Apa-I polymorphism in VDR gene is related to metabolic syndrome in polycystic ovary syndrome: a cross-sectional study

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a common endocrine disorder determined by polygenic traits as well as environmental factors. Lower vitamin D levels have been detected in PCOS women and related to hormone and metabolic disturbances. Vitamin D acts in tissues through the vitamin D receptor (VDR). VDR gene variants have been associated with worse metabolic profile in the general population. We investigated the genotype and haplotype distribution of the Bsm-I (rs1544410), Apa-I (rs7975232), and Taq-I (rs731236) VDR gene polymorphisms in PCOS and non-hirsute women from southern Brazil. We further investigated the associations of these gene variants and their haplotypes with PCOS, vitamin D levels, and metabolic abnormalities, including the metabolic syndrome (MetS).

Methods: A group of 191 women with PCOS (Rotterdam criteria) and 100 non-hirsute controls with regular ovulatory cycles were genotyped for all polymorphisms by real-time PCR, with allelic discrimination assays. MetS and the cutoffs for its isolated components were defined in accordance with the Joint Scientific Statement.

Results: Women with PCOS were younger and had significantly higher BMI and total testosterone levels than controls (p < 0.05). The frequency of MetS in PCOS and controls was 26.5% and 4.8% respectively. The CC genotype of Apa-I entailed higher risk of MetS in PCOS (OR: 2.133; 95% CI 1.020–4.464, p = 0.042), and was associated with higher systolic blood pressure (p = 0.009), total cholesterol (p = 0.040), and LDL-cholesterol (p = 0.038) in both PCOS and control groups (two-way ANOVA). The frequencies of VDR haplotypes were similar in PCOS and control women.

Conclusions: The present results suggest that the Apa-I variant in VDR gene may be associated with MetS in southern Brazilian women with PCOS, and with blood pressure, total cholesterol, and LDL-c in women with and without PCOS.

Keywords: PCOS, Vitamin D receptor, Gene polymorphisms, Metabolic syndrome

Background

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 9 to 18% of women of reproductive age according to different diagnostic criteria [1–3]. While its etiology remains unclear, PCOS is considered a polygenic and multifactorial disease, with metabolic, endocrine, and reproductive alterations [4]. In PCOS women, evidence suggests that vitamin D levels may be decreased and related to hormone and metabolic disturbances [5, 6].

The vitamin D receptor (VDR) is expressed in many tissues and organs (such as those involved in calcium homeostasis mechanisms), in glucose metabolism, and in the reproductive system [7], and modulates vitamin D action in these systems. VDR gene (ID: 7421) polymorphisms have been investigated in PCOS as well as in...
disturbances of androgen secretion. A previous study has suggested an association between VDR gene variants and precocious pubarche (PP) [8]; in turn, data on PCOS risk are controversial, with a relationship between VDR gene variants and PCOS detected by some [9–12] but not all studies [13–15]. Regarding endocrine characteristics, VDR gene polymorphism has been associated with total testosterone in PCOS and PP populations [8, 13], with estradiol levels in PP girls [8], and with metabolic abnormalities in different non-PCOS populations [16–23].

Therefore, the aims of the present study were to assess the genotypic and allelic distribution of Bsm-I (rs1544410), Apa-I (rs7975232) and Taq-I (rs731236) polymorphisms of the VDR gene and to determine whether these gene variants are associated with 25-hydroxyvitamin D [25(OH)D] levels and with metabolic abnormalities, including MetS, in women with PCOS in comparison to non-hirsute, ovulatory control women.

Methods

Patients

This is a cross-sectional study including 191 patients with PCOS and 100 non-hirsute women with regular, ovulatory cycles, recruited by advertisement in the local media. The characteristics of the study sample have been described elsewhere [24]. PCOS was diagnosed according to Rotterdam criteria [25]. Neither PCOS nor control participants had received any drugs known to interfere with hormone levels (such as oral contraceptive pills, antiandrogens, metformin, fibrates, or statins) for at least 3 months before the study. The exclusion criteria were pregnancy and liver or kidney disease. Approval for this study was obtained from the Institutional Review Board and the local Ethics Committee at Hospital de Clínicas de Porto Alegre. Written informed consent was obtained from every subject.

Study protocol

Anthropometric measurements included body mass index (BMI) and waist circumference (measured at the midpoint between the lower rib margin and the iliac crest). Blood pressure was measured after a 10-min rest, with the patient seated, with both feet on the floor and the arm supported at heart level. Two measurements were obtained 10 min apart using an Omron HEM-7421NT automatic blood pressure monitor (Rio de Janeiro, Brazil) with the correct cuff size for the arm diameter [26–29]. MetS and the cutoffs for its isolated components were defined in accordance with the Joint Scientific Statement [30].

Laboratory measurements

All samples were obtained between the 2nd and 10th days of the menstrual cycle, or on any day if the patient was amenorrheic, between 8:00 and 10:00 am, after a 12-h overnight fast. Blood samples were drawn from an antecubital vein for determination of hormone levels. Blood samples were also collected for genomic DNA extraction.

Total cholesterol, high-density lipoprotein cholesterol (HDL-c), triglycerides, and glucose levels were determined by colorimetric-enzymatic methods (Bayer 1650 Advia System). LDL-cholesterol (LDL-c) was determined indirectly with the formula total cholesterol – HDL-c – triglycerides/5. Total testosterone levels were measured by chemiluminescence (Siemens Advia Centaur XP), with a sensitivity of 0.10 ng/mL and intra- and interassay coefficients of variation (CVs) of 3.3 and 7.5% respectively. Plasma insulin and sex hormone-binding globulin (SHBG) levels were measured by chemiluminescence (Siemens Advia Centaur XP), with a sensitivity of 0.50 U/mL and 0.035 nmol/L, respectively, with intra-assay CV < 3% and interassay CV < 5%. The free androgen index (FAI) was calculated as testosterone (nmol/L)/SHBG (nmol/L) × 100. The homeostasis model assessment index (HOMA index) was calculated by multiplying insulin (μIU/mL) by glucose (mmol/L) and dividing this product by 22.5 [31]. 25(OH)D levels were measured in a subset of 102 women (54 PCOS and 48 controls) by chemiluminescence (Liaison, DiaSorin), with intra-assay and interassay CV of 7.7 and 10.9% respectively.

Genotype analysis

Genomic DNA was extracted from peripheral blood leukocytes [32]. The DNA samples were diluted to 2 ng/mL. Molecular genotyping was performed through real-time polymerase chain reaction (7500 Fast Real-Time Polymerase Chain Reaction System, Applied Biosystems, CA, USA), using the allelic discrimination assay with TaqMan MGB primers and probes (Applied Biosystems, CA, USA).

For genotyping the single nucleotide polymorphisms (SNPs) Apa-I and Taq-I, the following were added: TaqMan Master mix (2.5 μL), TaqMan assay (0.25 μL), and H2O (1.25 μL), for a final volume of 4 μL per sample, followed by addition of 1 μL of DNA for a total reaction volume of 5 μL. To genotype SNP Bsm-I, TaqMan Master mix (5.0 μL), TaqMan assay (0.50 μL), and H2O (3.5 μL) were added for a final volume of 9 μL per sample, and 1 μL of DNA was added for a total reaction volume of 10 μL. Reaction conditions for all polymorphisms were: 10 min at 95 °C after 50 cycles of denaturation at 95 °C (15 s) and annealing at 60 °C (1 min). Endpoint fluorescent readings were performed in the 7500 Fast System Sequence Detection Software version 1.4 environment. The internal quality of genotype data was assessed by typing 10% of blinded samples in duplicate.
Table 1 Clinical and endocrine features of PCOS and control women

| Variable                  | PCOS (191) | Controls (100) | P value |
|---------------------------|------------|----------------|---------|
| Age (years)               | 22.89 ± 6.66 | 25.18 ± 7.72  | 0.013   |
| BMI ≥25(kg/m²)           | 72.6%      | 53.4%          | 0.002   |
| Metabolic syndrome        | 26.5%      | 4.8%           | < 0.001 |
| TT (ng/mL)                | 0.90 ± 0.41 | 0.54 ± 0.17    | < 0.001 |
| FAI                       | 16.52 ± 15.81 | 5.28 ± 3.41    | < 0.001 |
| SHBG (nmol/L)             | 29.18 ± 20.35 | 43.37 ± 19.37  | < 0.001 |
| 25(OH)D (ng/mL)          | 21.47 ± 7.61 | 21.50 ± 6.90   | 0.985   |

Data are expressed as means ± SD (Student's t test) or percentages (Pearson chi-square test). BMI body mass index, TT total testosterone, FAI free androgen index, SHBG sex hormone–binding globulin, 25(OH)D 25-hydroxyvitamin D.

Table 3. The genotype and allele distribution of all three polymorphisms was similar in PCOS and control groups.

Figure 1 shows the frequency of MetS in PCOS participants according to Apa-I genotypes. Individuals with the CC genotype had higher risk of MetS vs. the CA + AA genotype (OR: 2.133; 95% CI 1.020–4.464, p = 0.042). The CC genotype was also associated with higher systolic blood pressure (p = 0.009), total cholesterol (p = 0.040) and LDL-c (p = 0.038) in both PCOS and control groups. There was no interaction between genotypes and PCOS or control groups (p > 0.05) (Table 4).

The Bsm-I (G → A) polymorphism was in almost complete linkage disequilibrium with the Apa-I (C → A) polymorphism ([D'] = 1.00; r² = 1.00), and in partial linkage disequilibrium with Taq-I (A → G) ([D'] = 0.75; r² = 0.21). Apa-I (C → A) was also in partial linkage disequilibrium with Taq-I (A → G) ([D'] = 0.87; r² = 0.35).

Eight haplotypes were inferred in the sample: AAA, AAG, ACA, ACG, GAA, GAG, GCA, and GCG, with frequencies of 0.022, 0.340, 0.015, 0.004, 0.192, 0.019, 0.393, and 0.015 respectively. The first letter of each haplotype refers to Bsm-I, the second to Apa-I, and the third to Taq-I. Taking into consideration the results of individual polymorphism analyses, haplotypes were grouped according to the presence of the C allele of Apa-I (ACA + ACG + GCA + GCG vs. AAA + AAG + GAA + GAG). The frequency of combined haplotypes was similar in PCOS and control groups (p = 0.332).

Discussion

In the present study, despite the similar vitamin D levels detected in PCOS and control participants, the CC genotype of Apa-I SNP of the VDR gene was specifically related to higher risk of MetS in PCOS participants. Moreover, this same genotype was associated with higher blood pressure, total cholesterol, and LDL-c in both PCOS and control participants. To the best of our knowledge, this is the first report to show an association...
between Apa-I VDR gene polymorphism and MetS in a PCOS population. This observation is relevant because it may help explain the meaning of vitamin D level variation, which may not play a role per se, but rather reflect a putative gene-environment interaction in different populations.

The few available studies analyzing the influence of Apa-I VDR gene polymorphisms on metabolic variables in PCOS women have reported no association with insulin resistance [10, 13] or glucose and lipid abnormalities [10]. However, data from non-PCOS populations suggest that metabolic abnormalities, such as obesity, insulin resistance, low HDL-c, and type 2 diabetes are associated with the VDR gene [16–21].

In this sense, a recent meta-analysis comprising 9232 participants showed that the association between insulin resistance-related diseases and Apa-I and Bsm-I VDR gene variants was more pronounced in dark-pigmented Caucasians and Asians than in Caucasians with white skin. In the sub-group analysis, Bsm-I (GG genotype) was associated with MetS, and the Apa-I variant (CC genotype) was associated with insulin resistance-related diseases in a population living in a mid-latitude zone (30°–60°) [23], which is also the case of the present population (30°01′59″S).

While a functional role of VDR gene polymorphisms has not yet been established, the association between Apa-I gene variant and MetS observed in the present study could be assumed to be linked to disturbed VDR gene expression [33]. The Apa-I polymorphism is located at the 3′ untranslated region (3′UTR) of the VDR gene, which has been recognized as being involved in the modulation of gene expression, especially through the regulation of mRNA stability and efficiency of protein translation [34]. Moreover, the methylation levels of the VDR gene appear to be altered according to race and presence of the polymorphisms of the 3′UTR region of the gene [35]. Additionally, Apa-I is in strong linkage disequilibrium with other VDR gene polymorphisms in different populations [22, 36], which may be contributing to the general transcriptional activity of VDR in different biological processes. Importantly, the VDR gene regulates more than 200 genes, and mediates most effects of vitamin D on gene expression via formation of a heterodimer with the retinoid X receptor molecule, which binds to promoter regions of many target genes [37, 38].

In our study, lower 25(OH)D levels were associated with MetS and with its isolated components in PCOS women, such as higher glucose, waist circumference and triglycerides. In this sense, the present results are in agreement with a meta-analysis reporting that women with PCOS and vitamin D deficiency are more likely to have dysglycemia compared to those without vitamin D deficiency [5], and that in women with both PCOS and MetS, vitamin D levels are lower than in women with PCOS and without MetS [39].

Similar vitamin D levels were detected in the present study in PCOS and control participants regardless of the presence of Apa-I SNP. Interestingly,

### Table 2

| Status of MetS/components | 25(OH)D levels (ng/mL) | p     |
|--------------------------|------------------------|-------|
|                         | MetS                   | Glu ≥100 mg/dL | BP ≥130/85 mmHg | WC ≥88 cm | HDL-c < 50 mg/dL | Trig ≥2150 mg/dL |
| Yes                      | 17.17 ± 5.46           | 14.83 ± 6.24  | 23.25 ± 7.80   | 19.28 ± 5.92 | 21.50 ± 7.66 | 17.84 ± 4.37   |
| No                       | 22.83 ± 7.74           | 22.22 ± 7.47  | 20.78 ± 6.71   | 23.46 ± 8.47 | 21.42 ± 7.74 | 22.71 ± 7.85   |
| p value                  | 0.018                  | 0.025         | 0.318          | 0.040       | 0.974         | 0.011          |

Data are expressed as means ± SD. P value by Student t test. Glu: glucose; BP: blood pressure; WC: waist circumference; HDL-c: high-density lipoprotein cholesterol. Trig: triglycerides

### Table 3

| SNP | PCOS n (%) | Controls n (%) | p     |
|-----|------------|----------------|-------|
| Bsm-I |             |                |       |
| GG   | 74 (39.6)  | 41 (41.0)      | 0.147 |
| GA   | 76 (40.6)  | 48 (48.0)      |       |
| AA   | 37 (19.8)  | 11 (11.0)      |       |
| G    | 224 (60.0) | 130 (65.0)     | 0.231 |
| A    | 150 (40.0) | 70 (35.0)      |       |
| Apa-I |            |                |       |
| AA   | 61 (32.1)  | 36 (36.0)      | 0.516 |
| AC   | 88 (46.3)  | 48 (48.0)      |       |
| CC   | 41 (21.6)  | 16 (16.0)      |       |
| A    | 210 (55.3) | 120 (60.0)     | 0.275 |
| C    | 170 (44.7) | 80 (40.0)      |       |
| Taq-I |            |                |       |
| AA   | 70 (37.2)  | 40 (40.4)      | 0.493 |
| AG   | 87 (46.3)  | 48 (48.5)      |       |
| GG   | 31 (16.5)  | 11 (11.1)      |       |
| A    | 227 (60.4) | 128 (64.6)     | 0.318 |
| G    | 149 (39.6) | 70 (35.4)      |       |

Data are expressed as percentages; p value by Pearson’s χ² test
Fig. 1 Frequency of metabolic syndrome in PCOS women according to Apa-I genotypes. Data are expressed as percentages (Pearson chi-square test). Frequency values: Apa-I: No – CC: 61.0%; CA + AA: 76.9% / Yes – CC: 39.0%; CA + AA: 23.1%. OR: 2.133; 95% CI: 1.020–4.464

Table 4 Clinical, endocrine, and metabolic features of PCOS and control women according to presence or absence of Apa-I SNP

| Variable | PCOS (n = 190) | Controls (n = 100) | p_gen |
|----------|----------------|-------------------|-------|
| WC (cm)* | CC (41) | 91.97 ± 15.45 | CC (16) | 91.42 ± 13.08 | 0.149 |
|         | CA + AA (149) | 88.56 ± 14.96 | | 77.41 ± 11.20 | |
| SBP (mmHg)* | CC (16) | 113.83 ± 11.35 | 108.60 ± 13.10 | 0.009 |
|         | CA + AA (84) | 119.49 ± 13.69 b | | 108.60 ± 13.10 b | |
| DBP (mmHg)* | CC (16) | 72.77 ± 10.17 | 70.41 ± 9.24 | 0.079 |
|         | CA + AA (84) | 77.21 ± 10.84 a | | 70.41 ± 9.24 a | |
| Glucose (mg/dL) | CC (16) | 90.47 ± 7.13 | 88.08 ± 7.65 | 0.805 |
|         | CA + AA (84) | 89.14 ± 15.92 a | | 88.08 ± 7.65 a | |
| Insulin (μU/mL)* | CC (16) | 11.64 ± 5.78 | 12.03 ± 6.71 | 0.846 |
|         | CA + AA (84) | 22.41 ± 21.27 b | | 12.03 ± 6.71 b | |
| HOMA-IR* | CC (16) | 2.56 ± 1.45 | 2.50 ± 1.60 | 0.654 |
|         | CA + AA (84) | 5.12 ± 5.86 a | | 2.50 ± 1.60 a | |
| TC (mg/dL) | CC (16) | 183.80 ± 36.87 a | 167.26 ± 28.76 b | 0.040 |
|         | CA + AA (84) | 180.95 ± 37.35 a | | 167.26 ± 28.76 b | |
| HDL-c (mg/dL)* | CC (16) | 53.73 ± 11.21 | 52.65 ± 12.55 | 0.529 |
|         | CA + AA (84) | 49.59 ± 10.86 a | | 52.65 ± 12.55 a | |
| LDL-c (mg/dL)* | CC (16) | 102.52 ± 31.69 b | 99.56 ± 24.48 b | 0.038 |
|         | CA + AA (84) | 111.99 ± 32.73 a | | 99.56 ± 24.48 b | |
| Trig (mg/dL)* | CC (16) | 118.90 ± 99.18 | 75.28 ± 41.89 | 0.149 |
|         | CA + AA (84) | 104.29 ± 62.18 b | | 75.28 ± 41.89 b | |
| 25(OH)D (ng/mL) | CC (16) | 21.31 ± 7.16 | 21.52 ± 7.16 | 0.399 |
|         | CA + AA (84) | 19.41 ± 11.13 a | | 21.52 ± 7.16 a | |

WC waist circumference, SBP systolic blood pressure, DBP diastolic blood pressure, HOMA homeostasis model assessment index, TC total cholesterol, HDL-c high-density lipoprotein cholesterol, LDL-c low-density lipoprotein cholesterol, Trig triglycerides, 25(OH)D 25-hydroxyvitamin D

Values are expressed as means ± SD (two-way ANOVA). Different superscript letters indicate statistical difference for comparisons between genotypes, grouped by the absence or presence of the polymorphic allele, in PCOS and control groups. * p < 0.005 for comparisons between PCOS (CC and CA + AA) and control (CC and CA + AA) groups
while two meta-analyses [5, 6] comprising 3182 and 2262 women respectively showed that serum 25(OH) D concentrations were lower in PCOS compared to controls, the reported standardized mean difference between the groups in both studies seems of little clinical relevance – only 0.74 ng/mL (95%IC: -1.26 to –0.22) [5] and 0.64 ng/mL (95%IC: -1.12 to –0.15) [6]. In turn, the fact that our PCOS patients with MetS had lower vitamin D levels and higher frequency of CC polymorphism compared to those without MetS suggests that the Apa-I gene variant might impact vitamin D levels in PCOS with MetS. In fact, vitamin D status is influenced by many factors, especially dietary pattern, season, and genetic traits [40]. A better understanding of the genetic factors that may be involved in vitamin D level variation and metabolic disturbances could shed some light on hypothetical gene-environment interactions of vitamin D. Further studies with larger PCOS populations and higher proportion of MetS are needed in order to confirm this hypothesis.

We did not find any association between genotypes or haplotypes of VDR gene variants in PCOS participants. Only a few studies are available in the literature assessing VDR gene polymorphism and risk of PCOS, with uncertain conclusions, which vary according to the studied sample. While some studies show an association between at least one VDR gene polymorphism and PCOS [9–12, 41], others report similar distributions of Bsm-I, Apa-I and Taq-I polymorphisms in PCOS and control women [13–15]. Also, regarding haplotypes of VDR gene variants, no definitive data are available, with few reports of distinct haplotypes of VDR gene polymorphisms presenting slightly higher frequency in PCOS women when compared to controls [10, 12, 14]. These unclear data may be, at least in part, attributed to ethnic differences in the studied populations and to the polygenic condition of PCOS. Yet, other studies have reported an association of VDR gene polymorphisms with PP [8] and diabetes [42–45].

One strength of our study is the focus on a less well represented ethnic group, PCOS women from southern Brazil, with assessment of gene variants which may be contributing to this polygenic and multifactorial disease. Furthermore, we evaluated polymorphisms found in a genomic position that plays an important role in the modulation of gene expression. Limitations of the present study are the relatively small sample size of 291 participants (191 PCOS and 100 controls) and the low frequency of MetS in the control group, precluding complementary analyses correlating VDR gene polymorphisms and MetS in that group. In addition, further studies on functional evaluation of VDR SNPs are needed in order to deepen the understanding of findings.

Conclusions
Our results indicate that Bsm-I, Apa-I, and Taq-I polymorphisms in VDR gene are not related to PCOS. However, there seems to be an association of the CC genotype of Apa-I with MetS in PCOS women, and with blood pressure, total cholesterol, and LDL-c in women with and without PCOS. Despite the similarity in the vitamin D levels of PCOS and control participants, our study suggests that Apa-I impacts vitamin D levels in PCOS with MetS.

Abbreviations
25(OH)D: 25-hydroxyvitamin D; BMI: Body mass index; BP: Blood pressure; CV: Coefficient of variation; DBP: Diastolic blood pressure; FAI: Free androgen index; Glu: Glucose; HDL-c: High-density lipoprotein cholesterol; HOMA: Homeostasis model assessment index; LDL-c: Low-density lipoprotein cholesterol; MetS: Metabolic syndrome; PCOS: Polycystic ovary syndrome; PCR: Polymerase chain reaction; PP: Precocious pubarche; SBP: Systolic blood pressure; SHBG: Sex hormone-binding globulin; SNP: Single nucleotide polymorphism; TC: Total cholesterol; Trig: Triglyceride; TT: Total testosterone; VDR: Vitamin D receptor; WC: Waist circumference

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Availability of data and materials
All data generated or analyzed during this study are included in this manuscript and in a previous publication (Santos BR, et al. PLoS One. 2017;12(3):e0173695. https://doi.org/10.1371/journal.pone.0173695).

Authors’ contributions
BRS and PMS were involved in the conception and design of the study, BRS, SBL and PMS were involved in data collection and analysis. BRS and PMS drafted the article. All the authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Institutional Review Board and the local Ethics Committee at Hospital de Clínicas de Porto Alegre (FIP-E-HCPA 340/2004). Written informed consent was obtained from every subject.

Competing interests
The authors declare that they have no competing interests.

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