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# The role of rs267606943 polymorphism in the prolidase gene and plasma prolidase in polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is a relatively common reproductive/endocrine problem [1]. Altered levels of reproductive hormones are the main biochemical feature of PCOS, and if untreated, long-term effects include increased risk for dysfunctional uterine bleeding, infertility, endometrial cancer, hypertension, type II diabetes, cardiovascular disease, stress and depression [2–6]. Polycystic ovaries arise due to alteration in folliculogenesis, normally a highly regulated process in which various endocrine, paracrine and autocrine factors act in a spatial and temporal manner, and in which the extra-cellular matrix (ECM) is believed to play an essential role in regulation. The ECM of the ovarian theca and granulosa cells are chiefly made up of collagens type I and IV, whilst matrix metalloproteinases are thought to play an important role in matrix remodelling, wherein, collagen molecules degrade, and new molecules of collagen are formed. Prolidase, EC 3.4.13.9, also known as Xaa-pro dipeptidase, a cytosolic exopeptidase, catalyses the final step of collagen degradation and releases proline for the synthesis of new collagen molecules, thereby acting as a rate limiting step of collagen metabolism [7]. In this way prolidase is thought to regulate the process of remodelling and, ultimately, cyst formation [8].

A number of studies have characterised and purified prolidase in different expression systems, and levels are associated with disorders related to reproduction, collagen metabolism and various cancers [9,10]. A small study (33 cases, 28 controls) reported raised levels of prolidase in PCOS [11]. Polymorphism rs267606943 is a substitution missense mutation found in exon 8 of the prolidase gene, *PEPD*, located on chromosome 19 [12]. As this position is highly conserved in protein structure, substitution of their position might affect the physiological levels of protein. Therefore, we hypothesised a link between rs267606943 polymorphism and the presence of PCOS and with plasma levels of prolidase.

A case control study was carried out on 200 PCOS patients and 200 healthy controls aged 16–40. Diagnosis of PCOS was by the Rotterdam criteria [13]. Subjects were considered obese if the body mass index (BMI) was ≥23 kg/m$^2$, or normal if BMI was between 18.5 and 22.9 kg/m$^2$. The study was approved by the Institutional Ethical Committee and carried out according to the principles of the Declaration of Helsinki. Written informed consent was obtained for all patients and controls. Venous blood, anticoagulated with EDTA, remained at room temperature (3–4 h) before plasma separation, whereupon prolidase levels were measured by the method of Myara et al. [14]. Briefly, plasma was incubated at 37 °C for one hour with $Mn^{2+}$, then mixed with glycine–proline followed by incubation at 37 °C for another hour. The reaction was stopped by 0.45 mol/L trichloroacetic acid. A mixture of glacial acetic acid, orthophosphoric acid and ninhydrin was added followed by incubation at 90 °C for 10 min. Absorbance was taken at 515 nm and prolidase activity was calculated by using a proline standard. The intra-assay coefficient of variation (CV) was 3.7%. Fasting glucose, HDL, LDL, CRP, TSH, LH, SHBG, testosterone and prolactin were measured by standard routine pathology laboratory techniques.

Genomic DNA was isolated by the Genetix MiniPrep DNA isolation kit from blood. The sequence of exon 8 carrying the S202F polymorphism was amplified by PCR (Applied Biosystems, Foster, U.S.A.) using 2 µl of genomic DNA with 1 µl of both the forward and reverse primers, 2 µl of 25 mM $MgCl_2$, 2 µl of dNTPs and 1 µl of Taq polymerase with 5 µl of buffer supplied with Taq polymerase. The aliquot was brought to 50 µl with nuclease free water. Primers used for amplification were: forward primer 5′-GCAGCAGCTCTGCTCCCTGC-3′ and reverse primer 5′-GTGTCCACGACTGCATGATG-3′ respectively with annealing temperature at 66 °C for 30 s. Amplified PCR products were visualized on 2% agarose gel. The PCR

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**Table 1.** Clinical and biochemical data of PCOS and healthy controls.

| Parameter | PCOS (*N* = 200) | Control (*N* = 200) | *P* | PCOS CC (*N* = 102) | PCOS CT (*N* = 98) | *P* | Control CC (*N* = 109) | Control CT (*N* = 91) | *P* |
|---|---|---|---|---|---|---|---|---|---|
| *Clinical data* | | | | | | | | | |
| Age | 25.6 ± 2.3 | 25.2 ± 3.0 | 0.216 | 25.7 ± 2.4 | 25.9 ± 2.7 | 0.683 | 25.7 ± 2.6 | 25.7 ± 2.8 | 0.941 |
| BMI (kg/m$^2$) | 21.8 ± 3.4 | 20.6 ± 4.3 | 0.003 | 21.8 ± 6.6 | 22.6 ± 6.4 | 0.435 | 20.9 ± 4.4 | 21.5 ± 4.2 | 0.388 |
| BP (Systolic) | 112 ± 10 | 114 ± 10 | 0.121 | 113 ± 10 | 112 ± 10 | 0.469 | 114 ± 6 | 114 ± 7 | 0.880 |
| BP (Diastolic) | 70 ± 9 | 71 ± 10 | 0.496 | 71 ± 8 | 71 ± 9 | 0.698 | 73 ± 9 | 72 ± 7 | 0.552 |
| *Laboratory data* | | | | | | | | | |
| Fasting glucose (mmol/dl) | 5.7 ± 0.2 | 4.8 ± 0.2 | <0.0001 | 5.4 ± 1.7 | 5.6 ± 2.3 | 0.567 | 4.7 ± 0.5 | 4.6 ± 0.3 | 0.060 |
| HDL-C (mmol/dl) | 1.18 ± 0.19 | 1.63 ± 0.27 | <0.0001 | 1.19 ± 0.71 | 1.32 ± 0.22 | 0.136 | 1.50 ± 0.78 | 1.62 ± 0.83 | 0.364 |
| LDL-C (mmol/dl) | 2.6 ± 0.8 | 2.2 ± 0.6 | <0.0001 | 2.7 ± 0.9 | 2.9 ± 1.0 | 0.388 | 2.3 ± 0.7 | 2.3 ± 0.4 | 0.750 |
| CRP (mg/l) | 1.2 ± 0.4 | 0.4 ± 0.1 | <0.0001 | 1.3 ± 0.5 | 1.2 ± 0.3 | 0.471 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.394 |
| Prolidase (IU/l) | 927 ± 32 | 622 ± 24 | <0.0001 | 929 ± 31 | 929 ± 31 | 0.919 | 623 ± 23 | 623 ± 23 | 0.920 |
| TSH (µIU/ml) | 2.3 ± 1.2 | 2.5 ± 1.3 | 0.0975 | 2.4 ± 1.0 | 2.3 ± 1.6 | 0.494 | 2.5 ± 1.8 | 2.2 ± 1.3 | 0.195 |
| FSH (mIU/ml) | 6.6 ± 2.4 | 5.0 ± 2.5 | <0.0001 | 6.1 ± 2.6 | 6.5 ± 3.0 | 0.397 | 4.4 ± 2.1 | 4.9 ± 2.5 | 0.614 |
| LH (mIU/ml) | 7.8 ± 0.6 | 3.1 ± 2.0 | <0.0001 | 7.9 ± 2.7 | 7.4 ± 1.7 | 0.124 | 3.3 ± 1.4 | 3.2 ± 1.2 | 0.623 |
| SHBG (nmol/l) | 37.8 ± 27.6 | 61.2 ± 30.8 | <0.0001 | 37.8 ± 23.1 | 35.9 ± 25.9 | 0.430 | 65.7 ± 29.8 | 61.8 ± 29.5 | 0.189 |
| Free testosterone (nmol/ml) | 0.8 ± 0.3 | 2.8 ± 0.9 | <0.0001 | 0.8 ± 0.4 | 0.8 ± 0.5 | 0.267 | 2.7 ± 0.4 | 2.8 ± 0.7 | 0.188 |
| Prolactin (ng/ml) | 16.2 (16.2–16.4) | 19.7 (19.6–19.8) | <0.0001 | 17.4 (17.4–17.4) | 15.2 (15.2–15.3) | 0.375 | 20.7 (18.4–22.0) | 19.8 (19.8–19.9) | 0.216 |

Notes: Data mean (SD) or median (IQR). BMI = body mass index, BP = blood pressure, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, CRP = c-reactive protein, TSH = thyroid stimulating hormone, FSH = follicle stimulating hormone, LH = luteinising hormone, SHBG = sex hormone binding globulin. PCOS = polycystic ovary syndrome.

**Table 2.** Allelic and genotype frequency of C/T polymorphism of prolidase gene in PCOS and the control population, normal and obese BMI.

| Genotype | PCOS *N* (200) | PCOS % | Control *N* (200) | Control % | *P* | Obese BMI *N* (107) | Obese BMI % | Normal BMI *N* (393) | Normal BMI % | *P* |
|---|---|---|---|---|---|---|---|---|---|---|
| CC | 102 | 51 | 109 | 54.5 | 0.547 | 59 | 55.14 | 219 | 55.72 | 0.941 |
| CT | 98 | 49 | 91 | 45.5 | | 48 | 44.8 | 174 | 44.27 | |
| TT | 0 | | 0 | | | 0 | | 0 | | |
| C | 302 | 75.5 | 309 | 77.25 | 0.671 | 166 | 77.57 | 612 | 77.86 | 0.927 |
| T | 98 | 24.5 | 91 | 22.75 | | 48 | 22.42 | 174 | 22.13 | |

Notes: PCOS = polycystic ovary disease, BMI = body mass index.

product was then subjected to RFLP analysis by enzyme MboII (Fast digest, Fermentas, Thermo Fischer Scientific, U.K.) and was observed on 3% agarose gel. PCR amplification of the sequence was carried out encompassing the S202F mutation and visualized on 2% agarose gel using ethidium bromide. Size of amplicon was approximately 520 bp. The S202F mutation corresponding to C/T transition at c605 position creates a site for MboII digestion and abolishes the native site. Three different genotypes were assigned: CC (homozygous wild type, showed by two bands of 335 and 164 bp), heterozygote CT (3 bands of 335, 226 and 164 bp) and variant TT genotype (2 bands of 226 and 164 bp). Continuously variable data are shown as mean with standard deviation (SD) or median with interquartile range. One-sample Kolmogorov–Smirnoff test was used to test the normality of distribution for continuous variables. Chi-squared analysis was used to determine whether the genotype distribution conformed to Hardy–Weinberg equilibrium and to compare differences in allelic frequencies.

Clinical and laboratory data are given in Table 1. No significant difference was found in age or blood pressure of the PCOS and control group. As expected, there were also differences in numerous biochemical indices (such as raised LH, fasting glucose and decreased SHBG), reflecting the pathophysiology of the disease, with increased BMI. Plasma prolidase levels were significantly higher in PCOS than in the controls. The homozygous CC genotype was present in 52% of the total population, that being 102 PCOS and 109 controls, the heterozygous CT genotype in 48%, that being 98 and 91, respectively. There were no differences in any measured laboratory index when patients and controls were classified by CC/TC status. Further aspects of allelic and genotype frequency of C/T polymorphism of prolidase gene in PCOS and the control population, normal and obese BMI are shown in Table 2. The heterozygous TC genotype was present in 49 and 45.5% of PCOS and control population, respectively, and homozygous TT genotype was not found in any of the subject. However, there were no significant differences in genotype distribution of CC and TC genotype in PCOS and control groups. Allelic frequency of the C allele was 75.5 and 77.25%, and of the T allele was 24.5 and 22.75% in PCOS and control population respectively, a difference that was not statistically different, with no significant deviations from Hardy–Weinberg

equilibrium in allelic frequencies observed. The study group was classified by BMI to determine any association with S202F polymorphism. Both obese and normal groups showed similar genotypic and allelic frequencies of C and T allele.

We tested the hypothesis that circulating prolidase levels differ between PCOS and a control population. As a single nucleotide polymorphism has the potential to affect structure, function and stability of protein, the effect of the rs267606943 polymorphism on prolidase levels was also studied. We found that prolidase levels are significantly higher in PCOS (confirming a much smaller study [11]), but that rs267606943 is not associated with prolidase levels. Moreover, no allelic frequency and genotype difference was observed between the PCOS and the control group, providing an insight that rs267606943 is also not associated with the risk of PCOS. It follows that other factors must be responsible for raised prolidase in PCOS, although we cannot speculate that such levels are linked directly to the other biochemical abnormalities. Further studies are required to determine the cause, and consequence (if any), of raised prolidase.

This work represents an advance in biomedical science because it shows that the rs267606943 polymorphism is not associated with plasma prolidase levels, BMI or PCOS.

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No potential conflict of interest was reported by the authors.

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