0.029
Diastolic blood pressure [mmHg]	-0.219	0.015
Metabolic biochemical characteristics		
Fasting glucose [mg/dl]	-0.210	0.005
1h glucose [mg/dl]	-0.291	<0.001
2h glucose [mg/dl]	-0.230	0.003
HOMA-IR	-0.327	<0.001
Fasting insulin [µU/ml]	-0.320	<0.001
1h insulin [µU/ml]	-0.215	0.007
2h insulin [µU/ml]	-0.180	0.026
AUCins	-0.227	0.005
PTH [pg/ml]	-0.309	0.004
Free testosterone [pg/ml]	0.004	0.953
FAI	-0.089	0.238
SHBG [nmol/l]	0.195	0.009
Testosterone [ng/[l]	0.050	0.509
Cholesterol [mg/dl]	0.141	0.065
Triglycerides [mg/dl]	-0.231	0.002
HDL [mg/dl]	0.278	<0.001
QChol/HDL	-0.231	0.002
LDL [mg/dl]	0.059	0.568
CRP [mg/l]	-0.298	0.005
Endocrine biochemical characteristics		
Testosterone [ng/[l]	0.050	0.509
Free testosterone [pg/ml]	0.004	0.953
SHBG [nmol/l]	0.195	0.009
FAI	-0.089	0.238

Data are given as Pearson's correlation coefficient r.

In addition, we found a significant correlation between 25(OH)D and HOMA-IR (table 3), QUICKI (figure 6) as well as HOMA-β (figure 7). To explore the association of 25(OH)D and HOMA-IR, we performed a multivariate regression analysis including HOMA-IR as dependent variable and BMI, age, 25(OH)D, and season as explanatory variables. In this analysis, 25(OH)D ($p=0.036$) was a significant and independent predictor for HOMA-IR, along with BMI ($p<0.001$). Furthermore, we performed a multivariate regression analysis including QUICKI as dependent variable and BMI, age, 25(OH)D, and season as explanatory variables. 25(OH)D ($p=0.047$) and BMI ($p<0.001$) were significant predictors of QUICKI, along with the other covariates explaining 18% of variation in QUICKI.

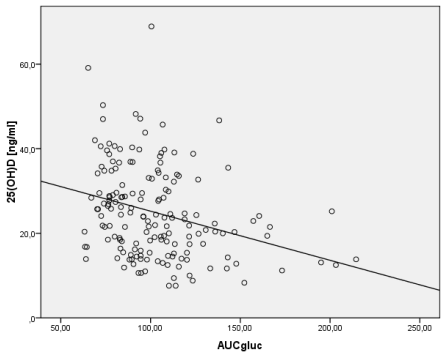
Figure 4-7: Correlation between 25(OH)D and BMI, QUICKI, AUCgluc, and HOMA-β (Pearson correlation).

Figure 4: Correlation between 25(OH)D and BMI.



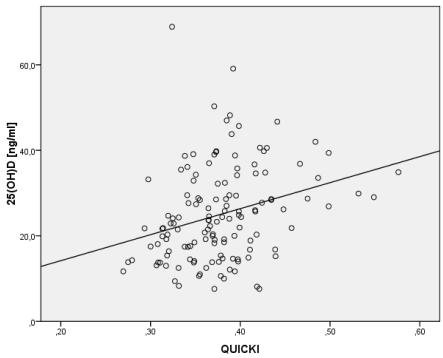
R=-0.346, $p<0.001$

Figure 5: Correlation between 25(OH)D and AUCgluc.



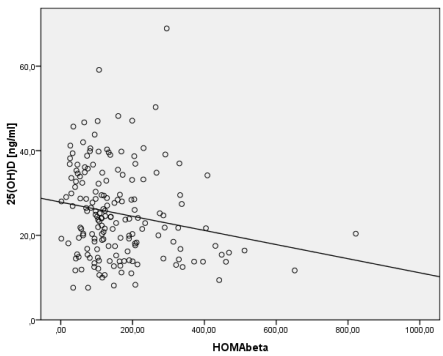
R=-0.333, p<0.001

Figure 6: Correlation between 25(OH)D and QUICKI.



R=0.327, p<0.001

Figure 7: Correlation between 25(OH)D and HOMA-β.



R=-0.212, p=0.005

30 PCOS women (15%) presented with impaired glucose tolerance. Women with impaired glucose tolerance had significantly lower levels of 25(OH)D than women with normal glucose tolerance (20.2 and 25.6 ng/ml, respectively) (p=0.026). In PCOS women with vitamin D deficiency (<20 ng/ml), 16 women (20.0%) had impaired glucose tolerance, in PCOS women with vitamin D insufficiency (20-29.9 ng/ml) 12 women (17.1%) showed impaired glucose tolerance, and in PCOS women with sufficient 25(OH)D levels (>30 ng/ml) 2 women (3.6%) had impaired glucose tolerance.

To address the heterogeneity of PCOS, subgroup analyses of PCOS women with and without a family history of type 2 diabetes were performed (46, 47). Data on family history of type 2 diabetes were available in 158 PCOS patients. Family history of type 2 diabetes was present in 47 (29.7%) out of 158 PCOS patients, and these PCOS women had significantly lower 25(OH)D levels when compared to PCOS women without a family history of type 2 diabetes (21.9 vs 24.5, p=0.049). Moreover, PCOS women with a family history of type 2 diabetes had significantly higher BMI, waist and hip circumference, fasting insulin, HOMA-IR, and QChol/HDL, and significantly lower QUICKI and SHBG than PCOS women without a family history of type 2 diabetes (p<0.05 for all, data not shown).

No significant correlations were found between 25(OH)D levels and endocrine parameters such as testosterone, free testosterone, and FAI. Hirsutism score correlated significantly negative (r=-0.267; p=0.001) and SHBG correlated significantly positive (table 3) with 25(OH)D. This correlation of 25(OH)D with SHBG was abolished, whereas the correlation of 25(OH)D with hirsutism score remained significant when controlling for BMI (r=-0.367, p=0.002). Further, hirsute PCOS women had significantly lower 25(OH)D levels (21.4 ng/ml) than PCOS women without hirsutism (26.8 ng/ml) (p=0.001).

Levels of PTH were significantly positive correlated with AUCgluc (r=0.235; p=0.036) and diastolic blood pressure (r=0.278; p=0.006).

8.1.2 Metabolic syndrome

Presence of metabolic syndrome was analysed in a subgroup of 174 out of 206 PCOS women (85.4%). Metabolic syndrome was evident in 25 women (12.2%). 18 out of 25 PCOS women with metabolic syndrome (72%) presented with deficient 25(OH)D levels, 7 women with metabolic syndrome (28%) had insufficient 25(OH)D levels, and none had a sufficient 25(OH)D level (>30 ng/ml). PCOS women with the metabolic syndrome had significantly lower 25(OH)D levels than PCOS women without the metabolic syndrome (17.3 versus 25.8 ng/ml, respectively) ($p<0.001$).

In logistic regression analyses, metabolic syndrome was associated with BMI (OR 1.28, 95% CI [1.15-1.42], $p<0.001$) and 25(OH)D (OR 0.86, 95% CI [0.75-0.98], p=0.019).

8.1.3 Findings stratified by vitamin D sufficiency and hypovitaminosis D

Clinical and biochemical characteristics of PCOS women with vitamin D sufficiency (≥30ng/ml) and hypovitaminosis D (<30 ng/ml) are shown in table 2. PCOS women with hypovitaminosis D had significantly higher age, weight, BMI, waist and hip circumference, WHR, and systolic blood pressure than women with vitamin D sufficiency ($p<0.05$ for all). Furthermore, the hypovitaminosis D group had significantly higher levels of 1h glucose, AUCgluc, HOMA-IR, HOMA-β, fasting insulin, 1h insulin, triglycerides, and CRP and

significantly lower levels of QUICKI, HDL and SHBG than the vitamin D sufficient group ($p<0.05$ for all).

Furthermore, we performed subgroup analyses of lean (BMI ≤25, n=116, 56.3%) and obese (BMI >25, n=90, 43.7%) PCOS women. When lean PCOS women were analysed separately, we observed no significant differences for all parameters included in table 1 between PCOS women with hypovitaminosis D and vitamin D sufficiency (data not shown). We found a significant positive correlation of 25(OH)D with HDL in lean PCOS women ($p<0.05$, data not shown), whereas all other correlations were not statistically significant. In the subgroup of obese PCOS women, we found significantly increased HbA1c levels and waist circumference in the hypovitaminosis D group when compared to the vitamin D sufficiency group ($p<0.05$ for all, data not shown), whereas all other parameters were not significantly different between groups. In obese PCOS women, there were significantly negative correlations of 25(OH)D with 1h glucose, 2h glucose, AUCgluc, HbA1c, HOMA-β, Fasting insulin, 1h insulin, and AUCins and significantly positive correlations of 25(OH)D with QUICKI and HDL ($p<0.05$ for all, data not shown).

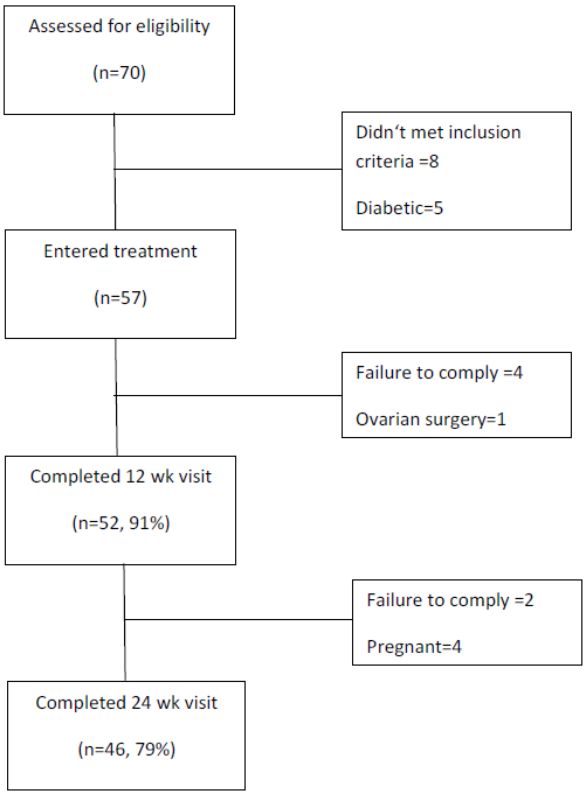
8.2 Intervention

8.2.1 Baseline characteristics

Seventy PCOS patients were screened and 57 PCOS women aged 26±6 years were included and received treatment. Forty-six of 57 PCOS patients (81%) completed the study per protocol (figure 8). At wk 12, 5 women dropped out (4 due to compliance [2 did not take medication, 2 were lost to follow up], 1 underwent laparoscopic ovarian surgery). At wk 24,

another 2 women dropped out (did not take medication). In 4 subjects metabolic assessment was not available because of (intended) pregnancy status.

Figure 8: Subject flow chart



Baseline characteristics of 52 PCOS women who completed 12 weeks of the study are shown in table 4. Median (interquartile range) vitamin D intake was 1210 IU (590-2360) per month.

Table 4: Characteristics of PCOS subjects at baseline and after vitamin D treatment.

	Baseline (n=52)		**12 weeks (n=52)**		**Baseline vs 12 weeks**	**24 weeks (n=46)**		**Baseline vs 24 weeks**
	mean	SD	mean	SD	p-value	mean	SD	p-value
Endocrine parameters								
25(OH)D [ng/ml]	28.0	11	51.3	17.3	<0.001	52.4	21.5	<0.001
$1,25(OH)_2D_3$ [pmol/l]	113	47.3	140.0	53.6	<0.001	134	64	<0.001
PTH [pg/ml]	37.9	40.3	31.1	11.7	<0.001	33.7	9.1	<0.001
Free testosterone [pg/ml]	2.83	0.99	2.72	0.99	0.416	2.72	0.99	0.800
SHBG [nmol/l]	48.6	33.2	53.7	44.42	0.312	45.2	21.07	0.337
Testosterone [ng/l]	0.64	0.22	0.61	0.21	0.15	0.61	0.22	0.787
FAI	6.5	4.3	5.8	3.4	0.090	6.2	4.6	0.980
Estradiol [pg/ml]	66.7	62.9	44.6	34.3	0.022	49.7	53.8	0.099
Calcium [mmol/l]	2.36	0.07	2.34	0.06	0.124	2.37	0.08	0.190
Anthropometric parameters								
Weight [kg]	71	18	71	19	0.757	71	20	0.549
BMI [kg/m^2]	25.4	6.6	25.7	6.6	0.712	25.3	6.9	0.486
WC [cm]	82	16	83	15	0.338	82	16	0.656
HC [cm]	106	12	107	11	0.602	103	11	0.014
WHR	0.78	0.09	0.77	0.07	0.749	0.80	0.14	0.920
BPsys [mmHg]	125	20	120	23	0.453	120	23	0.852
BPdias [mmHg]	85	15	82	17	0.518	82	17	0.144
Oral glucose tolerance test								
Fasting glucose [mg/dl]	87	7	88	7	0.735	84	7	0.010
Glucose 30 min [mg/dl]	137	25	139	21	0.443	128	23	0.083
Glucose 1h [mg/dl]	130	35	123	35	0.185	109	36	0.043
Glucose 2h [mg/dl]	105	26	99	24	0.133	92	21	0.001
AUCgluc	114.8	17.4	112.3	17.5	0.477	103.0	18.3	0.002
Fasting insulin	7.2	6.5	7.15	5.72	0.82	7.4	8.3	0.965

[µU/ml]								
Insulin 30 min [µU/ml]	64.7	55.4	55.5	45.01	0.184	64.9	59.1	0.525
Insulin 1h [µU/ml]	67.3	49	67.51	48.19	0.9	60	59.5	0.319
Insulin 2h [µU/ml]	46.6	39.3	47.64	45.53	0.762	47.7	57.3	0.725
HOMA-IR	1.58	1.49	1.59	1.36	0.613	1.60	1.92	0.200
HOMA-beta	101.0	77.5	103.0	73.8	0.698	117.5	108.3	0.485
C-Peptid fasting [ng/ml]	5.2	3	3.58	2.94	<0.001	1.5	0.8	<0.001
C-Peptid 30 min [ng/ml]	11.9	4.3	9.26	4.18	<0.001	5.5	3.06	<0.001
C-Peptid 1h [ng/ml]	13.6	3.3	11.12	3.5	<0.001	6.8	3.4	<0.001
C-Peptid 2h [ng/ml]	13	3.5	9.89	4.1	<0.001	6.2	3.41	<0.001
Lipids								
Cholesterol [mg/dl]	172	30	179	30	0.008	179	38	0.057
Triglycerides [mg/dl]	91	53	83	19	0.214	64	24	0.001
HDL [mg/dl]	70	19	69	25	0.722	73	18	0.289
LDL[mg/dl]	93	23	101	25	0.005	102	33	0.026
Fertility								
Regular menses	6/52	11.5 %	20/52	38.5%	nd	23/46	50.0 %	nd
Seeking pregnancy	16/52	30.8 %						

8.2.2 Changes with drug treatment

At baseline, 61.5% of our study subjects had 25(OH)D levels below 30 ng/ml, which indicates an insufficient vitamin D status. We observed a significant increase of 25(OH)D levels from 28±11 ng/ml to 51.3±17.3 and 52.4±21.5 after 12 and 24 weeks of weekly oral administration of 20.000 IU cholecalciferol, respectively ($p<0.001$). After 12 weeks of vitamin D treatment, one PCOS woman had 25(OH)D concentrations >100 ng/ml (105.8 ng/ml and 107.6 ng/ml, respectively), after 24 weeks another PCOS woman had a 25(OH)D level >100 ng/ml. Both

women were normocalcemic (serum calcium < 2.65 mmol/l). In addition, all other patients were normocalcemic at baseline as well as after 12 and 24 weeks of vitamin D treatment.

PTH levels significantly decreased and 1,25-dihydroxyvitamin D levels increased after 12 and 24 weeks of vitamin D treatment (table 4). We observed no significant changes in BMI, waist circumference, and blood pressure after 12 and 24 weeks of vitamin D treatment. After 24 weeks of vitamin D treatment, hip circumference significantly decreased.

We observed no clinically significant adverse event during the study.

8.2.2.1 Metabolic changes

Fasting and stimulated glucose and C-peptide levels significantly decreased after vitamin D 24 weeks of vitamin D treatment (figure 9-12). Moreover, we observed a significant decrease in triglyceride levels, whereas total cholesterol and LDL cholesterol levels significantly increased after vitamin D treatment (table 4). When women with 25(OH)D levels <20 ng/ml at baseline were analysed separately, results did not materially change.

Figure 9-12: Fasting and 2h glucose and C-peptide levels at baseline and after 12 and 24 weeks of orally vitamin D3 supplementation.

(Paired student`s T-test)

§, $p<0.001$ for baseline vs 12 weeks

# $p<0.02$ for baseline vs 24 weeks

Figure 9: Fasting glucose levels at baseline and after 12 and 24 weeks of orally vitamin D3 supplementation.

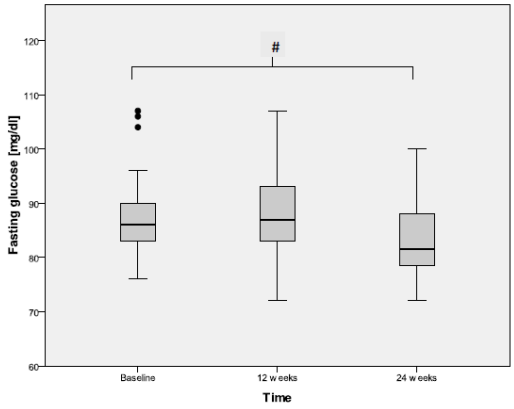


Figure 10: Glucose 2h levels at baseline and after 12 and 24 weeks of orally vitamin D3 supplementation.

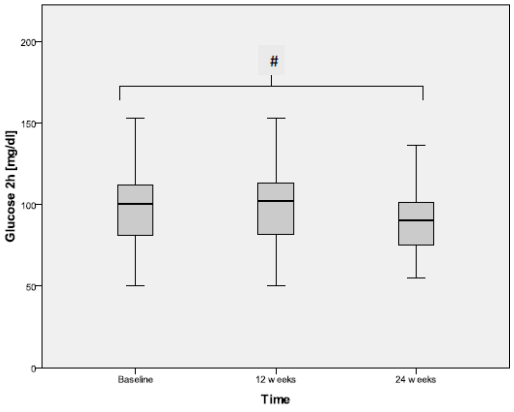


Figure 11: Fasting C-peptide levels at baseline and after 12 and 24 weeks of orally vitamin D3 supplementation.

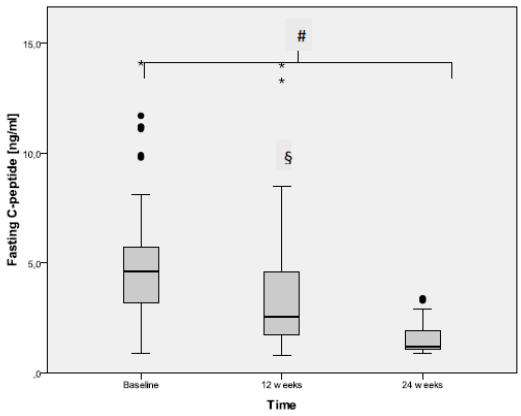
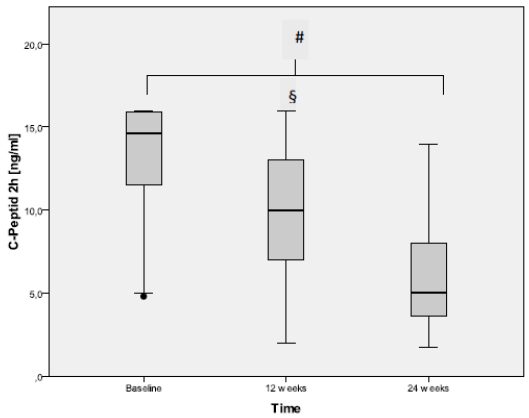


Figure 12: C-peptide 2h levels at baseline and after 12 and 24 weeks of orally vitamin D3 supplementation.



8.2.2.2 Endocrine parameters

Endocrine parameters such as testosterone, free testosterone and SHBG remained unchanged after 12 wk and 24 wk of vitamin D treatment (table 4). We observed a significant reduction of estradiol levels after 12 weeks of vitamin D treatment and a trend towards lower estradiol levels after 24 weeks and lower FAI levels after 12 weeks.

8.2.2.3 Menstrual regularity

At baseline, 6 out of 52 women (11.5%) reported regular menses whereas 46 out of 52 women (88.5%) had oligo- or amenorrhoea. After 12 weeks of vitamin D treatment, 14 out of 46 PCOS women previously affected by menstrual disturbances (30.4%) reported normalization or improvement of regularity of menses. After 24 weeks, 23 out of 46 women (50.0%) who were oligo- or amenorrhoeic at baseline reported normalization or improvement of menstrual pattern.

Overall, 4 out of 16 women seeking pregnancy at baseline conceived (25%).

8.3 Genetics

Table 5 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 amenorrhoea was present in 475 out of 545 PCOS women (87%) and polycystic ovaries were found in 264 women (48%).

Table 5: Baseline characteristics of PCOS and control women.

	PCOS women (n=545)		**Control women (n=145)**		
	Median	Interquartile Range	Median	Interquartile Range	p-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 2h [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 2h [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
Androstenedione [ng/ml]	2.8	2.0-4.2	1.5	1.0-2.5	<0.001

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 ($p<0.001$). 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.

We found a significantly positive correlation of 25(OH)D levels with SHBG, HDL, QUICKI, and MATSUDA and significantly negative correlations of 25(OH)D levels with BMI, WHR, WC, PTH, fasting insulin, HOMA-IR, HOMA- β, HbA1c, triglycerides, QChol/HDL, and hirsutism-score in PCOS women.

8.3.1 Genotyping

Allelic frequencies of VDR, GC, CYP2R1, and GC variants are shown in table 6. None of the genotype frequency distributions deviated significantly from the Hardy-Weinberg equilibrium ($p>0.05$). There was no difference in genotype frequencies between PCOS and control women.

Table 6: Allelic frequencies of VDR and vitamin D level associated variants.

	PCOS women	Control women	p-value
VDR *Cdx-2*	*(n=545)*	*(n=145)*	0.420
GG	346 (0.64)	96 (0.66)	
GA	176 (0.32)	46 (0.32)	
AA	23 (0.04)	3 (0.02)	
VDR *Fok-I*	*(n=538)*	(n=135)	0.954
AA	82 (0.15)	22 (0.16)	
AG	241 (0.45)	60 (0.45)	
GG	215 (0.40)	53 (0.39)	
VDR *Bsm-I*	*(n=537)*	(n=137)	0.622
GG	216 (0.40)	49 (0.36)	
GA	244 (0.45)	66 (0.48)	
AA	77 (0.14)	22 (0.16)	
VDR *Apa-I*	*(n=543)*	(n=135)	0.155
AA	142 (0.26)	48 (0.33)	
AC	274 (0.51)	60 (0.41)	
CC	127 (0.23)	37 (0.26)	
VDR *Taq-I*	*(n=536)*	(n=137)	0.335
TT	226 (0.42)	49 (0.36)	
TC	238 (0.44)	65 (0.47)	
CC	72 (0.14)	23 (0.17)	
GC	*(n=448)*	*(n=145)*	0.836
TT	221 (0.50)	67 (0.46)	
TG	172 (0.38)	61 (0.42)	
GG	55 (0.12)	17 (0.12)	
DHCR7	*(n=447)*	*(n=145)*	0.440
TT	227 (0.51)	77 (0.53)	
TG	185 (0.41)	52 (0.36)	
GG	35 (0.08)	16 (0.11)	
CYP2R1	*(n=450)*	*(n=145)*	0.121
GG	172 (0.38)	64 (0.44)	
GA	212 (0.47)	52 (0.36)	
AA	66 (0.15)	29 (0.20)	

8.3.2 VDR Cdx-2

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 towards lower AUCins levels in PCOS women carrying the AA genotype (table 7). In multivariate adjusted analyses, results remained materially unchanged for fasting insulin, QUICKI, and MATSUDA but were attenuated for HOMA-IR.

We did not observe an association of *Cdx-2* polymorphism with endocrine parameters or 25(OH)D levels.

Table 7: Metabolic parameters according to VDR *Cdx-2* genotype.

	GG		**GA**		**AA**			
	Median	Interquartile	Median	Interquartile	Median	Interquartile	p-	p-
Age [years]	27	23-31	26	22-29	28	25-31	0.111	n.a.
BMI [kg/m²]	24.5	21.5-29.4	23.7	20.8-28.7	22.5	20.6-23.9	0.167	n.a.
Calcium [mmol/l]	2.35	2.30-2.41	2.34	2.30-2.41	2.34	2.27-2.38	0.637	n.a.
25(OH)D [ng/ml]	26.6	18.5-34.9	24.0	16.7-34.8	21.9	14.1-36.5	0.309	n.a.
PTH [pg/ml]	36.1	27.9-46.2	33.6	28.7-45.2	31.5	22.7-43.4	0.810	n.a.
Fasting glucose [mg/dl]	87	81-92	86	80-91	86	81-90	0.350	n.a.
Glucose 30 min [mg/dl]	134	119-152	134	119-154	147	117-162	0.874	n.a.
Glucose 1h [mg/dl]	119	96-147	123	98-148	122	100-152	0.734	n.a.
Glucose 2h [mg/dl]	98	84-118	98	85-118	99	77-118	0.717	n.a.
AUCgluc	178.5	155.5-200.0	171.0	153.0-198.3	173.6	153.0-207.5	0.674	n.a.
Fasting insulin [µU/ml]	6.4	4.0-11.1	6.3	3.3-9.5	5.2	2.9-7.7	0.039*	0.045*
Insulin 30 min [µU/ml]	42.7	29.1-69.1	47.5	29.3-79.7	36.2	14.1-54.2	0.353	n.a.
Insulin 1h [µU/ml]	51.6	34.0-85.4	45.4	28.2-72.9	36.0	27.3-57.6	0.315	n.a.
Insulin 2h [µU/ml]	35.6	22.8-61.3	35.1	20.4-56.6	17.2	11.9-60.0	0.256	n.a.
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*	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*	0.031*
MATSUDA	7.79	4.71-11.0	7.85	4.9-14.1	10.59	6.24-19.7	0.003*	0.015*

[a]Adjusted for age, BMI, 25(OH)D, month of blood sampling, serum calcium

*Significant for GG vs AA

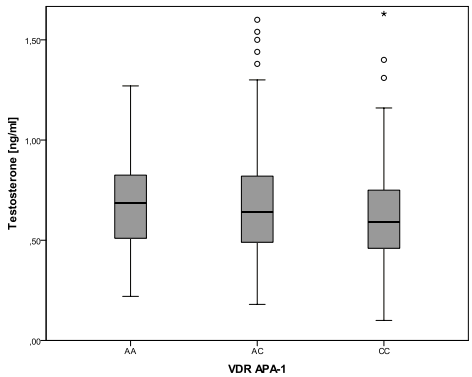
To convert 25(OH)D levels into nanomoles per liter, multiply by 2.5.

8.3.3 VDR Apa-I

In PCOS women, androgen levels were significantly different according to VDR *Apa-I* genotypes. In detail, testosterone (p=0.028; figure 13) and androstenedione (p=0.062; figure 14) levels were significantly higher in women carrying the AA allele when compared to PCOS women with the CC genotype (0.69 [0.51-0.82] ng/ml vs 0.59 [0.46-0.75] ng/ml and 3.0 [2.2-4.6] ng/ml vs 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.

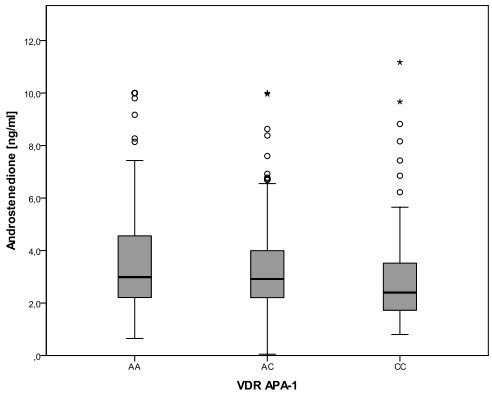
We did not observe an association of *Apa-I* polymorphism with metabolic parameters or 25(OH)D levels.

Figure 13: Testosterone levels in PCOS women according to Apa-I polymorphism.



p=0.028 for AA vs CC

Figure 14: Androstenedione levels in PCOS women according to Apa-I polymorphism.



p=0.028 for AA vs CC

8.3.4 VDR Bsm-I, Fok-I, Taq-I

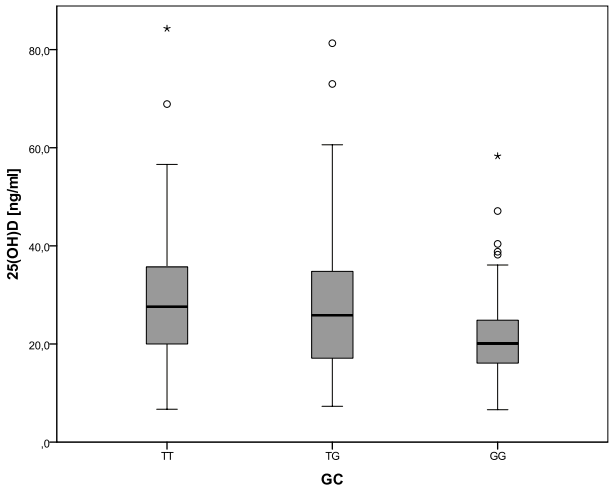
There was no association of VDR *Bsm-I*, *Fok-I*, and *Taq-I* polymorphisms with anthropometric, endocrine or metabolic parameters.

8.3.5 GC

We observed a significant association of GC polymorphism with 25(OH)D levels in crude (figure 15) 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 (figure 16). In binary logistic regression analyses calculating ORs for combined vitamin D insufficiency and deficiency (25[OH]D levels <30ng/ml), we found a significant higher risk in PCOS women carrying the GG allele when compared to TT carriers in the crude model (OR 2.47 [1.12-5.47]; p=0.026) as well as in the

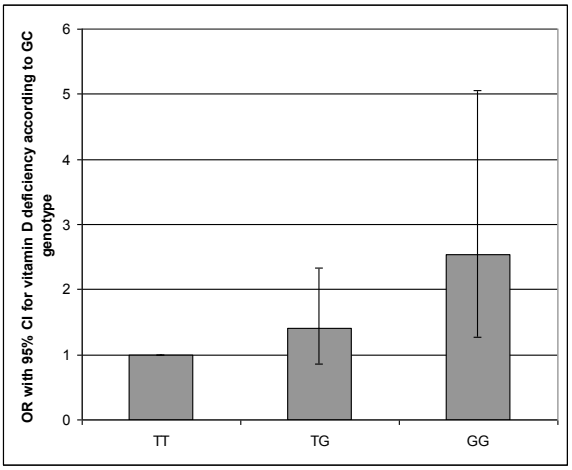
multivariate analysis adjusting for age, BMI and month of blood sampling (OR 2.36 [1.03-5.39]; p=0.042)

Figure 15: 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, respectively; p=0.027 for TT vs GG

Figure 16: ORs for vitamin D deficiency (<20ng/ml) according to GC genotype.



TT reference, OR 2.53 [1.27-5.06], p=0.009, adjusted for age, BMI, month of blood sampling

Moreover, weight, height, BMI, WC, TC, HDL, QChol/HDL and AUCins levels were significantly different within GC genotypes (table 8). 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, serum calcium).

Table 8: Anthropometric and metabolic parameters according to GC genotype.

	TT		**TG**		**GG**			
	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	p-value	p-value[a]
Weight [kg]	65	57-78	70	60-83	68	58-81	0.008*	0.023*
Height [cm]	165	162-170	168	163-171	165	161-170	0.027*	0.021*
BMI [kg/m²]	23.3	20.7-28.0	25.3	21.6-29.9	24.6	20.8-30.9	0.049*	0.056[b]
Waist circumference [cm]	79	72-90	81	73-96	83	74-101	0.047[#]	0.030[b#]
Hip circumference [cm]	103	95-111	105	99-114	106	96-115	0.094	n.a.
WHR	0.77	0.74-0-83	0.78	0.73-0.87	0.82	0.74-0.90	0.065	n.a.
PTH [pg/ml]	34.6	28.2-44.4	35.5	26.3-48.0	37.4	29.9-45.9	0.582	n.a.
Cholesterol [mg/dl]	182	158-204	174	155-197	171	152-152	0.008[#]	0.008[#]
Triglycerides [mg/dl]	81	56-116	76	57-101	77	56-95	0.462	n.a.
HDL [mg/dl]	67	56-79	60	51-76	68	62-76	0.005*[§]	0.030*[§]
QChol/HDL	2.70	2.10-3.50	2.80	2.35-3.4	2.50	2.10-3.00	0.017[§]	0.011[§]
AUCins	56. 8	35.9-88.5	65.3	47.9-100.6	48.6	36.9-36.9	0.048[§]	0.380[§]

[a]Adjusted for age, BMI, 25(OH)D, month of blood sampling, serum calcium

[b]Adjusted for age, 25(OH)D, month of blood sampling, serum calcium

*TT vs TG

[#]TT vs GG

[§]TG vs GG

8.3.6 DHCR7

We found a significant association of DHCR7 variants with 25(OH)D levels in crude (figure 17) as well as in multivariate adjusted analyses (adjusted for age, BMI, 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 (figure 18). In binary logistic regression analyses calculating ORs for combined vitamin D insufficiency and deficiency (25[OH]D levels <30ng/ml), we found no significant association with DHCR7 genotype.

Moreover, hip circumference, fasting glucose, glucose 2h, fasting insulin, insulin 2h, TC, TG, HOMA-IR, MATSUDA, QUICKI, and serum phosphate levels were significantly different within DHCR7 genotypes (table 9).

Table 9: Metabolic parameters according to DHCR7 genotype.

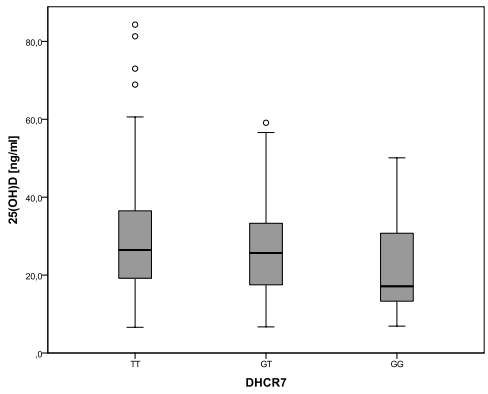
	TT		TG		GG		
	Median	Interquartile	Median	Interquartile	Median	Interquartile	p-value
Age [years]	27	23-31	27	24-31	28	22-31	0.914
Weight [kg]	65	58-78	69	60-82	63	57-80	0.121
Height [cm]	168	162-170	165	163-170	163	160-170	0.126
BMI [kg/m²]	23.80	20.55-27.7	24.77	21.88-30-11	23.71	20.96-28.2	0.073
Waist circumference [cm]	80	71-91	82	74-97	82	72-95	0.127
Hip circumference [cm]	102	96-110	105	99-116	103	96-112	0.025*
WHR	0.77	0.73-0.84	0.78	0.73-0.86	0.79	0.75-0.87	0.782
PTH [pg/ml]	33.20	26.90-44.2	37.50	29.50-46.8	38.10	28.9-48.8	0.240
Fasting glucose [mg/dl]	85	79-90	88	82-94	87	81-90	0.036*
Glucose 30 min [mg/dl]	137	119-153	133	117-150	137	107-154	0.363
Glucose 1h [mg/dl]	117	96-142	126	99-154	115	102-127	0.116
Glucose 2h [mg/dl]	94	81-108	103	88-122	100	86-123	0.010*
AUCgluc	170.3	150.8-195.3	181.0	159.0-207.0	171.0	154.3-184.8	0.117
Fasting insulin [µU/ml]	6.0	3.4-9.3	7.0	4.6-12-5	6.0	5.0-10.7	0.027*
Insulin 30 min [µU/ml]	43.6	32.9-69.1	37.7	26.7-70.1	48.1	17.8-83.7	0.630
Insulin 1h [µU/ml]	46.2	31.1-77.6	49.8	31.0-79.5	55.2	33.8-107.5	0.412
Insulin 2h[µU/ml]	28.5	19.0-56.6	38.8	24.0-65.4	47.0	29.3-67.1	0.006*
AUCins	61.2	39.9-83.1	59.2	36.6-92.3	68.1	40.9-108.2	0.879
HOMA-IR	1.23	0.73-1.95	1.62	0.93-2.81	1.46	1.05-2.38	0.020*
MATSUDA	9.09	5.80-13.77	6.91	4.00-10.38	6.71	4.43-12.3	0.006*
QUICKI	0.37	0.35-0.40	0.36	0.33-0.39	0.36	0.34-0.38	0.039*
HOMA-β	99.6	58.4-153.9	108.3	72.3-182.8	101.3	70.9-174.6	0.302
Calcium [mmol/l]	2.35	2.30-2.40	2.34	2.30-2.41	2.35	2.29-2-41	0.778
Phosphate [mmol/l]	1.09	0.94-1.19	1.03	0.89-1.14	1.05	0.96-1.17	0.022*
Cholesterol [mg/dl]	178	159-202	177	154-199	174	155-201	0.029#
Triglycerides [mg/dl]	76	55-96	82	58-121	63	56-100	0.024*
HDL [mg/dl]	68	56-77	64	52-77	63	52-83	0.353
QChol/HDL	2.70	2.20-3-30	2.70	2.20-3.50	2.60	2.10-3-30	0.598
LDL [mg/dl]	98	83-117	96	79-116	88	75-116	0.435

*TT vs TG

#TT vs GG

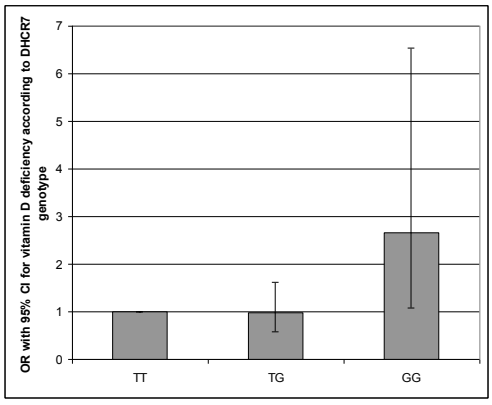
In multivariate adjusted analyses (age, BMI, PTH, month of blood sampling, serum calcium), the association of DHCR7 with 2h insulin remained significant, whereas the other association (fasting glucose, fasting insulin, MATSUDA, HOMA-IR, and QUICKI) lost significance.

Figure 17: 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, respectively; p=0.006 for TT vs GG.

Figure 18: ORs for vitamin D deficiency (<20ng/ml) according to DHCR7 genotype.



TT reference, OR 2.66 (1.08-6.55), p=0.033, adjusted for age, BMI, month of blood sampling.

8.3.7 CYP2R1

25(OH)D levels were similar within 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 towards an increased risk for vitamin D deficiency in PCOS women carrying the GG genotype when compared to 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 <30ng/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).

8.3.8 Combined analyses

We did 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 women presented with all 3 risk genoytpes, we combined PCOS women with 2 (8.3% of all women) and 3 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.

9 Discussion

9.1 Cross-sectional

Our results indicate that low 25(OH)D levels are significantly associated with components of the metabolic syndrome and insulin resistance in women with PCOS. 25(OH)D was an independent predictor of insulin resistance and insulin sensitivity in a multivariate regression analysis.

Low 25(OH)D levels have been linked to an increased risk for cancer (48), autoimmune diseases, diabetes and cardiovascular diseases (15, 48, 49) indicating the importance of sufficient 25(OH)D levels. Although there is no consensus on optimal levels of 25(OH)D, a level of 30ng/ml can be considered to indicate sufficient vitamin D status (15). In our study, 72.8% of PCOS women showed 25(OH)D values below this recommended level.

Our data demonstrate a significant association of low 25(OH)D levels with increased levels of fasting and stimulated glucose, AUCgluc, HOMA-IR, and fasting and stimulated insulin. Accordingly, Hahn et al. reported an association of low 25(OH)D levels with insulin resistance in 120 PCOS women (50). Apart from these cross-sectional findings, there is one small prospective intervention study with vitamin D supplementation that demonstrates beneficial effects of vitamin D on insulin secretion and serum lipids in PCOS women (51). In non-PCOS cohorts including subjects with various BMI, vitamin D concentration was inversely related to the prevalence of diabetes (52), plasma concentrations of glucose (53), insulin resistance (53, 54) and the metabolic syndrome (54, 55). Besides, the risk of future hyperglycemia and insulin resistance was associated with hypovitaminosis D (56).

The mechanisms underlying the association of low 25(OH)D levels and insulin resistance 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 (55). The vitamin D responsive element is present in the promoter of the human insulin gene (57) and the transcription of the human insulin gene is activated by $1,25(OH)_2D_3$ (58). Second, vitamin D regulates extracellular and intracellular calcium which is essential for insulin-mediated intracellular processes in insulin-responsive tissues such as skeletal muscle and adipose tissue (55). Moreover, alterations in calcium flux can have adverse effects on insulin secretion, which is a calcium-dependent process (59). Finally, as vitamin D has a modulating effect on the immune system (60), hypovitaminosis D might induce a higher inflammatory response, which is again associated with insulin resistance (61). This hypothesis is supported by the results of our study indicating an association of low 25(OH)D with increased CRP levels .

In turn, an additional mechanism might be seen in impaired beta cell function in PCOS women. This is underlined by our finding of a negative association of 25(OH)D levels and HOMA-β and the inverse association of stimulated glucose levels and AUCgluc with 25(OH)D.

A new finding in our study was the association of increased triglycerides and QChol/HDL with low 25(OH)D levels. Moreover, we found low 25(OH)D levels associated with low levels of HDL, confirming previous results in PCOS women (50), but contrasting the lack of association in a recent study in a cohort of healthy young women (62). In a pilot study, Kotsa et al. showed an improvement of HDL and triglycerides after treatment with vitamin D in a small cohort of PCOS women (51). Since dyslipidemia should be considered as an additional

therapeutic target in PCOS (63), vitamin D might be useful in the complex treatment of PCOS women.

These parameters, i.e. low HDL and elevated triglycerides, are central features of the metabolic syndrome. For the first time, we observed an association of low 25(OH)D levels with the metabolic syndrome independently from BMI, age, and seasonal variation of 25(OH)D in PCOS women.

Nevertheless, our data suggest a strong relationship of 25(OH)D and BMI in PCOS women which is in agreement with previous studies (50, 64). So far, it is not clear whether vitamin D insufficiency results from obesity and/or if obesity is a consequence of vitamin D insufficiency. On the one hand, obesity may contribute to low circulating vitamin D levels by trapping vitamin D in fat tissues. Wortsman et al. demonstrated that the increase of 25(OH)D levels 24 h after whole-body UV-light exposure was 57% lower in obese than in nonobese subjects (65). On the other hand, obese patients may avoid sunlight, which is necessary for the synthesis of vitamin D in the skin (66). This might be especially the case in hirsute PCOS women, who tend to hide from the public due to their appearance. There is evidence that low vitamin D levels are associated with obesity (50) and vice-versa low vitamin D intake might be an independent predictor of obesity (67).

Recent data from an overweight/obese women's study suggested that women with high 25(OH)D levels respond more positively to hypocaloric diets and lose more body fat than women with low 25(OH)D levels (68). There is evidence that weight loss is probably the most effective treatment of PCOS women at the moment (69). Thus, vitamin D supplementation might be an element in the complex treatment of PCOS women. This hypothesis is supported

by the findings of our study indicating the association of low 25(OH)D levels with obesity, insulin resistance, and the metabolic syndrome in PCOS women.

In our study, there was no correlation of 25(OH)D levels with FAI, total testosterone or free testosterone. Our observations are in line with a previous study that did not find any differences among PCOS and healthy control women with respect to 25(OH)D levels (70). One previous report on a correlation between vitamin D levels and FAI (50) could be mediated through the obesity induced reduction of SHBG. On this note, the significant correlation between SHBG and 25(OH)D in our patients was abolished when controlling for BMI. However, we found an inverse correlation of 25(OH)D with hirsutism score that was independent of BMI, which is in line with previous studies (50, 71). In addition, 25(OH)D levels were significantly lower in hirsute women, which is consistent with the results from a small study in hirsute women (71). This association might be caused by various mechanisms. First, the cosmetic distress may cause hypovitaminosis D due to the decreased sun exposure of hirsute women, as mentioned above. Second, the vitamin D receptor is found in keratinocytes of the outer root sheath as well as in cells of the bulge indicating an important role of vitamin D in hair follicle cycling (60). However, the mechanism by which the vitamin D receptor regulates hair follicle cycling and its potential role in hirsutism remains unclear.

To the best of our knowledge, this is the first study describing an inverse association of low 25(OH)D levels with impaired β cell function, impaired glucose tolerance, and the metabolic syndrome in PCOS women. Further, we confirm previous findings reporting the relation of low 25(OH)D levels with obesity and insulin resistance women with PCOS. To prove these findings and to find new therapeutic approaches, intervention trials with vitamin D supplementation are warranted in PCOS women.

9.2 Intervention

Our 24 week pilot trial demonstrated that weekly supplementation of 20000 IU vitamin D3 orally administered results in a significant improvement of glucose metabolism and menstrual pattern in a relatively large cohort of PCOS women. Moreover, triglyceride levels significantly decreased whereas total cholesterol and LDL cholesterol increased after vitamin D treatment.

Low 25(OH)D levels have been linked to adverse health outcome (15, 48, 49) indicating the importance of sufficient 25(OH)D levels. In line with epidemiological findings, first interventional studies demonstrate that vitamin D supplementation may reduce various chronic diseases and mortality (72-74). Moreover, there is evidence suggesting that vitamin D and calcium deficiency influences postprandial glycemia and insulin response while supplementation may be beneficial in optimizing these processes (55). In a study cohort including patients with impaired fasting glucose, combined vitamin D3 and calcium carbonate supplementation attenuated the increase in fasting glycemia and insulin resistance that normally occurs in that population over time (75). In contrast, others failed to show an improvement of glycemia after vitamin D treatment (44, 76).

Apart from cross-sectional findings (50) demonstrating an association of hypovitaminosis D and insulin resistance, one small prospective intervention study with alphacalcidol administration demonstrates a beneficial effects of vitamin D on first phase insulin secretion and serum lipids in PCOS women (51). Another small study in 11 obese insulin resistant PCOS women, supplementation with a single dose of 300.000 IU vitamin D3 leads to a significant decrease of HOMA-IR (77), whereas our data indicates a significant decrease in

glucose and C-peptide levels without a change in HOMA-IR. However, our study participants had a lower BMI and lower HOMA-IR levels at baseline.

The role of vitamin D supplementation in lipid metabolism is ambiguous. We observed an improvement of triglyceride levels, which is in line with a small pilot study showing an improvement of HDL and triglycerides after treatment with vitamin D in a cohort of PCOS women (51). This might be explained by the suppressive effect of vitamin D on serum PTH concentrations. Because elevated PTH concentrations are accompanied by a decrease in plasma postheparin lipolytic activity (78), the reduction in serum PTH in our study may have decreased serum triglycerides via increased peripheral removal. In contrast, there was an adverse change in total and LDL cholesterol levels, which has also been reported during a study investigating the effect of vitamin D during weight loss (44). However, data from the Women's Health Initiative indicate that Vitamin D plus calcium supplementation is not associated with lipid changes over 5 years (79).

There is accumulating evidence that vitamin D plays an important role in reproductive processes, which is further supported by our data showing an improvement of menstrual pattern in about 50 % of PCOS women. In a study among 84 infertile women undergoing in vitro fertilization, women with higher levels of 25(OH)D in serum and follicular fluid were significantly more likely to achieve clinical pregnancy following in vitro fertilization and high vitamin D levels were significantly associated with improved parameters of controlled ovarian hyperstimulation (29). In a small intervention study including 13 premenopausal women with chronic anovulation and hyperandrogenism, vitamin D repletion with ergocalciferol combined with calcium administration resulted in normalization of menstrual cycles in 7 women and 2 became pregnant (28). In contrast, in a pilot study among 13 women with PCOS, the

administration of the single dose of 300.000 units of vitamin D3 orally did not significantly change the levels of total and free testosterone (77), which is in line with data from our study. Interestingly, we observed a trend towards decreased FAI levels. One might speculate that the current study is underpowered to detect small effects of vitamin D treatment on androgen status in PCOS women. This notion is supported by the fact that vitamin D supplementation leads to an improvement of acne vulgaris in a small intervention study including 13 premenopausal women with chronic anovulation and hyperandrogenism (28), indicating an influence of vitamin D on androgen status. Moreover, in a study among 100 women with PCOS, the authors observed a correlation of 25(OH)D levels with testosterone and DHEAS levels (80). Further, a significant association of 25(OH)D levels with hirsutism score (81) and FAI (50) in PCOS women has been described. This association might be driven by the relation of vitamin D with insulin resistance and obesity; the exact underlying mechanisms, however, remain to be explored. Interestingly, in our study estradiol levels significantly decreased after 12 weeks of vitamin D treatment, confirming data from a study including 101 young volunteer women taking vitamin D supplements (82). Importantly, low vitamin D levels are associated with a high risk of breast cancer (83), that is hormonally related and likely to be influenced by estrogens (84).

Our data showing an improvement of menstrual frequency and fertility of PCOS women after vitamin D treatment are supported by several facts. VDR has been found in ovary, endometrium and placenta (21). It has been shown that vitamin D deficiency reduces mating success and fertility in female rats (22), both VDR and 1α-hydroxylase knockout female mice are infertile and present with uterine hypoplasia, impaired folliculogenesis, and anovulation (21, 23, 24). Moreover, vitamin D is the key regulating hormone in calcium homeostasis. It

has been shown that calcium plays a role in oocyte activation and maturation resulting in the progression of follicular development (27).

Our study has several limitations that should be noted. The main limitation of our work is the lack of a placebo group in this is uncontrolled pilot trial, thus the results have to be interpreted with caution. Second, our study population was relatively lean without severe insulin resistance and our findings may therefore not be generalizable to other PCOS cohorts including women who are more obese or insulin resistant. As obesity is associated with hypovitaminosis D (81), the impact of vitamin D treatment might be stronger in an obese PCOS cohort. Moreover, 25(OH)D levels were high at baseline which might attenuate the effects of vitamin D replacement. Further, we did not perform quantified food records or structured interviews and earlier fat intake in a 24-hour-time might have influenced lipid parameters and might explain the relatively great SD of triglycerides at baseline.

In conclusion, our study results suggest that vitamin D treatment might improve glucose metabolism and fertility patterns in PCOS women. Further randomized controlled trials are warranted to confirm our findings and to evaluate the role of vitamin D in glucose metabolism and female reproduction in PCOS women.

9.3 Genetics

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 *Cdx-2* and DHCR7 gene are associated with insulin resistance 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. 25(OH)D levels were inversely associated with insulin resistance and positively associated with insulin sensitivity.

Polymorphisms of the VDR gene might be associated with PCOS and biochemical markers related to PCOS (85, 86). 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 (87). In contrast, *Cdx-2* acts as a transcription factor of VDR (88) and VDR regulates the transcription of about 3% of the human genome (15). Yamamoto et al. (89) first described a functional binding site for the intestinal-specific transcription factor *Cdx-2* in the 1a promoter region of the VDR gene. The G to A substitution, that was investigated in our study, was first described by Arai et al (88) and was found to modulate the intestine specific transcription of the VDR gene. The *Cdx-2* 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 (15, 55).

Whereas the VDR *Cdx-2* polymorphism has been shown to be associated with BMD and fracture risk (87), the association with metabolic parameters has not been investigated so far. It can be hypothesized that VDR *Cdx-2* A allele carriers have higher intestinal calcium absorption, because of the elevated expression of intestinal calcium channel proteins (90). 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 insulin resistance and type 2 diabetes in PCOS (50, 81) as well as in other cohorts (55). Likewise, we observed a correlation of 25(OH)D levels with insulin and glucose metabolism related parameters in this study. The exact mechanisms underlying the association of vitamin D and insulin resistance are not fully understood.

Interestingly, Mahmoudi et al. (85) 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. Notably, 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 (81) and others (50) showed an association of 25(OH)D and free androgen index levels in PCOS. Of note, 25(OH)D levels are correlated with androgen levels in men and display a similar seasonal variation (91). Thus, one might speculate on an association of vitamin D with androgens. The underlying mechanisms, however, remain to be explored.

Although there is no consensus on optimal levels of 25(OH)D, a level of 20ng/ml has been suggested to cover the requirements of at least 97.5% of the population according to the latest public health report (92). Others (93), 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 (94), we observed a strong independent association of GC “GG” and DHCR7 “GG” genotype with risk for vitamin D deficiency. The detection of those harmful 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 (95). 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 personalised approach to therapy.

GC gene encodes a multifunctional plasma protein GC, also known as group-specific component, or 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 (96). 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 (96). 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 (81) as well as in other cohorts (41), the relationship with VDBP is, however, less clear. Taes et al (97) 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-dehydrocholestererol (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 (98). 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 insulin resistance 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 (99). Whereas results from the large genome-wide association study (94) 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 in 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 Cdx-2 and DHCR7 gene are associated with insulin resistance and insulin sensitivity and VDR Apa-I variants are associated with testosterone levels in PCOS women. Moreover, we confirmed 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 (81).

10 References

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