

CLINICAL STUDY

Parameters for calcium metabolism in women with polycystic ovary syndrome who undergo clomiphene citrate stimulation: a prospective cohort study

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Abstract

Objective: To evaluate whether parameters for calcium metabolism were associated with characteristics of polycystic ovary syndrome (PCOS).

Design: A prospective cohort study.

Methods: Ninety-one anovulatory, infertile women with PCOS patients underwent clomiphene citrate (CC) stimulation. Main outcome measures were parathyroid hormone (PTH); 25-hydroxyvitamin D3 (25OHD3); serum levels of calcium, phosphorus, magnesium, albumin, and total protein; the serum calcium–phosphorus product; LH; FSH; sexual hormone binding globulin; testosterone; and androstenedione.

Results: PTH correlated inversely with serum calcium ($r = -0.235$; $P = 0.004$) and 25OHD3 ($r = -0.664$; $P < 0.001$), whereas positive correlations were found between PTH and body mass index (BMI; $r = 0.270$; $P = 0.010$) and between PTH and testosterone ($r = 0.347$; $P = 0.001$). After stimulation with 50 mg CC, 57.1% (52/91) developed a follicle, whereas 26.4% (24/91) became pregnant. In a multivariate model to predict both follicle development and pregnancy, BMI and 25OHD3 deficiency were significant predictive parameters.

Conclusions: 25OHD3 deficiency was an independent predictive parameter of CC stimulation outcome, in terms of follicle development and pregnancy. Our results suggest a substantial role of vitamin D in PCOS and infertility treatment in these patients.

European Journal of Endocrinology 166 897–902

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common female endocrinopathies (1). It has recently been demonstrated that elevated levels of phosphorus and parathyroid hormone (PTH) might be involved in the pathogenesis of the syndrome, possibly through their effects on insulin levels and insulin resistance. In women with PCOS, phosphorus was correlated negatively with insulin and insulin resistance and positively with 25-dihydroxyvitamin D3 (25OHD3) (2). Recently, it has been demonstrated that other parameters of bone metabolism, namely lower osteocalcin and elevated serum levels of its carboxylated form, were associated with androgen levels, insulin resistance, and ovarian morphology in PCOS women (3). These findings suggest a potential interaction between bone-derived markers and the metabolic/hormonal abnormalities observed in PCOS.

For infertile, anovulatory PCOS patients, clomiphene citrate (CC) is typically the first-line therapy (4). Several factors have been reported to predict fertility success

after CC stimulation, including body mass index (BMI) and Hashimoto's thyroiditis, among others (5, 6). However, it has not yet been clarified why some patients respond to CC while others do not.

Thus, the objective of our study was to confirm previous observations that parameters for calcium metabolism were associated with characteristics of PCOS. This included outcome after CC stimulation, i.e. follicle maturation and pregnancy.

Materials and methods

Study population

In a prospective cohort study, we evaluated possible associations between parameters for calcium metabolism and the characteristics of anovulatory women with PCOS who were undergoing CC stimulation. In this analysis, we included 91 anovulatory, infertile women with PCOS who were undergoing 123 cycles of CC stimulation at our department from December 2010

until May 2011. PCOS was diagnosed according to the revised European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) criteria of 2004 (4). Exclusion criteria were: previous CC stimulation or laparoscopic ovarian drilling; hyperprolactinemia; manifest hyperparathyroidism; previous thyroid or parathyroid surgery, as normal PTH levels do not exclude postoperative parathyroid dysfunction in these patients (7); current vitamin D or calcium supplementation; and concomitant male, tubal, and uterine factors of infertility. All women had undergone at least one cycle of monitoring, as described below, to document complete anovulation as part of the clinical routine at our department.

Study design

As the main outcome measures, we included parameters of calcium metabolism (PTH; 25-hydroxyvitamin D3 (25OHD3) with 25 nmol/l defining the lower limit of adequacy of vitamin D status (8) and 30 nmol/l defining a second cutoff point (9); serum calcium; serum phosphorus; the serum calcium-phosphorus product, as circulating calcium and phosphate concentrations are correlated inversely and the serum calcium-phosphorus product/precipitation of calcium and phosphate have been mentioned as a clinically important parameter in bone and calcium metabolism (10); serum magnesium; serum albumin; and total serum protein) and the PCOS (LH; FSH ratio; sexual hormone binding globulin (SHBG); testosterone; androstenedione; and insulin resistance as calculated from the indices homeostatic model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI)). The calculation of indices HOMA-IR and QUICKI was based on the equations: $HOMA-IR = \text{fasting insulin} \times \text{fasting glucose} / 22.5 \times 18$; $QUICKI = 1 / \log(\text{fasting insulin}) + \log(\text{glucose})$ (11, 12). Patients with both $HOMA-IR \geq 2.5$ and $QUICKI \leq 0.333$ were considered insulin resistant ≤ 0.333 (13). Blood was drawn on the third to the fifth day of the menstrual cycle before the initiation of CC stimulation. All serum parameters were determined using commercially available assays.

As possible predictive factors for CC stimulation outcome, the following parameters were included: age, BMI, insulin resistance, testosterone, and androstenedione (14); the LH:FSH ratio, PTH, serum calcium, 25OHD3, and 25OHD3 deficiency were new candidates for predicting the success of CC stimulation.

Menstrual bleeding was induced with oral administration of 20 mg dydrogesterone for 10 days. CC stimulation began with a dose of 50 mg/day from the fifth to the ninth day of the menstrual cycle. Beginning with the ninth day of the menstrual cycle, all patients underwent daily monitoring (including vaginal ultrasound for detection of follicular cysts and determination

of morning LH, FSH, estradiol, and progesterone for documentation of ovulation) until a postovulatory rise in progesterone was seen. If ovulation did not occur despite the development of a large follicle, follicle rupture was induced using human chorionic gonadotrophin (15). The success of CC stimulation was defined as the development of a large follicle with a minimum diameter of ≥ 18 mm. The study was approved by the Institutional Review Board of the Medical University of Vienna (IRB number: 755/2010). Written informed consent was obtained from all patients.

Statistical analyses

Variables are described by median and ranges. For all tests, the open source statistical package R (R Project for Statistical Computing, Vienna, Austria) was used. To compare the two groups of both outcome variables (development of a follicle after CC stimulation, pregnancy), continuous variables were tested using Shapiro Wilk test of normality and, in case of normality, a Welch two-sample *t*-test was performed, whereas the Wilcoxon signed rank test with continuity correction was used in non-normal cases. To evaluate the influence of abnormal insulin resistance and hypovitaminosis D, Fisher's exact test was used. Factors that were found to differ significantly for one of the two outcome variables were additionally tested in a multivariate logistic regression model to predict both outcome parameters. Nagelkerke's R^2 test was used to assess goodness-of-fit, that is, how well the data were described by the model tested. It corresponds to a scaled Cox and Snell R^2 . Nagelkerke R^2 can range from 0 to 1. To compare different multivariate models, the likelihood ratio test was used. For correlations, a Spearman's rank test was used.

Patients were subdivided according to the following weight categories: underweight (BMI < 18.50 kg/m²), normal weight (BMI 18.50–24.99 kg/m²), overweight (BMI 25.00–29.99 kg/m²), and obese (BMI ≥ 30.0 kg/m²; World Health Organization, BMI classification; available online at http://apps.who.int/bmi/index.jsp?introPage=intro_3.html). Differences in calcium metabolism parameters between these groups were calculated using the Cochran–Armitage test for a linear trend in proportions, the Jonckheere–Terpstra test for increasing trend or decreasing trend.

Differences were considered statistically significant if $P < 0.05$.

Results

Patient characteristics and basic findings

Details about patient characteristics at the initial visit are provided in Tables 1 and 2. All patients revealed PTH, serum calcium, phosphorus, serum calcium-phosphorus product, serum magnesium, serum albumin, and total

Table 1 Patient characteristics at initial visit.

Characteristics	Values
Total number of patients (<i>n</i> (%))	91
Age (years; median (range))	29 (19–38)
Body mass index (kg/m ² ; median (range))	27.3 (17.7–40.6)
Duration of infertility (months; median (range))	26.5 (12.0–57.0)
Patients with primary/secondary infertility (<i>n</i> (%))	55 (60.4)/36 (39.6)
Insulin-resistant patients (<i>n</i> (%))	33 (36.3)
Obese patients (BMI ≥30 kg/m ² ; <i>n</i> (%))	19 (20.9)
Patients on metformin treatment (<i>n</i> (%))	91 (100.0)
Patients with polycystic ovaries in ultrasound (<i>n</i> (%))	24 (26.4)

serum protein within the institutional normal ranges. 25OHD3 levels below the normal range (i.e. <25 nmol/l) were found in 39 patients (42.9%). PTH correlated inversely with serum calcium ($r = -0.235$; $P = 0.004$), 25OHD3 ($r = -0.664$; $P < 0.001$), and SHBG ($r = -0.337$; $P = 0.005$), whereas positive correlations were found between PTH and BMI ($r = 0.270$; $P = 0.010$), as well as between PTH and testosterone ($r = 0.347$; $P = 0.001$). No significant correlations were found between PTH and androstenedione, LH, or FSH ($P > 0.05$).

When comparing insulin-resistant (33/91, 36.3%) with noninsulin-resistant patients (58/91, 63.7%), no differences were found for PTH (45.6 (16.9–63.5) vs 39.2 (20.9–64.8) pg/ml; $P = 0.370$), 25OHD3 (12.0 (0–63.8) vs 26.2 (0–106.6) nmol/l; $P = 0.206$), and serum phosphorus levels (1.1 (0.9–1.5) vs 1.1 (0.8–1.3) mmol/l; $P = 0.172$). However, serum calcium was higher in the noninsulin-resistant than in the insulin-resistant group (2.5 (2.3–2.7) vs 2.4 (2.3–2.5) mmol/l; $P = 0.043$).

Patients were subdivided according to their BMI (Table 3). Overweight and obese patients were significantly more often insulin resistant and revealed significantly higher PTH and lower 25OHD3 levels.

Prediction of outcome after CC stimulation

After stimulation with 50 mg CC, 57.1% (52/91) developed a follicle, whereas 26.4% (24/91) became pregnant. When comparing women who had developed a follicle with those who were resistant in univariate analysis, significant differences were found for age, 25OHD3 levels, and 25OHD3 deficiency <25 nmol/l (Table 4). Differences between pregnant and non-pregnant women were found for age, BMI, LH:FSH ratio, 25OHD3 levels, and 25OHD3 deficiency <25 and 30 nmol/l (Table 3).

These parameters were included in multivariate models to predict both follicle development and

pregnancy. To test the influence of the vitamin D status, 25OHD3 deficiency <25 nmol/l was chosen (Table 5). BMI and 25OHD3 deficiency contribute to follicle development, whereas age, BMI, and 25OHD3 deficiency were predictive for pregnancy. In Nagelkerke's R^2 , the proportion of variability explained by the models was 0.27 for follicle development and 0.37 for pregnancy.

For both outcome parameters, the above-mentioned multivariate analyses were compared with multivariate models including only age and BMI (data not shown). In likelihood ratio tests, significant differences were found for follicle development ($P = 0.047$) and pregnancy ($P = 0.030$), indicating that 25OHD3 deficiency provides significant explanatory values and, thus, cannot be omitted.

Discussion

The inverse correlation between PTH and serum calcium, as well as the fact that all other calcium-linked parameters apart from 25OHD3 were within the normal ranges, indicated normal function of calcium metabolism in all patients at the initial visit. Notably, PTH was inversely correlated with SHBG. The same was found for SHBG and BMI (data not shown), whereas PTH and BMI were correlated positively. It has already been mentioned that SHBG is influenced by the BMI (16). Our data partly confirm previous observations in PCOS women, including the positive correlation between PTH and testosterone as well as the fact that overweight and obese patients revealed significantly lower 25OHD3 levels (2, 17). However, increased PTH levels were not associated with insulin resistance in our data, in contrast to lower serum calcium levels. This might be explained by the fact that we did not analyze normal-weight patients separately. Although not statistically significant, lower 25OHD3 levels were found for insulin-resistant compared with noninsulin-resistant women. One might consider

Table 2 Biochemical profile of patients at initial visit. The data are presented as median (range).

Parameters	Normal range	Value
LH (IU/l)	^a	7.8 (0.7–18.7)
Testosterone (ng/ml)	<0.5	0.5 (0.1–1.1)
Androstenedione (ng/ml)	0.3–3.3	2.8 (1.0–4.2)
Parathyroid hormone (pg/ml)	15.0–65.0	41.5 (16.9–64.8)
25-OHD3 (nmol/l)	28.0–107.0	34.7 (0–106.6)
Serum calcium (mmol/l)	2.2–2.7	2.5 (2.3–2.7)
Total serum protein (g/l)	66–83	67.5 (49.2–91.7)
Serum albumin (g/l)	34.0–48.0	45.1 (40.2–52.9)
Serum magnesium (mmol/l)	0.7–1.0	0.9 (0.8–1.0)
Serum phosphorus (mmol/l)	0.8–1.5	0.9 (0.8–1.5)
Calcium–phosphorus product (mmol ² /l ²)	–	2.2 (1.7–3.7)

^aCycle dependent.

Table 3 Parameters of calcium metabolism according to BMI category. Weight categories are defined as follows: underweight, BMI < 18.50 kg/m²; normal weight, BMI 18.50–24.99 kg/m²; overweight, BMI 25.00–29.99 kg/m²; and obese, BMI ≥ 30.0 kg/m².

	Underweight (n=2)	Normal weight (n=25)	Overweight (n=44)	Obese (n=20)	P
Insulin resistance (n, %)	0 (0) ^a	1 (4.0) ^a	19 (43.2) ^a	13 (65.0) ^a	<0.001 ^b
Parathyroid hormone (pg/ml)	43.2 (38.9–47.4) ^c	35.6 (16.9–64.6) ^c	42.6 (17.1–64.8) ^c	46.9 (25.5–63.4) ^c	0.006 ^d
Serum calcium (mmol/l)	2.4 (2.3–2.5) ^c	2.5 (2.3–2.6) ^c	2.5 (2.3–2.7) ^c	2.4 (2.3–2.6) ^c	0.606 ^d
25-OHD3 (nmol/l)	68.9 (28.8–105.0) ^c	39.0 (0–106.6) ^c	24.0 (0–103.0) ^c	26.4 (0–47.8) ^c	0.016 ^e
25-OHD3 < 25 nmol/l	0 (0) ^a	8 (32) ^a	23 (52.3) ^a	8 (40.0) ^a	0.193 ^b
25-OHD3 < 30 nmol/l	1 (50.0) ^a	11 (44.0) ^a	29 (43.2) ^a	12 (60.0) ^a	0.135 ^b

^aValues are given as median and ranges.^bCochran–Armitage test for a linear trend in proportions.^cValues are given as absolute numbers and percentage.^dJonckheere–Terpstra test for increasing trend.^eJonckheere–Terpstra test for decreasing trend.

a larger number of subjects necessary in order to demonstrate a significant association between these groups of patients.

However, in our data set, PTH and serum calcium were not predictive for CC stimulation outcome. Instead, 25OHD3 seems to play a major role: low 25OHD3 levels and 25OHD3 deficiency (< 25 nmol/l) were found to be associated with lower rates of follicle development and pregnancy after stimulation with 50 mg CC. To the best of our knowledge, we are the first to demonstrate this phenomenon. The physiological mechanisms are unclear. Experimental studies have demonstrated that the ovary is a target organ for 1,25-dihydroxyvitamin D3 raising the possibility that this active metabolite of vitamin D3 might play a role in modulating ovarian activity (18). It has also been proposed that variants in the vitamin D receptor through their effect on LH, SHBG levels and testosterone are involved in the pathogenesis of PCOS (19, 20).

The fact that higher 25OHD3 levels were found for women who had developed a follicle and for women who became pregnant is concordant with the observation that patients who had received supplementation with calcium and vitamin D were more likely to

spontaneously develop large dominant follicles (21). Moreover, it has been shown, in a small sample size, that PCOS-associated signs of hyperandrogenism were alleviated after administration of high doses of vitamin D (22). Recently, serum calcium levels have been suggested to play a possible role in follicle selection, as calcium receptors have been found to be expressed in preovulatory granulosa explants (23). It has also been mentioned that higher vitamin D levels in serum and follicular fluid would be associated with increased clinical pregnancy rates in women who undergo *in vitro* fertilization (24). As recently reviewed by Lerchbaum & Obermayer-Pietsch, animal studies have demonstrated that 1,25-dihydroxyvitamin D3 significantly increases uterine weight. Moreover, it is thought to play a physiological role in endometrial cell differentiation into decidual cells, which is crucial in the process of blastocyst implantation (25). Taken together, these findings suggest a possible role of vitamin D supplementation in infertile PCOS women who undergo ovarian stimulation.

Other factors predictive for CC stimulation outcome were a lower BMI and an older age. This is in accordance with previous observations (13, 26). It has

Table 4 Variables associated with induction of follicle development and gravidity in univariate analysis.

Variable	Follicle development			Pregnancy		
	Yes (n=52)	No (n=39)	P	Yes (n=24)	No (n=67)	P
Age (years)	30 (19–38) ^a	27 (19–38) ^a	0.007	30 (21–37) ^a	28 (19–38) ^a	0.008
Body mass index (kg/m ²)	26.1 (17.7–39.5) ^a	27.5 (19.5–40.6) ^a	0.065	26.2 (17.8–39.5) ^a	27.4 (17.7–40.6) ^a	0.017
Insulin resistance	17 (32.7)	16 (41.0)	0.510	7 (29.2)	26 (38.8)	0.465
LH:FSH ratio	1.2 (0.2–4.5) ^a	1.6 (0.3–3.9) ^a	0.060	0.9 (0.3–4.4) ^a	1.4 (0.3–4.5) ^a	0.043
Testosterone (ng/ml)	0.4 (0.1–0.8) ^a	0.5 (0.2–1.1) ^a	0.360	0.5 (0.2–0.7) ^a	0.4 (0.1–1.1) ^a	0.736
Androstenedione (ng/ml)	2.9 (1.0–5.95) ^a	2.7 (1.4–4.2) ^a	0.259	2.3 (1.01–5.95) ^a	2.9 (1.4–4.2) ^a	0.090
Parathyroid hormone (pg/ml)	41.3 (16.9–64.8) ^a	43.4 (17.8–63.5) ^a	0.411	38.9 (17.7–60.7) ^a	44.9 (16.9–64.8) ^a	0.059
Serum calcium (mmol/l)	2.5 (2.3–2.7) ^a	2.5 (2.3–2.6) ^a	0.101	2.5 (2.3–2.7) ^a	2.5 (2.3–2.6) ^a	0.929
25-OHD3 (nmol/l)	29.9 (0–106.6) ^a	16.5 (0–99.7) ^a	0.014	44.3 (10.8–106.6) ^a	20.4 (0–99.7) ^a	< 0.001
25-OHD3 < 25 nmol/l	15 (28.8) ^b	24 (61.5) ^b	0.003	4 (16.7) ^b	35 (52.2) ^b	0.003
25-OHD3 < 30 nmol/l	26 (50.0) ^b	27 (69.2) ^b	0.086	9 (37.5) ^b	44 (65.7) ^b	0.029

P values in bold indicate statistical significance.

^aValues are given as median and ranges.^bValues are given as absolute numbers and percentages.

Table 5 Variables associated with induction of follicle development and gravidity in multivariate analysis.

Variable	Follicle development		Pregnancy	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Age	1.15 (1.02–1.29)	0.059	1.18 (1.05–1.40)	0.018
Body mass index	0.85 (0.76–0.96)	0.024	0.84 (0.73–0.98)	0.009
LH:FSH ratio	0.60 (0.31–1.17)	0.359	0.67 (0.29–1.21)	0.221
25-OHD3 <25 nmol/l	0.33 (0.13–0.85)	0.022	0.24 (0.07–0.84)	0.026

95% CI, 95% confidence interval. P values in bold indicate statistical significance.

been suggested that the association of hypovitaminosis D with features of PCOS may be associated with obesity but not with the presence of PCOS (27). In our data set, 25OHD3 deficiency remained significant in multivariate analyses. Furthermore, likelihood tests proved that 25OHD3 deficiency significantly increased the predictive values of the multivariate models. This suggests 25OHD3 as an independent predictive factor for CC stimulation outcome. All in all, the explained deviances according to Nagelkerke's R^2 were 27% for follicle development and 37% for pregnancy. This indicates that a large fraction of the deviance was explained by the logistic regression models using only three parameters, i.e. age, BMI, and 25OHD3 deficiency. We consider the model consistent. This is substantiated by the similar odds ratios of univariate and multivariate analyses.

In conclusion, our data confirm the positive correlation between PTH and testosterone in anovulatory patients with PCOS. 25OHD3 deficiency was an independent predictive parameter of CC stimulation outcome, in terms of follicle development and pregnancy. Our results suggest a substantial role of vitamin D in PCOS and infertility treatment in these patients. However, one might argue that actually a relationship between calcium metabolism and fecundability rather than PCOS *per se* was observed. Thus, the impact of vitamin D and calcium metabolism should also be evaluated in non-PCOS women who undergo CC stimulation. Further studies are warranted to clarify the underlying mechanisms and evaluate possible associated therapeutic options. One treatment approach would be supplementation of vitamin D in deficient patients. This needs to be evaluated in the future.

Declaration of interest

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

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement

All authors substantially contributed to the conception and design of the project and manuscript. J Ott, L Wattar, E Vytiska-Binstorfer, and R Seemann contributed to the acquisition of data. J Ott, C Kurz, J C Huber, and K Mayerhofer drafted and revised the article for intellectual content. All authors approved the final version to be published.

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Received 14 December 2011

Revised version received 4 February 2012

Accepted 13 February 2012