LIST OF PUBLICATIONS


TECHNICAL REPORT


Single-nucleotide polymorphism of *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G* and *CAPN10* genes in the pathogenesis of polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is the most common and a complex female endocrine disorder, and is one of the leading causes of female infertility. Here, we aimed to investigate the association of single-nucleotide polymorphism of *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G* and *CAPN10* gene in the pathogenesis of PCOS. A hospital-based, observational case-control study was carried on 169 PCOS and 169 control women in the southern region of India. Genotype was carried out by real-time polymerase chain reaction. A chi-square ($\chi^2$) test was performed and the genotypes were verified to comply with the Hardy-Weinberg equilibrium. Odds ratio and 95% confidence interval were calculated to assess the relative risk. Comparison of clinical characteristics of women with PCOS and controls reveal an increase in body mass index (BMI), luteinizing hormone/follicle stimulating hormone (LH/FSH) ratio, glucose levels, insulin, testosterone, hirsutism and atrial follicular count in PCOS women. The variant rs1801278 ($P = 0.002$; OR = 2.88; 95% CI = 1.43, 5.80) show an association with PCOS. In the genotypic ($P = 0.0002$) and allelic models ($P = 0.0000$), significance persisted even after Bonferroni correction. The genotypes of SNPs strongly influence BMI, LH, LH/FSH ratio, ovarian volume and atrial follicular count in PCOS women. The study results were suggestive of a positive association between Gly972Arg of *IRS1* and PCOS in the South Indian population, while *INS*, *IRS2*, *PPAR-G* and *CAPN10* failed to show any association with PCOS in our studied population. Further studies focussing the role of *IRS1* are warranted to delineate its implication towards PCOS.

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Introduction

Polycystic ovary syndrome (PCOS), the most common and highly complex endocrinopathy affects the female population of reproducing age at an increasing prevalence of about 4–8% (Aziz et al. 2004). PCOS, the leading cause of oligo-anovulatory infertility is characterized by insulin resistance, hyperinsulinaemia (found in 50–70% of women diagnosed) (Navaratnarajah et al. 2008; De Franca Neto et al. 2010) and many other metabolic abnormalities that include obesity and hyperandrogenism which has resulted in increased risk of diabetes mellitus (DM), dyslipidaemia, atherosclerosis and also endometrial carcinoma (Carmina et al. 2006; Orso et al. 2006; Giallauria et al. 2008). Further, women with PCOS have expressed several interrelated features, including chronic anovulation, polycystic ovaries and oligomenorrhea which are coupled with anomalous androgen and

insulin-related parameters irrespective of other standard reproductive factors (Adams et al. 2004). Various studies have also suggested that women with PCOS are at a higher risk of gestational diabetes, miscarriages, preeclampsia and preterm labour (Boomsma et al. 2006). The genetic basis of the disease is still not clearly understood owing to the difficulties in determining the inheritability of PCOS. The genes that regulate insulin secretion and action, ovarian and adrenal steroidogenesis are considered as candidate genes which determine the expression of several integral phenotypes of PCOS. Insulin gene thought to play a functionally central role in insulin secretion and/or action and also in the signalling pathways. The insulin gene to the 5’-flanking region has variable number of tandem repeats and has shown to influence transcriptional activity of gene in vitro (Paquette et al. 1998). The insulin receptor gene (*INSR*) is a tetradecameric complex containing 22 exons. Exons 17–21 encode tyrosine kinase domain which is essential for insulin signal transduction. Any polymorphisms in *INSR* gene can introduce

Keywords. anovulation; hyperandrogenism; infertility; polycystic ovary syndrome; single-nucleotide polymorphism; real-time polymerase chain reaction.

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Changes in insulin receptor function and may predispose to the development of PCOS (Siegel et al. 2002). Insulin receptor substrate 1 (IRS1) and 2 (IRS2) involved in the insulin signalling by activating phosphatidylinositol-3 kinase, reported to be associated with insulin resistance, type 2 DM and PCOS (Dilek et al. 2005). Peroxisome proliferator receptor gamma (PPAR-G) is a nuclear receptor and plays a critical role in carbohydrate, lipid metabolism and adipocyte differentiation by regulating multiple genes (Gonzalez Sanchez et al. 2002). The calpain 10 (CAPN10) gene is calcium dependent and is expressed at mRNA and protein levels by several tissue types.

This gene was recognized as type 2 DM susceptibility loci and is allied to proinsulin processing, insulin secretion and insulin resistance (Bairer et al. 2000; Horikawa et al. 2000).

In the present study, we tried to investigate the role of single-nucleotide polymorphisms (SNPs) of INS, INSNR, IRS1, IRS2, PPAR-G and CAPN10 genes in Indian population which were previously studied in different populations with consid- eration of utmost link towards PCOS. Polymorphism of the above said genes in link with PCOS has been reported by several studies in different populations but a very few studies have been reported in the Indian population. INS-VNTR in link with PCOS is studied for the first time in Indian population. The SNP rs1805897 of IRS2 and rs2975766, rs7607759 of CAPN10 gene related to PCOS is also studied for the first time in Indian population. This study will shed light on the polymorphic pattern in the selected population and its association towards the syndrome.

**Materials and methods**

**Study subjects**

This study was conducted altogether on 338 women, comprising 169 cases (PCOS) and 169 controls. PCOS subjects were selected based on observation of oligoamenorrhea/anovulation, clinical or biochemical evidence of hyperandrogenism and/or polycystic ovaries on ultrasonography (The Rotterdam criteria 2003) and normal, unaffected, age-matched fertile women with regular menstrual cycles (interval of 28–35 days) and with normal ovaries from the same geographical region were included in the study as controls. Women with galactorrhea, hyperthyroidism, any systemic disease that affects their reproductive physiology, or any medication which interferes with the normal function of the hypothalamic–pituitary–gonadal axis were excluded from the study. The age of subjects ranged from ≥20 to ≤40 y.

This study was approved by the institutional ethical committee of Sri Ramachandra University. Written informed consent was collected from all the subjects enrolled for the study. Subject’s history and other anthropometric assessments were carried out.

**Biochemical and hormonal analyses**

Blood samples were taken from the subjects after 8-h fast through vein puncture to carry out the biochemical and hormonal analyses on day 2 (D2) or 3 (D3) of the follicular phase. Tests were conducted using Siemens-ADVIA Centaur Automated System and were assayed by chemiluminescent immuno assay (CLIA). Hirsutism was calculated using the modified Ferriman–Gallwey scoring method (Ferriman and Gallwey 1961). Insulin resistance (IR) was assessed using the homeostatic model assessment (HOMA-IR), calculated as (fasting insulin $\times$ fasting glucose)/22.5 (Li et al. 2014). Polycystic ovary (PCO) was confirmed by ultrasound assessments by means of a transvaginal ultrasonography with a transvaginal probe of curved array 5.0–2.0 MHz (for ovary) with a frequency of 5.9 MHz using diagnostic ultrasound system, Sonoscopic, Guangdong, China; with 12 or more follicles in each ovary, measuring 2–9 mm in diameter and/or increased ovarian volume (10 cm$^3$).

**Molecular analysis**

DNA was isolated from peripheral blood using modified salting-out method. Genotyping of SNPs was carried out with real-time polymerase chain reaction technology (Taqman SNP Genotyping Assay, Applied Bioystems, Carlsbad, USA). Each reaction mixture consists of a final volume of 5μL (2.50μL of 2× Taqman Genotyping Master Mix, 0.25μL of 20× Taqman Drug Metabolism Genotyping Assay mix and 2.25μL (3–20ng of genomic DNA) genomic DNA diluted in distilled water). Thermal Cycle reaction with initial denaturation of 95°C for 10 min followed by 40 cycles of denaturation 95°C for 15 s, annealing/extension of 60°C for 1 min was carried out in a 384 well-optical plate on a 7900HT fast real time PCR machine. The Taqman Drug Metabolism Genotyping assay mix contains the primers and fluorescent probes. The alleles were labelled with VIC®-dye and FAM™ dye.

**Statistical analysis**

The continuous variables were expressed as mean ± standard deviation. All statistical analysis were performed using the SPSS statistical software ver. 9.0. Hardy–Weinberg equilibrium (HWE) tests were performed by comparison of observed and expected genotype frequencies using $\chi^2$ goodness-of-fit test. The genotype and allele frequencies of each polymorphism was compared between subjects with PCOS and the controls by the $\chi^2$ test. The odds ratio and 95% confidence interval were calculated using wild-type genotypes or alleles as the reference group. $P < 0.05$ was considered to be statistically significant. Significant values were further confirmed by multiple testing using Bonferroni correction to address the multiple comparisons problem. Pair-wise linkage disequilibrium (LD) was computed as both $D'$ and $r^2$ for the PPAR-G and CAPN10 genes using Haploview ver. 4.1. SNP–SNP interactions among variants of PPAR-G gene were assessed by nonparametric multifactor dimensionality reduction (MDR) analysis using software ver. 3.0.2.
Results

The anthropometric and clinical parameters between cases and controls are measured and presented in table 1. The mean age of PCOS and control women were 26.92 and 27.52, respectively. BMI between PCOS and control women were more or less similar with mean 25.14 and 24.18, respectively. The mean LH level in controls was 4.68 and a two-fold increase in mean value was observed in PCOS women (9.12). However, a decrease in the FSH levels were observed in PCOS (6.18) compared to controls (7.11). Also the mean LH/FSH ratio was increased among PCOS women (1.62) compared to controls (0.71). A highly significant difference was observed in the mean testosterone levels of PCOS women (71.65) compared to their controls (28.23). Correspondingly, the mean hirsutism scores of PCOS women (4.65) were higher than the controls (1.83). The mean insulin and HOMA-IR levels in PCOS and controls were 13.77, 10.76 and 3.08, 2.28, respectively. Glucose levels were elevated in PCOS women. The unilateral right and left ovarian

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Case</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>27.52</td>
<td>26.91</td>
</tr>
<tr>
<td></td>
<td>26.91</td>
<td>27.58</td>
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<tr>
<td><strong>BMI</strong></td>
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</tr>
<tr>
<td></td>
<td>24.68</td>
<td>25.14</td>
</tr>
<tr>
<td><strong>Waist/hip</strong></td>
<td>0.73</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>LH</strong></td>
<td>4.68</td>
<td>4.32</td>
</tr>
<tr>
<td></td>
<td>5.04</td>
<td>9.12</td>
</tr>
<tr>
<td><strong>FSH</strong></td>
<td>7.11</td>
<td>6.75</td>
</tr>
<tr>
<td></td>
<td>7.46</td>
<td>6.18</td>
</tr>
<tr>
<td><strong>LH-FSH</strong></td>
<td>0.71</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>0.77</td>
<td>1.62</td>
</tr>
<tr>
<td>Estradiol</td>
<td>60.23</td>
<td>56.93</td>
</tr>
<tr>
<td></td>
<td>63.53</td>
<td>64.72</td>
</tr>
<tr>
<td><strong>T. testosterone</strong></td>
<td>28.23</td>
<td>26.23</td>
</tr>
<tr>
<td></td>
<td>30.22</td>
<td>71.65</td>
</tr>
<tr>
<td><strong>Hirsutism</strong></td>
<td>1.82</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>2.06</td>
<td>4.65</td>
</tr>
<tr>
<td>Insulin</td>
<td>10.76</td>
<td>10.12</td>
</tr>
<tr>
<td></td>
<td>11.42</td>
<td>13.77</td>
</tr>
<tr>
<td><strong>P</strong> (mg/dL)</td>
<td>85.74</td>
<td>84.47</td>
</tr>
<tr>
<td></td>
<td>87.00</td>
<td>89.54</td>
</tr>
<tr>
<td><strong>P</strong>-pg (mg/dL)</td>
<td>110.19</td>
<td>108.56</td>
</tr>
<tr>
<td></td>
<td>111.84</td>
<td>116.90</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>2.28</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td>2.43</td>
<td>3.08</td>
</tr>
<tr>
<td><strong>OV</strong> (right)</td>
<td>4.79</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>5.39</td>
<td>10.33</td>
</tr>
<tr>
<td><strong>OV</strong> (left)</td>
<td>4.33</td>
<td>3.71</td>
</tr>
<tr>
<td></td>
<td>4.94</td>
<td>9.26</td>
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<tr>
<td><strong>AFC</strong> (right)</td>
<td>6.07</td>
<td>5.71</td>
</tr>
<tr>
<td></td>
<td>6.42</td>
<td>10.43</td>
</tr>
<tr>
<td><strong>AFC</strong> (left)</td>
<td>5.78</td>
<td>5.43</td>
</tr>
<tr>
<td></td>
<td>6.13</td>
<td>10.27</td>
</tr>
</tbody>
</table>

BMI, body mass index; LH, luteinizing hormone; FSH, follicle stimulating hormone; T, testosterone, total testosterone; Pg, preprandial glucose; P-pg, postprandial glucose, HOMA-IR, homeostatic model assessment of insulin resistance; OV, ovarian volume; AFC, antral follicular count; statistical analysis was performed using the SPSS statistical software version 9.0. The continuous variables are expressed as mean ± standard deviation; the significant difference obtained in PCOS subjects and controls were calculated and compared using ANOVA. P < 0.05 was considered to be statistically significant; *P < 0.05; **P < 0.01.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Control (n = 169)</th>
<th>Case (n = 169)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS</td>
<td>rs6889</td>
<td>TT</td>
<td>124 (73.7)</td>
<td>131 (77.51)</td>
<td>0.667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA</td>
<td>41 (24.26)</td>
<td>35 (20.71)</td>
<td>0.81 (0.48-1.35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>4 (2.37)</td>
<td>3 (1.78)</td>
<td>0.71 (0.16-3.23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAA</td>
<td>45 (26.63)</td>
<td>38 (22.49)</td>
<td>0.81 (0.49-1.31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HWD</td>
<td>0.780</td>
<td>0.711</td>
<td></td>
</tr>
<tr>
<td>Allele frequency</td>
<td>T</td>
<td>289 (85.8)</td>
<td>297 (87.87)</td>
<td>Reference</td>
<td>0.365</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>49 (14.0)</td>
<td>41 (12.13)</td>
<td>0.81 (0.52-1.31)</td>
</tr>
</tbody>
</table>

A χ² test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HW; OR and 95% CI were calculated to assess the relative risk; P < 0.05 was considered to be statistically significant.

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volume in PCOS and control were 10.33, 9.26 and 4.79, 4.33 and the unilateral right and left AFC in PCOS and control were 10.43, 10.27 and 6.07, 5.78, respectively.

All the genotype frequencies were distributed according to the HWE in both case and control subjects except for rs1801278 of IRS1 gene (cases only). In the rs689 variant of INS, T alleles were more compared to A alleles in both cases and controls. The minor allele frequency was 14% in control and 12% in PCOS case groups (table 3). The genotypic distribution between PCOS and control subjects did not show any significant association. In the INS gene (rs1799817), the frequency of T allele was found to be more (66.27%) than the C allele (33.73%), in both cases and controls (table 3). No significant association was observed at the dominant or allelic model. In the SNP rs1801278 of IRS1 gene (table 4), the minor allele frequency among cases and controls was 53% and 43%. Significant difference was observed between PCOS and control subjects in genotypes (GA, 0.002; AA, 0.002) of dominant (0.001) and allelic model (<0.001). This significance was maintained after Bonferroni correction for multiple testing (GA, 0.0002; AA, 0.0002; GA+AA, 0.0001). Frequency of A allele (53.25%) was more compared with the G allele (46.75%) in PCOS women and the frequency of G allele (57.40%) was more compared to A allele (42.60%) in control women. Polymorphism of rs1805097 of IRS2 gene is depicted in table 5. The frequency of homozygous GG was 49.11% and 49.70% in PCOS and control, while the homozygous AA was lesser in both PCOS and control with 8.88% and 11.83%. No significant difference was seen in the genotypic, dominant and allelic models for the variant rs1805097. Table 6 provides explanation for two variants rs1801282 and rs3856806 of PPAR-G gene. The genotype distribution frequency for rs1801282 among cases and controls for homozygous GG were higher 76.33 and 73.96% compared to the homozygous CC which was 2.37 and 1.78%. The genotype distribution of rs3856806 for cases and controls for genotypes CC, CT and TT are 72.78, 23.67, 3.55 and 69.23, 28.99, 1.78. The minor allele frequency for cases and control subjects were more or less similar for both the variants. The variants rs7607759 and rs2975766 of CAPN10 gene are illustrated in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet. No significant difference was observed in the genotype distribution, dominant and allelic models between cases and controls for both.

Table 3. Allele and genotype frequencies of INS rs1799817 in PCOS and control groups.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Control (n = 169)</th>
<th>Case (n = 169)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS</td>
<td>rs1799817</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>22 (13.02)</td>
<td>19 (11.24)</td>
<td>Reference</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>67 (37.64)</td>
<td>76 (44.97)</td>
<td>1.31 (0.65–2.63)</td>
<td>0.442</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>80 (47.34)</td>
<td>74 (43.79)</td>
<td>1.07 (0.53–2.14)</td>
<td>0.845</td>
</tr>
<tr>
<td></td>
<td>CT+TT</td>
<td>147 (86.98)</td>
<td>150 (88.76)</td>
<td>1.18 (0.61–2.27)</td>
<td>0.617</td>
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<tr>
<td></td>
<td>HW:P</td>
<td>0.188</td>
<td>0.938</td>
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<td></td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>111 (32.84)</td>
<td>114 (33.73)</td>
<td>Reference</td>
<td>0.806</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td>227 (67.16)</td>
<td>224 (66.27)</td>
<td>0.96 (0.69–1.32)</td>
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<tr>
<td>MAF</td>
<td></td>
<td>0.67</td>
<td>0.66</td>
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</table>

A χ² test was performed to evaluate the association between SNP and PCOS; the genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; P < 0.05 was considered to be statistically significant.

Table 4. Allele and genotype frequencies of IRS1 rs1801278 in PCOS and control groups.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Control (n = 169)</th>
<th>Case (n = 169)</th>
<th>OR (95% CI)</th>
<th>P value</th>
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<td>IRS1</td>
<td>rs1801278</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>59 (29.59)</td>
<td>25 (14.79)</td>
<td>Reference</td>
<td>0.003**</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>90 (55.62)</td>
<td>106 (63.91)</td>
<td>2.36 (1.32–4.00)</td>
<td>0.002**</td>
</tr>
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<td></td>
<td>AA</td>
<td>25 (14.79)</td>
<td>36 (21.30)</td>
<td>2.88 (1.43–5.80)</td>
<td>0.002**</td>
</tr>
<tr>
<td></td>
<td>GA+AA</td>
<td>119 (71.41)</td>
<td>144 (85.21)</td>
<td>2.42 (1.41–4.14)</td>
<td>0.001**</td>
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<tr>
<td></td>
<td>HW: P</td>
<td>0.0742</td>
<td>&lt; 0.001</td>
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<td>Allele frequency</td>
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</tr>
<tr>
<td>G</td>
<td></td>
<td>194 (57.40)</td>
<td>158 (46.75)</td>
<td>Reference</td>
<td></td>
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<tr>
<td>A</td>
<td></td>
<td>114 (42.60)</td>
<td>189 (53.25)</td>
<td>1.94 (1.41–2.66)</td>
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<tr>
<td>MAF</td>
<td></td>
<td>0.43</td>
<td>0.53</td>
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</table>

A χ² test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; **P < 0.01; P < 0.05 was considered to be statistically significant.
Table 5. Allele and genotype frequencies of IRS2 rs1805097 in PCOS and control groups.

<table>
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<th>Gene</th>
<th>Genotype</th>
<th>Control (n = 169)</th>
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<td>rs1805097</td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>84 (49.70)</td>
<td>83 (49.11)</td>
<td>Reference</td>
<td>0.611</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>65 (38.46)</td>
<td>71 (42.01)</td>
<td>1.11 (0.70–1.74)</td>
<td>0.664</td>
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<td>AA</td>
<td>20 (11.83)</td>
<td>15 (8.88)</td>
<td>0.76 (0.36–1.58)</td>
<td>0.461</td>
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<td>AA=AA</td>
<td>85 (50.30)</td>
<td>86 (50.89)</td>
<td>1.02 (0.67–1.57)</td>
<td>0.913</td>
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<tr>
<td></td>
<td>HW/P</td>
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<td>0.973</td>
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<td>Allele frequency</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>233 (68.93)</td>
<td>237 (71.12)</td>
<td>Reference</td>
<td>0.738</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>105 (31.07)</td>
<td>101 (28.88)</td>
<td>0.95 (0.68–1.31)</td>
<td>0.728</td>
</tr>
<tr>
<td></td>
<td>MAF</td>
<td>0.31</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A χ² test was performed to evaluate the association between SNP and PCOS. The genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; P = 0.05 was considered to be statistically significant.

Table 6. Results of association test with PPAR-G gene polymorphisms rs1801282 and rs3856806 in PCOS and control groups.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Control (n = 169)</th>
<th>Case (n = 169)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-G</td>
<td>rs1801282</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>3 (1.8)</td>
<td>4 (2.37)</td>
<td>Reference</td>
<td>0.576</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>41 (24.26)</td>
<td>36 (21.30)</td>
<td>0.65 (0.39–1.14)</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>125 (73.94)</td>
<td>129 (74.33)</td>
<td>0.77 (0.50–1.20)</td>
<td>0.740</td>
</tr>
<tr>
<td></td>
<td>GG+GG</td>
<td>166 (98.22)</td>
<td>165 (97.63)</td>
<td>0.75 (0.50–1.20)</td>
<td>0.702</td>
</tr>
<tr>
<td></td>
<td>HW/P</td>
<td>0.86</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>47 (13.91)</td>
<td>44 (13.02)</td>
<td>Reference</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>291 (86.01)</td>
<td>294 (86.98)</td>
<td>1.07 (0.69–1.68)</td>
<td>0.729</td>
</tr>
<tr>
<td></td>
<td>MAF</td>
<td>0.86</td>
<td>0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3856806</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>117 (69.23)</td>
<td>123 (72.78)</td>
<td>Reference</td>
<td>0.357</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>40 (23.89)</td>
<td>40 (23.87)</td>
<td>0.78 (0.48–1.20)</td>
<td>0.694</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>15 (8.88)</td>
<td>13 (7.35)</td>
<td>1.50 (0.37–7.8)</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td>CT:TT</td>
<td>52 (30.77)</td>
<td>46 (27.22)</td>
<td>0.84 (0.53–1.35)</td>
<td>0.471</td>
</tr>
<tr>
<td></td>
<td>HW/P</td>
<td>0.045</td>
<td>0.237</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>283 (83.73)</td>
<td>286 (84.62)</td>
<td>Reference</td>
<td>0.751</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>55 (16.27)</td>
<td>52 (15.38)</td>
<td>0.94 (0.62–1.41)</td>
<td>0.729</td>
</tr>
<tr>
<td></td>
<td>MAF</td>
<td>0.16</td>
<td>0.15</td>
<td></td>
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</tbody>
</table>

A χ² test was performed to evaluate the association between SNP and PCOS; the genotypes were verified to comply with the HWE; OR and 95% CI were calculated to assess the relative risk; P = 0.05 was considered to be statistically significant.

the SNPs but increased risk was observed in GG genotype (P = 0.558; OR = 1.37; CI: 0.47–3.98) of rs7607759 and genotypes GA (P = 0.121; OR = 1.83; CI: 0.84–3.98), AA (P = 0.531; OR = 2.12; CI: 0.19–23.65), dominant model (P = 0.099; OR = 1.85; CI: 0.88–3.91) and allelic model (P = 0.086; OR = 1.32; CI: 0.91–1.66) of rs2975766. However, neither of these findings remain significant. The clinical and hormonal variables were compared with the genotypes of all the SNPs and are presented in tables 2-9 in electronic supplementary material. The genotypes of rs689 showed increase in waist/hip ratio, LH, LII/FSH and right ovarian volume in PCOS women. Post-hoc test was conducted to find out as which specific groups differed, and the results revealed that the TT/TA combination differed significantly (table 2 in electronic supplementary material). The rs1799871 of INSR gene, increase in LH (P = 0.049) was observed in PCOS women with CC/CT combination (table 3 in electronic supplementary material). In rs1801278 of IRS1 gene and rs3856806 of PPAR-G gene, the phenotypic variables did not show any significant difference in PCOS group but unilateral left antral follicular count was significantly more in the control groups with AA, GA combinations (P = 0.036) in rs1801278 (table 4 in electronic supplementary material) and CC, TT combinations (P = 0.020) in rs3856806 (table 7 in electronic supplementary material) showing risk. In IRS2 gene, an increase in BMI was seen in PCOS women.

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Discussion

Several biochemical and hormonal variables were checked to find the difference in PCOS and control women. Not much difference was observed in our population in the mean values of BMI in PCOS (25.14) and controls (24.18) but waist/hip ratio was observed to be highly significant in PCOS women. Waist/hip ratio could perhaps be a better indicator risk than BMI. LH levels were higher in PCOS women and lower in control and FSH levels were higher in control and lower in PCOS women. A decrease in FSH levels disrupts follicular growth. Also, a significant difference was observed in the testosterone levels of PCOS women compared to that of controls, supporting with the hypothesis that an increase in testosterone level leads to suppression of normal menstruation and ovulation. The augmented GnRH pulse frequency is hugely associated with hyperandrogenism and increased ovarian volume. An increase in testosterone levels above a normal range leads to an unusual increase in hair growth (hirsutism). An increased conversion of testosterone to dihydrotestosterone through the enzyme 5 alpha reductase within the pilosebaceous unit may be one of the root causes for hirsutism. As testosterone increases, a decline in the serum prolactin levels and FSH levels were observed which possibly lead to the suppression of normal menstruation and ovulation. A significant difference in the glucose levels, fasting insulin levels and HOMA-IR index were found between PCOS and control women with an elevation in PCOS women. We also found that the unilateral antral follicular count and ovarian volume of the right ovary showed a marginally higher mean value than that of the left in women with PCOS.

The variable number tandem repeats positioned at −23 bp in the 5'-flanking region of INS gene is considered to be a susceptible loci and acts as a surrogate marker. The rs689 polymorphism has shown association with PCOS by the mutant class III alleles also presenting with increased serum insulin levels and BMI in PCOS women (Waterworth et al. 1997). The frequency of class III allele (12%) reported in our population was much lower compared to the Japanese, European, Korean and Han Chinese which was 97, 30, 94 and 93.5% accordingly. The homozygous class I alleles in our study were higher in both PCOS and control. This variant rs689 of INS gene was not associated with PCOS in the present study. But the heterozygote TA and homozygous AA genotypes showed an increase in waist/hip ratio (P = 0.020), increase in luteinizing hormone levels (P = 0.020) and right ovarian volume (P = 0.042) in PCOS women. Alterations in insulin action and β-cell function may lead to augmented GnRH pulse frequency which increases LH secretion (Li et al. 2014) and is largely associated with hyperandrogenism and increased ovarian volume (Kralovickova 2006). Studies carried out on anovulatory women with PCOS selected based on criteria proposed by National Institute of Health (NIH) in Czech women also reported a negative association with OR 1.44 (0.50–4.18; P = 0.59) (Vankova et al. 2002). Another
study with 216 PCOS and 192 non-PCOS women in Han Chinese population reported no significant difference between cases and control groups either in allele ($P = 0.096$) or genotype ($P = 0.802$) frequencies (Xu et al. 2009); also a family based association study reported no excess transmission of alleles of rs689 VNTR polymorphism, regardless of parent of origin (Powell et al. 2005).

In our population, the rs1799817 polymorphism of INSR gene shows a negative association towards PCOS women but the genotypes influenced the phenotypic expression. Women with T1 genotype of His1058His showed an increase in insulin, endometrial thickness, ovarian volume and right antral follicular count and women with CC genotype showed an increase in testosterone, hirsutism grade and preprandial glucose in PCOS women. Insulin resistance might upregulate testosterone production in theca cells, LH secretion in pituitary which may perhaps disturb follicular maturation and lead to PCOS (Le Fur et al. 2006). The rs1799817 polymorphism influenced an increase in LH levels ($P = 0.049$) in PCOS women. The frequencies of CC genotypes (PCOS, 11.24%; control, 13.02%) were much lower in our population. While CC genotype frequency in other population reported: PCOS, 43.9%; control, 52.8%; PCOS, 34%; control, 27.66% (Mulderjee et al. 2009; Bagheri et al. 2015). Lack of association of His1058His was also reported in other studies (Tebrit et al. 2013; Urbaneck et al. 2005; Bagheri et al. 2015). Two GWAS studies by Chen et al. (2011) and Shi et al. (2012) reported an association of rs1799817 of INSR gene in PCOS women. Another study reported an increase in frequency of uncommon T allele in lean PCOS women with body mass index (BMI) $< 27$ kg/m$^2$ compared with lean controls (Siegel et al. 2002). Yet another study reported that genetic variation in exon 17 of INSR is associated with insulin resistance and hyperandrogenemia among lean Indian women with polycystic ovary syndrome but not obese women. The difference in phenotypic expression and association of exon 17 polymorphism towards PCOS in other populations could be due to the frequency of distribution of genotypes TT, CT and CC across population where the effect of genotype influences suppression or increased expression of phenotypic change.

In rs1801278 of IRS1 gene, 21% PCOS subjects showed glycine to arginine substitution whereas in control 15% had this substitution. The Gly972Arg polymorphism towards susceptibility of PCOS reveal that the heterozygous Gly/Arg genotype may increase the risk of developing PCOS by 2.30 times ($P = 0.002$) and having Arg/Arg genotype which is the mutant in our population might increase a risk 2.88 times in development of PCOS ($P = 0.002$). These findings remained significant even after Bonferroni correction for multiple comparisons ($P = 0.0002$). The dominant model (Gly/Arg+Arg/Arg) did contribute to a relative risk of 2.42 ($P = 0.001$), Bonferroni corrected value ($P = 0.0001$). And the allele Arg showed a risk of 1.94 in developing PCOS ($P < 0.001$), Bonferroni corrected value ($P = 0.0001$).

But Gly972Arg polymorphism did not show a change in the phenotypes except for the left antral follicular count in control subjects ($P = 0.036$). Consistent with our results, a study from the Japanese population reported significantly more IRS-1 972Arg carriers among PCOS women compared to healthy controls (10.6% versus 4.8%, $P = 0.029$), and had a significantly increased risk of developing PCOS (OR: 3.31, 95% CI: 1.49–7.35). They also found that the IRS1 polymorphism was not associated with its phenotypes such as BMI, insulin resistance or androgen levels in PCOS women (Baba et al. 2007). Also, women carrying IRS-1 Gly/Arg had an increased risk of PCOS (OR = 2.49, 95% CI: 1.16–5.37, $P = 0.019$) (Lin et al. 2014). Two recent meta-analysis on PCOS women reported that Gly/Arg and Gly/Gly genotypes are significantly associated with risk of developing PCOS (OR = 3.31; 1.49–7.35) which is chiefly mediated by higher levels of fasting insulin (Ruan et al. 2012). The IRS-1 variant allele occurs significantly more frequently among PCOS patients than among healthy women (Sir-Petermann et al. 2001). On the other hand Spanish, Croatian, Chilean, Taiwanese, Caucasian, Slovak and Iranian populations did not find any association of Gly972Arg towards PCOS. However a study analysing 250 PCOS and 299 controls reported IRS-1 polymorphic alleles having a similar distribution between cases and controls, and seems to have a probable protective role against development of specific PCOS subphenotypes (Dasgupta et al. 2012).

In rs1805097 of IRS2, we report that Asp was the minor allele in our studied population and the Caucasians and the African Americans also presented Asp as the minor allele (San Millán et al. 2004). Nevertheless, Lin et al. (2014) reported Gly as the minor allele and association of Asp/Asp genotype with higher fasting insulin and HOMA index (Goodarzi et al. 2005). While Ehrmann and his team reported association of Gly/Gly with higher 2h OGTT levels (Ehrmann et al. 2002). The variant rs1805097 (Gly1057Asp) of IRS2 gene was not associated with PCOS in the studied population but this polymorphism may possibly have an effect on weight gain in PCOS women. The nonassociation was confirmed in Spanish and Greek populations as well. IRS1 and IRS2 polymorphisms influence glucose homeostasis and could influence obesity, regardless of women presented with PCOS (Taylor 1997). Levels of both IRS-1 and IRS-2 are increased in theca cells of women with PCOS, which leads to proliferation and increased androgen synthesis (Yen et al. 2004).

The Ala variant of rs1801282 of PPAR-G gene was found to be higher in PCOS and controls. The Ala/Ala variant tendency to decrease waist/hip ratio, lower LH levels, LH/FSH ratio, right OV and AFC in PCOS women in our population. Ala variant reduces the risk of PCOS with decline in levels of BMI and fasting insulin (Xu et al. 2009).

Polymorphism of rs1801282 was not associated with PCOS in the studied population. Similarly, a negative association was observed in Caucasian and Greek populations and a positive association was seen in Italy and Korea. Earlier
studies have also confirmed that the PPPARG Pro12Ala variant allele association with higher insulin sensitivity, high-density lipoprotein (HDL) levels (Deazh et al. 1998), decreased risk of T2DM (Hara et al. 2000) and decreased incidence of cardiovascular disease (CVD) (Doney et al. 2004). A meta-analysis reported lower insulin levels in Ala carriers considering PCOS patients and controls as an entire group (San-Millan and Escobar-Morreale 2010). Two additional meta-analyses determined that PPPARG Pro12Ala variant may result in lower insulin levels but exerts no effect on HOMA-IR in PCOS patients (He et al. 2012; Zhang et al. 2012). One article published in India found an association towards PCOS at the allelic level (Dasgupta and Reddy 2013) but another study from Mumbai, India showed significant difference in both allelic and genotypic frequency between PCOS and controls with reduced susceptibility to PCOS (Shaikh et al. 2013). Therefore, the Ala variant of PPPAR-G is thought to be associated with reduced transcriptional activity which improves insulin action towards suppression of lipolysis in turn reducing the production of free fatty acids and enables their storage in adipocytes (Taylor et al. 1999).

The variant rs3856806 located in exon 6 of PPPAR-G is a functional synonymous SNP. For rs3856806 of PPPAR-G (His447His), neither the genotypes nor the alleles show any association towards PCOS but the mutant homozygous TT genotype showed an increase in the right antral follicular count (P = 0.020) in control women. Also to see the effect of genotypes of SNPs together, MDR analysis was carried out. The best MDR models for the studied SNP rs1801282 and rs3856806 markers had a testing accuracy (TA) of 0.541 and cross-validation consistency (CVC) of 10/10. However, this model was not significantly associated with PCOS (P = 0.755). Each cell is labelled as high risk if the ratio of the affected individual to the unaffected individual exceeds a threshold of 1 and low risk if the threshold does not exceed it. The higher risk group contain combinations of (corresponding to SNP rs3856806 and rs1801282) CC–CC, CC–CG, CT–CG, CT–GG, TT–CG. The homozygous GG of rs180282 signifies no risk and conterls protection towards PCOS over the other two genotypes. The pairwise LD values (D’ = 0.280 and r² = 0.22) between rs1801282 and rs3856806 revealed that these two SNPs are not in strong LD.

The minor allele frequency of rs7607759 of CAPN10 gene was 17.75% while Whites, Blacks, Hispanics, Asian/Pacific islanders reported 15.5, 6.73, 11.5 and 9.06% respectively (Wu et al. 2000). Even though the frequency is high in Indian population, we found a negative association of rs7607759 genotype and allele frequency of CAPN10 towards PCOS. We say that the SNP rs7607759 is not a susceptible locus linked to PCOS in our study population. When genotypes of PCOS and control were compared with the phenotypes, we found that the hirsutism grades in control women with presence of AA/AG combination did show any significance and also no phenotypic relation was observed in PCOS women. The genotypes of SNP rs2975766 did not show any association towards PCOS. The mutant homozygous AA genotype was observed in two women with PCOS and one in the control group. This homozygous variant and the heterozygous variant renders risk 2.12 (0.19–23.65); P = 0.531 but was not significant enough to show an association. When the genotypes of rs2975766, compared to the biochemical and other hormonal features of PCOS it was revealed that increased levels of LH and LH/FSH ratio was observed in women with AA genotype compared to the other genotypes. The mutant AA genotype may possibly indicate changes at the phenotype level in PCOS women. Studies from meta-analysis reported no association with the above studied variants of CAPN10 gene towards PCOS (Shen et al. 2013).

The SNP’s rs760759 and rs2975766 of CAPN10 gene are the first of its kind to be studied in the Indian population. The pairwise LD values D’ = 0.211 and r² = 0.87, between rs7607759 and rs2975766 of CAPN10 gene revealed no significant LD between SNPs.

Conclusion

Of all the SNPs studied, Gly972Arg of IRS1 gene showed an association with PCOS. Polymorphism of Gly972Arg could play a contributory role in the pathophysiology and risk of PCOS. Further analysis of this SNP variant should be evaluated in larger populations and the same variant should be checked in different population groups to confirm its significance. Also, additional SNPs of IRS1 gene has to be studied to confirm the role of insulin receptor substrate 1 gene towards PCOS. Apart from rs1801278, the other SNPs failed to show any association with PCOS in the population we studied. The genotypes of analysed SNPs strongly influenced the change in phenotype by increasing weight gain, LH levels, LH/FSH ratio, ovarian volume and antral follicular count in PCOS women.

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References


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Polycystic Ovary Syndrome: Causes and Contributions.

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Abstract:
Polycystic ovary syndrome (PCOS) is a significant clinical condition as it is a major affliction of women in the reproductive age globally. The incidence rates are steadily increasing with more environmental, physiological and genetic parameters being associated with the condition. Interactions among various variables that make up one's life style and an enhanced multi-parametric effect from various sources are making PCOS more complicated than it was a few decades ago. The important factors that are implicated for PCOS include conditions that induce obesity, the formation of advanced glycation end products, deficiency of certain vitamins, an imbalance in the levels of endocrine disrupting chemicals, etc. Also, the drugs commonly used for a variety of lifestyle diseases such as obesity and type 2 diabetes can have implications in PCOS. In this review, we present the multi-parametric causes and contributions to PCOS and the interrelationship among some of these factors.

Keywords: Advanced Glycation End Products, Endocrine Disrupting Chemicals, Vitamin D, Polycystic Ovary Syndrome.

Introduction:
Polycystic Ovary Syndrome (PCOS) has become commonplace in today's world [1]. The prevalence of this heterogeneous disorder has been found to be partially under the influence of the diagnostic criteria used. For instance, in a study conducted to assess the prevalence of metabolic syndrome in 282 PCOS women in Southern Italy, 8.2% and 16% of women were diagnosed with metabolic syndrome in accordance with the Adult Treatment Panel III (ATP III) and WHO criteria respectively [2]. Also, a recent report consisting of 827 women with WHO class II oligo-ovulation, 55% of them were diagnosed to have PCOS by the NIH 1990 criteria while 91% were diagnosed using the Rotterdam Criteria. Furthermore, a study conducted in Iran demonstrated that the prevalence values estimated using the Rotterdam criteria were double those of the NIH criteria [3]. The population under evaluation also plays a significant role while calculating the prevalence. It has been reported that 5-10% of Chinese women, 6.5% of Caucasian women from Spain and 6% in Mexican women were diagnosed with PCOS [4-6]. Hence, an absolute value for prevalence cannot be estimated. Characterized by chronic anovulation, hyperandrogenism, insulin resistance, hirsutism and obesity, the clinical presentations (including obesity and insulin resistance) have been found to fluctuate among different ethnic and racial groups within India. For instance, in a study conducted on 460 students of a residential college in Andhra Pradesh, 6.30% of the girls under study were found to present with oligomenorrhea and polycystic ovaries (PCO) while only 2.39% of girls were found to present with hirsutism in addition to the above mentioned findings [7]. In another study conducted on 120 newly diagnosed, post - pubertal women with PCOS in Western Maharashtra, it was observed that 65% of women affected presented with oligomenorrhea, 44.68% of married women complained of infertility, 44.16% presented with hirsutism and were positive for Acanthosis nigricans [8].
Furthermore, a study conducted in Lucknow to assess the prevalence of PCOS in North India studied a total of 1520 girls, out of which, 75 girls were examined for further evaluation. Twenty one girls were diagnosed with PCOS and consequently the prevalence was extrapolated to be 3.7% [9]. The above statistical data suggests that there is no definite value for the prevalence of PCOS in India. However, it is found to range between 2% - 26% [7].

The reason for variation in populations belonging to different geographical areas can be attributed both at the genetic and the environment level. The multifarious and complex network of interwoven causes makes it difficult to comprehend the dynamic variables involved. Nevertheless, interplay between the environmental and genetic causes has been postulated. For instance, in the context of gaining the Western lifestyle, the Indian population has undergone a revolution in such a way that is conductive to the increase in prevalence of PCOS among young girls [10]. Poor dietary choices coupled with a sedentary lifestyle leads to obesity that has been observed in women affected with PCOS. In addition, it has been found that Asian Indians are more susceptible to insulin resistance and cardiovascular risks at a low Body Mass Index (BMI) because of the presence of higher percentage of body fat and abnormal adiposity [9]. Obesity has been linked to chronic anovulation and hyperinsulinemia, which in turn, has a negative effect on the ovulatory process by causing an increase in the levels of Leutening Hormone (LH), thereby leading to premature cessation of ovulation. Insulin resistance (IR) is an intrinsic factor that has been observed in 50 – 70 % of the cases [11]. IR induced hyperinsulinemia drives the production of excess androgen which causes hirsutism observed in many patients.

At the genetic level, various genes have been hypothesized to play a role in PCOS by the candidate gene approach. Variants of many genes involved in obesity like FTO gene, PPARY, and MCR4 are found to be significantly associated to increased susceptibility. Among the genes involved in the biosynthesis and metabolism of androgens, there are no studies that clearly delineate the exact role of CPA1, CPA17 and CPA19 genes. In the process of folliculogenesis, follicle-stimulating hormone (FSH) is observed to be one of the strongest candidate genes to be involved in PCOS. Genes involved in the biosynthesis of ovarian androgens like Sex Hormone Binding Globulin (SHBG) and Androgen Receptor (AR) gene are found to be insignificant. Six important genes have been linked to insulin resistance: the INS gene, IRS-1, IRS-2, IGFB2 and CAPN10 gene. Mutations of the IGFB2, IRS-1, IRS-2, and CAPN10 genes were not significant enough to cause PCOS [12, 13]. However, there is evidence that INS - VNTR is a strong susceptible locus for PCOS. In addition, genes that regulate Vitamin D levels are also associated with development of PCOS. Since the candidate gene approach is complex and requires prior knowledge of the pathogenesis, Genome Wide Association Study (GWAS) approach is being adopted [13-15].

As one cannot alter one's genetic makeup, it is only logical that simple changes in lifestyle can lead to the prevention of acquiring PCOS or at the minimum keep the harmful effects at bay. Hence, this paper delves into the various environment factors that have been found to influence PCOS.

Lifestyle modifications and other factors influence on PCOS:

Obesity:
A high prevalence of obesity is found to be observed in women with PCOS [16]. Obesity is found to worsen metabolic indices and amplify the clinical manifestations manifold [17]. Clinical features exacerbated by obesity include hyperandrogenemia and menstrual irregularities. Furthermore, their normal counterparts showed low body weight. The pattern of body fat distribution plays a major role in regulating the various levels of hormones [19]. A study conducted by Pasquali et al. examined 97 hyperandrogenic women with PCOS. They were grouped into three on the basis of waist to hip ratio. It was observed that compared to women with peripheral fat, women with central adiposity had higher levels of LH, oestrone and androstenedione, higher concentrations of fasting glucose, a higher prevalence of hirsutism, acanthosis nigricans and a more atherogenic lipid profile. Several studies have reported that an increase in metabolism of visceral fat leads to the accumulation of pro-inflammatory cytokines (due to the increased production of free fatty acids that accumulate in non-adipose tissues thus casing lipotoxicity), which in turn, lead to insulin resistance.

Compensatory hyperinsulinemia and insulin resistance are closely associated to obesity although the former is proved to occur independently. Hepatic insulin resistance is exclusive to obese women with PCOS [20]. Structural defects of the insulin receptor are absent in PCOS patients and hence it can be safe to conclude that post receptor defects must be involved in the development of insulin resistance [21]. Serine phosphorylation of IRβ subunit of the insulin receptor leading to subsequent inhibition of tyrosine autophosphorylation thus constituting a block in the signaling pathway is observed in most PCOS patients as the cause of insulin resistance. Early studies have clearly demonstrated that infertility and anovulation observed in women with PCOS can be attributed to hyperinsulinemia [22]. However, several studies have demonstrated the administration of insulin reducing agents have led to a reduction in the effects of
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Endocrine disrupting chemicals:
According to the US Environment Protection Agency, an endocrine disruptor is “an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are required for the maintenance of homeostasis, reproduction, development and/or normal behavior” [24]. A vast ocean of studies has been conducted to investigate the role played by these chemicals in disrupting the endocrine system.

Mechanism of action – Endocrine Disrupting Chemicals (EDCs) are observed to act in three ways:
1. by directly binding to Nuclear Receptors (NR) like Steroid Receptors, Androgen Receptors, non-nuclear steroid hormone receptors, non-steroid receptors (like dopamine receptor), orphan receptors (like aryl hydrocarbon receptor),
2. by competing with the original ligand for co-activators that would enable the activated receptor to transcribe the appropriate genes,
3. by increasing both hormone catabolism and production.

Various mechanisms have been demonstrated to play a major role in throwing the endocrine system off balance. Most notable of all EDCs is BisphenolA (BPA). A 228Da endogenic monomer used in the manufacturing of polycarbonate plastic, plastic water bottles, toys and as an additive in manufacturing polyvinylchloride (used in medical tubing). BPA is one of the most ubiquitous environmental pollutant associated with the pathogenesis of PCOS. Being one of the potent ligid for Estrogen Receptor (ER), it has been demonstrated that BPA interacts in a different manner than estradiol, has a higher affinity for ERβ than ERα and activates its co-regulators differently [25-29]. Apart from its estrogenmimetic properties, many studies have reported an increase in androgen levels. Increased androgen levels have found to down regulate uridinephosphate - glucuronosyltransferase activity, thereby leading to its decreased metabolic clearance. Furthermore, it is a potent ligand for Sex
Hormone Binding Globulin (SHBG) and consequently results in an increase in the free androgen levels [30]. Additionally, BPA is found to inhibit the release of adipokines from adipose tissues, thereby conferring an increased susceptibility to metabolic syndrome [26]. Organochlorines are potent EDCs owing to the absence of detoxification mechanisms and subsequent accumulation in lipid secreting cells. They have a strong carbon-chlorine bond which is resistant to cleavage by normal metabolic pathways. They are major components of pesticides and contaminate nearby water bodies, which upon ingestion, leads to hormonal imbalances. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is a commonly investigated organochlorine. It is postulated to act via the aromatic hydrocarbon receptor [27] and induce the expression of cytochrome P450, leading to its anti-estrogenic properties. In addition, in vitro studies have shed light on disruption of major pathways. Administration of human chorionic gonadotrophin and TCDD in immature female rats have been found to alter levels of estradiol, FSH and LH but no decrease in the estradiol levels were observed. In cynomolgus monkeys treated with TCDD, decreased concentrations of estradiol, progesterone and chorionic gonadotrophin was observed. Hexachlorobenzene (HCB) is a worldwide organic pollutant. When a pregnant woman is exposed to HCB, it is observed to be transmitted to the fetus by crossing the placenta prenatally and via the lactating milk after birth. Cynomolgus monkeys exposed to a concentration of 10mg/kg of body weight per day for three menstrual cycles decreased the levels of estradiol. Studies on rats indicate that HCB indirectly targets steroidogenesis. Studies demonstrating the effect of other organochlorines have been performed. Estrous irregularities were observed in rats treated with organochlorine pesticides like atrazine, hexachlor and methoxychlor. Women who consumed fish from Lake Ontario had a reduced menstrual length indicating a possible relationship between fish consumption and polychlorinated biphenyls (PCB) exposure. Many studies have demonstrated a decreased fecundity with exposure to different PCBs [32, 33]. PCBs disrupt follicular steroidogenesis by altering pattern of hormone synthesis, altering enzyme secretion, changing hormone affinities and mimicking natural hormones as an agonist [34]. Polychlorinated naphthalenes (PCN) have toxicological characteristics similar to that of PCB. It is postulated to be due to the inhibitory activity of the enzyme required for the conversion of testosterone to estradiol.

Geinstein is an isoflavonoid phytoestrogen which can be found in soy products, which are lactose-free substrate. An increase in anogenital distance (masculinization), accelerated puberty, and irregular estrous cycles in adult CD-1 mice was observed with neonatal administration of 0.5-50mg/kg of Geinstein. Batesman and Paul demonstrated that a short 2-day exposure of Geinstein caused dememinization of Hypothalamus Pituitary Gonadal (HPG) axis [35]. Endocrine disruptors have an effect on estrogen and steroid receptors and, in line, directly influence PCOS development. Studies have reported a positive association of BPA with PCOS. Women with PCOS showed higher levels of BPA and are strongly associated with metabolic and hormonal disturbances [36]. And also neonatal exposure to BPA results in PCOS development [37]. Studies also confirmed that excess fetal androgen exposure in female nonhuman primates may develop PCOS like symptoms in adulthood [38]. Thus, EDCs are environment pollutants which interfere with the functioning of normal metabolic pathways, culminating in hormonal imbalances and PCOS. The contributions of EDCs to PCOS and the pathways involved are depicted in Figure 2.

Figure 2: The mechanism of action of Endocrine disruptors.
Vitamin D:
Many studies have been conducted to evaluate the relationship between vitamin D levels and PCOS [39-43]. In tandem, a study involving 206 women with PCOS observed that 72.8% of them had insufficient vitamin D levels and demonstrated that almost three out of every four PCOS women may have vitamin D deficiencies [43]. About 60 – 75% of women with PCOS are found to have vitamin D levels of less than 20ng/ml [44]. Deficiency of vitamin D is found to exacerbate the symptoms of PCOS through genetic and cellular mechanisms which can be explained as follows: nuclear vitamin D receptors are found in a number of tissues like the parathyroid gland. These receptors are found to regulate the transcription of genes that are involved in the maintenance of LH, SHBG, and testosterone levels. So, a decrease in vitamin D levels leads to the improper transcription of the above mentioned genes which consequently affect their plasma levels, leading to observable symptoms. Furthermore, calcium is observed to regulate functions like egg activation, oocyte maturation, follicular development and mammalian ovary development and consequently a decrease in calcium levels leads to its improper control [45]. The authors of a study involving 13 women with PCOS (conducted by the Columbia University) hypothesize that a positive correlation exists between vitamin D levels and follicular development. Of the 13 subjects, five of them were found to have obvious vitamin D deficiency and three of them showed borderline deficiency. They were administered with vitamin D2 at a dose of 50,000 IU once or twice a week and also received an additional 1500mg of supplemental calcium per day. After the treatment period, seven subjects experienced a regular cycle within two months and two of them became pregnant [46]. In another study, observational studies show lower 25OHD levels were associated with insulin resistance, ovulatory and menstrual irregularities, lower pregnancy success, hirsutism, hyperandrogenism, obesity and elevated cardiovascular disease risk factors [44]. Hence, it is evident from a number of studies that administration of adequate doses of vitamin D is beneficial to the management of PCOS [47]. Also, vitamin D deficiency in PCOS women is associated with increased oestrogen production from ovary due to its control over the aromatase gene. Many studies have expressed the confounding role of insulin resistance, which is one of the most commonly observed phenotypes in women with PCOS [44]. However, recent studies indicate that individuals with increased vitamin D levels are 40% less likely to develop diabetes as this vitamin is found to increase insulin secretion, and reduce systemic inflammation [48]. Furthermore, a deficiency of vitamin D in PCOS women has found to be correlated with increased cardiovascular risk while a marked decrease in symptoms of depression has been observed in 50% of the patients when supplemented with vitamin D (More research is required in this area as the exact mechanism is not established) [49, 50]. Thus, vitamin D deficiency has a negative correlation with metabolic risk factors (insulin resistance, BMI, waist to hip ratio) and is found to aggravate symptoms of PCOS like hirsutism, hyperandrogenism, menstrual irregularities, obesity and increased risk for cardiovascular disease [51]. The contributions of vitamin D to PCOS and the pathways involved are depicted in Figure 3.

Figure 3: The role played by vitamin D in the maintenance of normal hormonal levels.
Implication of Genetic Factors Contributing to Insulin Resistance in the Pathogenesis of Polycystic Ovary Syndrome

Advanced glycation end products: Advanced glycation end products (AGEs) are produced when reducing sugars react non-enzymatically with the amino groups of protein (like lysine or arginine) to produce Schiff Bases and Amadori products, which consequently undergo reactions like rearrangement, dehydration and condensation to become heterogeneously linked AGEs [52]. They are involved in crosslinking of collagen by reducing enzyme activity and changing the biophysical properties to alter interactions with other enzymes. Immunohistochemical techniques have demonstrated the selective accumulation of AGEs in the ovaries of PCOS women [19]. The stroma of ovary is made up of collagen, so, both endogenous and exogenous AGEs (whose sources include smoking and improper cooking of food substances, consumption of pre-cooked fast food meals) can lead to dysregulation in the production of hormones and reproductive functions. Additionally, AGEs and their receptor RAGE have been localized in the granulosa layer of the ovarian tissue [30]. Furthermore, it has been observed that AGEs perpetuate insulin resistance by increasing the levels of protein kinase C. However, Phosphatidylinositol 3 kinase has been found to sequester AGEs through the Macrophage Scavenger Pathway (MSP) [17]. Consequently small tweaks in one’s diet can reduce the contribution of AGEs in exacerbation of the symptoms. The contributions of AGEs to PCOS and the pathways involved are depicted in Figure 4.

![Diagram of advanced glycation end products](image)

**Figure 4: The complex network of metabolism of Advanced Glycation End products (AGEs) - their production and mechanism of causing disruption in signaling pathways, thus culminating in PCOS.**

**Smoking:**
The physiological and genetic aspects of PCOS are extensively researched, however, very little has been done to study the psychological effects. It is observed that PCOS affected women are more prone to depression and anxiety, have a low self-esteem, and a negative body image and feminine identity [23]. However, smoking only deteriorates their quality of life. Women with PCOS become susceptible to cardiovascular risk [53, 54]. In a study by Morotti et al., 81 women were divided into three groups on the basis of the number of cigarette packets they smoked. It was demonstrated that the group that smoked more than 10 packets had a higher diastolic blood pressure, atherogenic index plasma and insulin sensitivity. Furthermore, smoking has profound negative effects on the hypothalamus – gonad axis that is found to operate by various mechanisms. Additionally, women with PCOS who smoke are susceptible to metabolic syndrome [55]. Also, many studies have demonstrated a positive correlation between smoking, delay in time of conception and decreased fecundity [56]. Hence, smoking by PCOS women must be avoided in order to improve the quality of life.

**Conclusion:**
Polycystic Ovary Syndrome is a disorder with a heterogeneous array of clinical features. It has not only been demonstrated to have a genetic basis, but the environmental factors implicated to play a major role have been researched. However, a lot of investigation must be performed to delineate the exact mechanism by which these factors affect the various physiologies of the reproductive pathways involved in PCOS. Many studies are a testament to the hypothesis that obesity exacerbates various clinical manifestations. Fortunately, there are many strategies to restore hormonal homeostasis. Exposure to various environmental pollutants at critical periods of development leads to the development of reproductive manifestations which present themselves at a later stage of life. Vitamin D levels play a major role at the
molecular level and consequently vitamin D supplements should be prescribed. As much the composition of one’s diet plays an important role in reducing weight, the style of cooking food influences its chemical nature, thereby leading to the formation of AGEs. Hence, appropriate cooking styles must be adopted. The hormonal imbalances evidenced in PCOS patients leads to emotional insecurities. These psychological side effects must be investigated and healthy ways of venting unlike smoking to obtain relief from stressors of life should be instituted. Thus, investigation from multiple perspectives is the need of the hour for the management of such a complex disorder as PCOS.

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Implication of Genetic Factors Contributing to Insulin Resistance in the Pathogenesis of Polycystic Ovary Syndrome
Implication of Genetic Factors Contributing to Insulin Resistance in the Pathogenesis of Polycystic Ovary Syndrome

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Implication of Genetic Factors Contributing to Insulin Resistance in the Pathogenesis of Polycystic Ovary Syndrome

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REVIEW

Genetic variants associated with insulin signaling and glucose homeostasis in the pathogenesis of insulin resistance in polycystic ovary syndrome: a systematic review

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Abstract Background Polycystic ovary syndrome must be recognized as a serious issue due to its implication on long term health regardless of an individual’s age. PCOS and insulin resistance are interlinked, as approximately 40 % of women with PCOS are insulin resistant. However, the detailed molecular basis for insulin resistance that is coupled with PCOS remains poorly understood. Objective To review the published evidence that polymorphisms in genes that are involved in insulin secretion and action are associated with an increased risk of PCOS. Methods We reviewed articles published through November 2012 which concerned polymorphisms of genes related to insulin signaling and glucose homeostasis as well as their associations with PCOS. The articles were identified via Medline searches. Conclusions No consistent evidence emerged of a strong association between the risk of PCOS and any known gene that is related to insulin signaling and glucose homeostasis. Moreover, recent genome-wide association studies are inconsistent in identifying the associations between PCOS and insulin metabolism genes. Many of the studies reviewed were limited by heterogeneity in the PCOS diagnosis and by not having a sufficient number of study participants. Further studies are warranted to determine predisposing risk factors which could modify environmental factors and thus reduce the risk of PCOS. Large genome-wide association studies devoted solely to PCOS will be necessary to identify new candidate genes and proteins that are involved in PCOS risk.

Keywords Insulin resistance · PCOS · SNP · Genetics

Introduction

Polycystic ovary syndrome (PCOS) must be considered a serious issue because of its implication on long term health regardless of a woman’s age. It needs to be seen as a lifelong condition, not one tied only to pregnancy. Polycystic ovary syndrome (PCOS) is a very common and complex female endocrine disorder. It affects women in their reproductive years with an estimated prevalence of 4–8 % [13]. The ESHRE/ASRM consensus conference held in Rotterdam in 2003 defined the syndrome as having two of the following three conditions diagnosed as PCOS: oligo-ovulation; clinical or biochemical evidence of androgen excess; and multicystic ovaries. The diagnostic criteria for defining the PCOS are as heterogeneous as the disease itself and have been amended in recent years. During the first international conference on PCOS held at the National Institutes of Health (NIH) in the US in 1990, three key features of PCOS were generally agreed on, including chronic anovulation; hyperandrogenism (clinical or laboratory evidence); and the absence of other endocrine disorders (e.g., congenital adrenal hyperplasia, hyperprolactinaemia or thyroid abnormalities) [46]. Polycystic ovary syndrome affects women of all races and nationalities. Indeed, this heterogeneous condition affects 7–10 % of women worldwide [10, 12] irrespective of their ethnic background [64]. An estimated prevalence of 20 % of the normal female population has polycystic ovaries [104]. PCOS is the most common cause of oligo-ovulatory infertility which is characterized by insulin resistance (IR), while hyperinsulinemia is found in 50–70 % of women diagnosed with PCOS. Women with PCOS are at increased risk for diabetes, dyslipidemia, atherosclerosis [22, 59, 117] as well as endometrial carcinoma.
Implication of Genetic Factors Contributing to Insulin Resistance in the Pathogenesis of Polycystic Ovary Syndrome

Furthermore, it is suggested that women with PCOS are at an increased risk for miscarriages, gestational diabetes, preeclampsia and preterm labour [19]. Due to the clinical and biochemical heterogeneity of PCOS, several studies have focused on the aspects of hormonal, genetic and environmental factors involved in the development of the syndrome. A study of PCOS subjects representing three different ethnic groups revealed that obesity and hirsutism varied with genetic and environmental factors. At the same time, the prevalence of adrenal androgen excess and insulin resistance among these subjects appeared fairly uniform [106]. More recently, DeUgarte [39] observed that ethnicity and PCOS were associated with independent and additive defects of insulin action in Caribbean-Hispanic PCOS women. However, women with PCOS undergo several interrelated features including ovarian hyperandrogenism, chronic anovulation, polycystic ovaries; these are coupled with anomalous androgen and insulin-related parameters irrespective of other standard reproductive factors [4]. The genetic basis of the disease is not clearly known, which is largely due to the difficulties in determining the inheritability of PCOS. The genes that regulate insulin secretion and action, ovarian and adrenal steroidogenesis and energy regulation act as candidate genes which determine the expression of several integral phenotypes of PCOS. The present review concentrates on the polymorphisms in the genes that are involved in insulin secretion and action (Table 1).

Insulin resistance and PCOS

PCOS and insulin resistance are interlinked, as approximately 40% of women with PCOS have been found to be insulin resistant [135, 162, 169]. Insulin resistance is a common feature in both polycystic ovary syndrome (PCOS) and non-insulin-dependent diabetes mellitus (NIDDM); however, persistent reproductive disturbances were limited to the PCOS, suggesting that insulin resistance in the ovary itself may be responsible for this susceptibility [173]. Insulin resistance refers to a state in which circulating insulin does not bind to the insulin receptors on the cell, or it does bind but its effects are deficient, thus giving a less than normal reduction of glucose to a given amount of insulin [30]. The pancreas then continues to secrete more insulin, leading to higher levels in the blood and ensuring normal glucose tolerance [58]. The association between insulin resistance and PCOS has provided significant insight into the pathogenesis of PCOS [141]. Several studies indicated altered insulin levels which can directly stimulate ovarian androgen production in PCOS [130, 137]. Hyperinsulinemia leads to hyperandrogenemia by stimulating ovarian androgen production [38, 63]. Insulin can also stimulate adrenal steroidogenesis by enhancing sensitivity to adrenocorticotrophic hormone (ACTH) and can increase pituitary LH release [45, 154]. Increased androgen levels lead to menstrual disturbances, development of ovarian cysts, hirsutism and other related disorders [22, 59, 117]. Important physiological processes including cellular glucose uptake [25, 133], metabolism [124, 133] and gene expression [109] are regulated by insulin. Specific abnormalities of insulin metabolism have been identified in PCOS. These include reduction in secretion, reduced hepatic extraction [116], impaired suppression of hepatic gluconeogenesis [44] and abnormalities in insulin receptor signaling [43].

Insulin signaling pathway

Insulin regulates both metabolism and gene expression. The insulin signal passes from the plasma membrane receptor to insulin-sensitive metabolic enzymes and then reaches the nucleus where it stimulates the transcription of certain genes. The insulin receptor is a heterodimeric complex consisting of 2 extracellular α-subunits and 2 transmembrane β-subunits. The α-subunit contains the insulin binding domain. The binding of insulin to the α subunit activates the tyrosine kinase activity of β subunit to transphosphorylate one another. This allows association of insulin receptor substrates, such as IRS-1 and IRS-2 a cascade of intracellular signaling proteins to the regulatory subunit of P13k kinase. The activated P13k further phosphorylates the membrane phospholipids and produces the phosphatidylinositol-3, 4, 5 triphosphate (PIP3). This PIP3 activates the enzyme protein kinase B (PKB: also known as Akt) which helps in the translocation of GLUT4 to the cell surface and results in the increased glucose uptake of the cells [2]. Defects in the insulin signaling system may result in insulin resistance, obesity, and type II diabetes [24, 95, 100]. Nevertheless, the detailed molecular basis for insulin resistance that is coupled with PCOS remains poorly understood.

Genes involved in insulin resistance

The majority of the evidence supports the finding that most women with PCOS have both insulin resistance and compensatory hyperinsulinemia. Insulin resistance in PCOS predisposes the individual to type 2 diabetes. The heritability of beta-cell dysfunction observed in families of women with PCOS demonstrated beta-cell dysfunction, a significant factor that predisposes to type 2 diabetes [31]. Therefore, several candidate genes involving signaling pathways (insulin secretion and action) are examined for PCOS.

Insulin gene (INS)

Insulin is composed of 2 dissimilar polypeptide chains, A and B, which are linked by 2 disulphide bonds. The gene coding for
### Table 1: List of polymorphisms that studied in different regions of the world

<table>
<thead>
<tr>
<th>Variants studied</th>
<th>Population</th>
<th>Study design</th>
<th>Samples</th>
<th>Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS-VNTR</td>
<td>United Kingdom</td>
<td>family based</td>
<td>17 Families</td>
<td>Yes</td>
<td>[168]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>European</td>
<td>family based</td>
<td>150 Families</td>
<td>No</td>
<td>[157]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>UK</td>
<td>family based</td>
<td>74 families</td>
<td>Yes</td>
<td>[105]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Czechoslovakia</td>
<td>case-control</td>
<td>38 cases, 22 controls</td>
<td>No</td>
<td>[159]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Spanish</td>
<td>case-control</td>
<td>96 cases, 38 controls</td>
<td>No</td>
<td>[20]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Irish</td>
<td>case-control</td>
<td>185 cases, 1,062 controls</td>
<td>No</td>
<td>[125]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>United Kingdom</td>
<td>family based association trios</td>
<td>255 parent-offspring trios</td>
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<td>[125]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Finnish</td>
<td>Cohort</td>
<td>1,599</td>
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<td>[125]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Estonian women</td>
<td>case-control</td>
<td>30 cases, 75 controls</td>
<td>No</td>
<td>[72]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Slovak</td>
<td>case-control</td>
<td>117 cases, 108 controls</td>
<td>No</td>
<td>[50]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Han Chinese</td>
<td>case-control</td>
<td>216 cases, 192 controls</td>
<td>No</td>
<td>[175]</td>
</tr>
<tr>
<td>INS-VNTR</td>
<td>Korean</td>
<td>case-control</td>
<td>218 cases, 141 controls</td>
<td>No</td>
<td>[176]</td>
</tr>
<tr>
<td>INSRR</td>
<td>United Kingdom</td>
<td>case-control</td>
<td>22 cases, 8 controls</td>
<td>No</td>
<td>[32]</td>
</tr>
<tr>
<td>Mutation scanning</td>
<td>United Kingdom</td>
<td>case-control</td>
<td>108 cases, 5 control</td>
<td>No</td>
<td>[148]</td>
</tr>
<tr>
<td>D19S884 &amp; other loci</td>
<td>European</td>
<td>family based</td>
<td>150</td>
<td>Yes</td>
<td>[157]</td>
</tr>
<tr>
<td>D19S884 &amp; other loci</td>
<td>Caucasian</td>
<td>case-control</td>
<td>85 cases, 87 controls</td>
<td>Yes</td>
<td>[155]</td>
</tr>
<tr>
<td>C/T -C10923T</td>
<td>US</td>
<td>case-control</td>
<td>99 cases, 136 controls</td>
<td>Yes</td>
<td>[138]</td>
</tr>
<tr>
<td>His1058 C/T</td>
<td>Chinese</td>
<td>No</td>
<td>[164]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hist1058 C/T</td>
<td>Korean</td>
<td>case-control</td>
<td>9 cases, 9 controls</td>
<td>No</td>
<td>[55]</td>
</tr>
<tr>
<td>Hist1058 C/T</td>
<td>Chinese</td>
<td>case-control</td>
<td>120 cases, 40 controls</td>
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<td>[26]</td>
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<tr>
<td>Cys1008</td>
<td>Chinese</td>
<td>case-control</td>
<td>109 cases, 107 controls</td>
<td>Yes</td>
<td>[83]</td>
</tr>
<tr>
<td>176447C&gt;T</td>
<td>Korean</td>
<td>case-control</td>
<td>134 cases, 100 controls</td>
<td>Yes</td>
<td>[94]</td>
</tr>
<tr>
<td>His1058 C/T</td>
<td>Indian</td>
<td>case-control</td>
<td>180 cases, 144 controls</td>
<td>Yes</td>
<td>[110]</td>
</tr>
<tr>
<td>INSRR exon17 C/T</td>
<td>Turkish</td>
<td>case-control</td>
<td>44 cases, 50 controls</td>
<td>No</td>
<td>[156]</td>
</tr>
<tr>
<td>IRS</td>
<td>France</td>
<td>case-control</td>
<td>53 cases, 102 controls</td>
<td>Yes</td>
<td>[50]</td>
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<tr>
<td>IRS1-Gly972Arg</td>
<td>Caucasians</td>
<td>case-control</td>
<td>69 cases, 15 controls</td>
<td>Yes</td>
<td>[172]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Chile</td>
<td>case-control</td>
<td>82 cases, 70 controls</td>
<td>Yes</td>
<td>[140]</td>
</tr>
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<td>IRS1-Gly972Arg</td>
<td>African-American</td>
<td>case-control</td>
<td>227 cases, 175 controls</td>
<td>No</td>
<td>[49]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Chile</td>
<td>case-control</td>
<td>143 cases, 97 controls</td>
<td>Yes</td>
<td>[139]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Turkish</td>
<td>case-control</td>
<td>60 cases, 60 controls</td>
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<td>[40]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>USA</td>
<td>case-control</td>
<td>114 cases, 95 controls</td>
<td>No</td>
<td>[171]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Spanish</td>
<td>case-control</td>
<td>103 cases, 48 controls</td>
<td>No</td>
<td>[163]</td>
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<tr>
<td>IRS2-Gly1057Asp</td>
<td>Germany</td>
<td>case-control</td>
<td>57 cases, 567 controls</td>
<td>No</td>
<td>[69]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Taiwanese</td>
<td>case-control</td>
<td>47 cases, 45 controls</td>
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<td>[97]</td>
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<tr>
<td>IRS1-Gly972Arg</td>
<td>Japanese</td>
<td>case-control</td>
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<td>Yes</td>
<td>[14]</td>
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<tr>
<td>IRS1-Gly972Arg</td>
<td>Chile</td>
<td>case-control</td>
<td>50 cases, 75 controls</td>
<td>No</td>
<td>[158]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Greece</td>
<td>case-control</td>
<td>183 cases, 88 controls</td>
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<td>[29]</td>
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<td>IRS2-Gly1057Asp</td>
<td>Italian</td>
<td>case-control</td>
<td>65 cases, 27 controls</td>
<td>Yes</td>
<td>[130]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Slovak</td>
<td>case-control</td>
<td>53 cases, 21 controls</td>
<td>No</td>
<td>[42]</td>
</tr>
<tr>
<td>IRS1-Gly972Arg</td>
<td>Greece</td>
<td>case-control</td>
<td>162 cases, 122 controls</td>
<td>No</td>
<td>[101]</td>
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<tr>
<td>IRS2-Gly972Arg</td>
<td>rs7887237, rs1865434</td>
<td>discovery cohort</td>
<td>273 cases, 173 controls</td>
<td>Yes</td>
<td>[65]</td>
</tr>
</tbody>
</table>
insulin is localized to 11p15.5 [74] and is located between the genes for tyrosine hydroxylase and the insulin-like growth factor-II (IGF-II) [84]. The human insulin gene contains three exons: exon 2 encodes the signal peptide, the B chain, and a fraction of the C peptide; while exon 3 encodes the balance of the C peptide and the A chain [144]. Insulin hormone is not synthesized as an active protein; insulin mRNA is initially translated into a single chain precursor called proinsulin. The proinsulin is 110 amino acids long and made up of a signal peptide, the A, B and C chains. The proinsulin enters
the endoplasmic reticulum and loses its signal peptide and converts into proinsulin which is 86 amino acids long. Later, the proinsulin is exposed to several specific endopeptidases and further loses the C chain; it is thus left with only the A and B chains, which is considered as insulin precursor [47]. Two compound heterozygote mutations which behave in a cis-dominant fashion to decrease mRNA transcription levels have been identified in the insulin-receptor gene of a patient with leprechaunism. Within this single allele there is a nonsense mutation at codon 897, while the other alleles map outside the coding sequence of the gene [167]. More recently, the direct sequencing of all 22 exons of the \textit{INSR} gene in three women with PCOS did not reveal any mutations [142]. The screening of 22 hyperinsulinemic patients for mutations of the insulin receptor gene revealed that these mutations are associated with decreased insulin resistance in UK PCOS subjects [32]. Furthermore, the screening of 24 severe insulin resistance patients revealed several mutations, but none of their missense or nonsense mutations contributed to the insulin resistance found in UK subjects with PCOS [148]. A His1058 C/T SNP at exon 17 of \textit{INSR} is not associated with decreased insulin resistance in Chinese [26, 164], Korean [55], or Turkish women with PCOS [156]. However, this polymorphism did show a significant association with the lean rather than the obese US [138] and Indian PCOS women [60]. A novel SNP in intron 21 (176477 C > T) of \textit{INSR} showed strong association with the pathogenesis of PCOS in the Korean population [142]. A meta-analysis of eight studies comprising 795 cases and 576 controls found no significant evidence for an association between PCOS and \textit{INSR} His1058 C/T polymorphism [107]. By contrast, linkage analysis using STRs encompassing the \textit{INSR} region of chromosome 19 did find evidence for an association between the D19S884 locus on \textit{INSR} [85, 150, 155]. Furthermore, the DNA sequence surrounding D19S884 conferred in vitro promotes activity in lymphoblastoid cell lines [11]. A recent study using pathway-based tagging SNP identifies new \textit{INSR} SNPs associated with PCOS; moreover, a large replication cohort confirmed association of PCOS with rs2252673 [152]. A family-based association study using 260 trios of Han Chinese origin did not reveal significant evidence of association or linkage of the \textit{INSR} gene to PCOS [78]. According to a Chinese study, a novel T/C SNP at codon Cys1008 of the \textit{INSR} gene is associated with decreased insulin sensitivity in Chinese PCOS women. The study found that the association is not caused by the change of synthesis or secretion of the \textit{INSR} beta-subunit, but most probably by the effects of this novel SNP on the function of the \textit{INSR} beta-subunit [83]. A novel SNP in the \textit{INSR} gene, +176477 C > T, was associated with the pathogenesis of PCOS in a Korean population [94]. The study found a significant association of C/T polymorphism at His1058 of \textit{INSR} with PCOS in lean rather than obese Indian women [110].

**Insulin receptor gene (\textit{INSR})**

The insulin receptor is a heterotetrameric glycoprotein with two alpha and beta units. Its gene is located at chromosome 19p13.2, spanning 120Kb with 22 exons [136]. The tyrosine kinase domain of the receptor, which is necessary for insulin signal transduction, is encoded by exon 17–21. Alpha and beta subunits of the insulin receptor were derived by the proteolytic processing of a common 1,382 amino acid preproreceptor [47]. Two compound heterozygote mutations which behave in a cis-dominant fashion to decrease mRNA transcription levels have been identified in the insulin-receptor gene of a patient with leprechaunism. Within this single allele there is a nonsense mutation at codon 897, while the other alleles map outside the coding sequence of the gene [167]. More recently, the direct sequencing of all 22 exons of the \textit{INSR} gene in three women with PCOS did not reveal any mutations [142]. The screening of 22 hyperinsulinemic patients for mutations of the insulin receptor gene revealed that these mutations are not involved in causing insulin resistance in UK PCOS subjects [32]. Furthermore, the screening of 24 severe insulin resistance patients revealed several mutations, but none of their missense or nonsense mutations contributed to the insulin resistance found in UK subjects with PCOS [148]. A His1058 C/T SNP at exon 17 of \textit{INSR} is not associated with decreased insulin resistance in Chinese [26, 164], Korean [55], or Turkish women with PCOS [156]. However, this polymorphism did show a significant association with the lean rather than the obese US [138] and Indian PCOS women [60]. A novel SNP in intron 21 (176477 C > T) of \textit{INSR} showed strong association with the pathogenesis of PCOS in the Korean population [142]. A meta-analysis of eight studies comprising 795 cases and 576 controls found no significant evidence for an association between PCOS and \textit{INSR} His1058 C/T polymorphism [107]. By contrast, linkage analysis using STRs encompassing the \textit{INSR} region of chromosome 19 did find evidence for an association between the D19S884 locus on \textit{INSR} [85, 150, 155]. Furthermore, the DNA sequence surrounding D19S884 conferred in vitro promotes activity in lymphoblastoid cell lines [11]. A recent study using pathway-based tagging SNP identifies new \textit{INSR} SNPs associated with PCOS; moreover, a large replication cohort confirmed association of PCOS with rs2252673 [152]. A family-based association study using 260 trios of Han Chinese origin did not reveal significant evidence of association or linkage of the \textit{INSR} gene to PCOS [78]. According to a Chinese study, a novel T/C SNP at codon Cys1008 of the \textit{INSR} gene is associated with decreased insulin sensitivity in Chinese PCOS women. The study found that the association is not caused by the change of synthesis or secretion of the \textit{INSR} beta-subunit, but most probably by the effects of this novel SNP on the function of the \textit{INSR} beta-subunit [83]. A novel SNP in the \textit{INSR} gene, +176477 C > T, was associated with the pathogenesis of PCOS in a Korean population [94]. The study found a significant association of C/T polymorphism at His1058 of \textit{INSR} with PCOS in lean rather than obese Indian women [110].

**Insulin receptor substrates (\textit{IRS})**

Tyrosine phosphorylation is the result of insulin binding to its receptor; this in turn leads to the phosphorylation of several
protein and insulin receptor substrates (IRS), including primarily IRS-1 and IRS-2, for initiating and coordinating multiple downstream pathways [145, 146]. A series of gene “knockout” experiments demonstrated the critical role of both IRS1 and IRS2 where both aid in activating multiple signaling pathways for the regulation of glucose homeostasis by insulin [8, 149]. The human IRS1 gene contains the entire 5' untranslated region and the protein coding region in a single exon and is localized on chromosome 2q36-37 by in situ hybridization [9]. The IRS2 gene is mapped on chromosome 13q34 [80]. The open reading frames of IRS1 and IRS2 predict a molecular weight of 131 and 136 kD. Arg972Gly, a common variant of IRS1, lies between two potential sites of tyrosine phosphorylation involved in binding the p85 subunit of the PI-3 kinase. Although the G972R variant is not associated with abnormal expression of the IRS-1 protein [147], it does impair signaling [6]. Asp1057Gly, a common IRS2 variant, has not been associated with changes in insulin sensitivity in lean or obese adults [5].

Although the initial study did not reveal any association of PCOS with the IRS1 gene [157], many subsequent studies have been concentrated on Arg972Gly and Asp1057Gly polymorphisms in PCOS; this continuing interest is due to the complementary role of IRS1 and IRS2 in insulin signaling. The higher frequency of the IRS1 variant was observed in adolescent girls with hyperandrogenism [172], but the G972R variant acted as a modifier locus among women who are heterozygous carriers of CYP21, which indicates its limited role in the development of PCOS [171]. Recently, attention has also been focused on insulin receptor substrates and the association with PCOS of SNPs at the IRS1 and IRS2 loci. The results, however, are contradictory. A slightly higher frequency of Arg972 was observed in PCOS women of Chilean [139, 140] and Turkish populations [40]. The IRS1 Gly972Arg polymorphism is significantly associated with PCOS in the Japanese [14] and Greek populations [29]. The IRS1 Gly972Arg has the highest frequency reported worldwide and is associated with insulin resistance and higher fasting insulin in Southern Italian women [120]. Furthermore, the IRS1 genotype also influenced the fasting insulin levels and HOMA indices in PCOS women on metformin therapy [52]. No significant association between insulin receptor substrate genes and PCOS was reported in the French [50], Spanish [163], German [69], Taiwanese [97], Chilean [158], Slovak [42], Greek [101], Indian [35] or Iranian populations [127]. Very few studies reported an association between IRS2 Gly1057Asp and PCOS. The Gly1057Asp polymorphism influenced blood glucose levels in nondiabetic Caucasian and African-American women with PCOS [49]. An analysis of US Caucasian women revealed three additional IRS2 SNPs that are associated with PCOS (rs7997595, rs7987237, rs1865434) [65]. A recent genome-wide association study (GWAS) of PCOS in Han Chinese women failed to detect associations between the polymorphism of the IRS gene and PCOS [27]. However, two independent meta-analyses suggest that IRS1 Gly972Arg polymorphism causes significant risk for PCOS, but that IRS2 Gly1057Asp polymorphism has not shown such risk [80, 131].

**Insulin-like growth factors (IGFs)**

The IGFs are peptide hormones secreted from many different cells and exhibiting a high sequence of similarity to insulin. There are two principal IGFs, known as IGF-1 and IGF-2. Their functions include: modulation of growth hormone action; stimulation of growth of cultured cells; stimulation of the action of insulin; and involvement in development and growth. Each of these has a number of variant forms, a result of the use of alternative gene promoters and alternative splicing. The gene IGF2 is located on chromosome 11p15.5 [115]. A single nucleotide polymorphism (SNP) in the 3' untranslated region of the IGF2 gene (ApaI; rs680) is known to increase IGF2 mRNA in leukocytes due to increased liver IGF2 expression and secretion. Together with IGF1 and IGF-binding proteins, IGF2 stimulates adrenal and ovarian androgen secretion. The association between PCOS and G alleles of the ApaI polymorphism (IGF2 3'UTR GA; rs680) was first established in Spanish women [134]. A subsequent study found that the ApaI polymorphism in the IGF2 cluster in combination with the −108 polymorphism (rs705379) in PON1 increased the risk of PCOS in German women [89]. A recent study showed a predominance of ApaI GA + AA genotypes in younger Brazilian women with PCOS [126].

**Peroxisome proliferator-activated receptor γ (PPARG)**

Peroxisome proliferator-activated receptors are members of the nuclear receptor super family of ligand-activated transcription factors [81]. The PPAR-γ2 is formed by an alternative mRNA splicing pathway and regulates the transcription and expression of numerous target genes. These genes have been shown to be involved in adipocyte differentiation, lipid and glucose metabolism, and atherosclerosis [96]. The gene coding for PPAR-γ is known to be expressed in the inner areas of fat cells and directs the transcription of a number of genes involved in the regulation of insulin sensitivity and glucose metabolism. Recent studies on PPAR-γ polymorphisms have revealed associations with reduced expression of insulin sensitivity [63]. The human PPAR-γ gene is composed of 9 exons; it spans more than 100 kb of genomic DNA [54]. A common C to G base exchange leads to the substitution of proline with alanine at codon 12, which has been associated with reductions in both DNA binding and transcriptional activity in vitro. Recent studies have indicated that the Ala12 allele is involved in increased insulin sensitivity by enhanced suppression of lipid oxidation, thereby permitting more efficient glucose disposal.

Several studies have found similar genotype and allele frequencies of the PPAR-γPro12Ala polymorphism in PCOS.
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women and healthy controls in Italy [118, 119], Spain [134], China [166], Turkey [153], Chile [68], Korea [23], Greece [28, 90, 174], Los Angeles [7], Germany [69, 88], Poland [18] and Slovenia [41]. Although PPAR-γPro12Ala polymorphism is equally distributed in PCOS women and healthy controls, it showed a modifier effect on insulin resistance in both German [71] and multi-ethnic populations [73]. Contrary to these findings, however, some studies have shown that the Pro12Ala polymorphism is significantly more frequent in control subjects when compared with PCOS women, indicating a protective effect by the Ala allele against the development of PCOS in Finland [92], Turkey [176], India [35], and Korea [67]. PCOS subjects carrying Pro12Ala showed higher leptin levels than the Pro12Pro and Ala12Ala genotypes, indicating that the single Ala12 allele may play a protective role in respect to hyperleptinemia [77]. Although the protective trend of the G allele existed, a recent meta-analysis did not show a significant association between Pro12Ala and PCOS [151]. A meta-analysis using 17 case-control studies from Europe and Asia supports the finding that the PPAR-γPro12Ala polymorphism is capable of reducing the PCOS in European but not in Asian women [179]. Yet another meta-analysis using 17 studies reported that the Ala12 variant would decrease the risk of PCOS and result in lower BMI and fasting insulin levels in Europeans, but would have no impact on HOMA-IR in PCOS patients [75].

Calpain-10 (CAPN10)

Calpains are calcium-dependent intracellular nonlysosomal proteases that are capable of hydrolyzing specific substrates involved in calcium-regulated signaling pathways [51]. Calpain-10 is an atypical member of the calpain family and is expressed at the mRNA and protein levels by several tissue types including pancreatic β islet cells; liver; skeletal muscle; and adipocytes [21, 123]. The gene encoding calpain-10 (CAPN10) consists of 15 exons and is located on chromosome 2q37.3. It was shown to be related to proinsulin processing, insulin secretion and insulin resistance [15, 181]. CAPN10 variants are known to influence cholesterol levels, blood pressure values, and insulin resistance phenotypes in the Spanish population [132]. Several SNPs in CAPN10 (UCSNP-63, −44, −43, −19) have been the focus of PCOS researches, however, the results are contradictory.

CAPN10 UCSNPs associated with PCOS varies in different populations. The CAPN10 UCSNP-44 allele showed significant association in the populations of Spanish [61, 62], Turkish [177], and Indian women [34]. A significant association between the UCSNP-43 polymorphism and the PCOS metabolic phenotype was found in hirsute southern Brazilian patients [170] as well as Chilean PCOS women [102]. The UCSNP-45 C allele is associated with ideopathic hirsutism in Spanish PCOS women [53]. The UCSNP-56 and insdel-19 are found to be in strong linkage disequilibrium and showed significant association with PCOS in German women. The TGG3AGCA and TGA2AGCA haplotypes showed both decreased and increased risk for PCOS [165]. The more common allele of UCSNP-63 showed evidence for excess transmission in a single-locus transmission disequilibrium analysis of European trios. However, this association was not replicated in the case-control study from that region [70]. In contrast to these associations, none of the CAPN10 polymorphisms were associated with PCOS in German Caucasians [69] or Turkish adolescent girls [156]. Although there is no significant association between individual polymorphisms of CAPN10 and PCOS, neither the haplotype nor the diplootypes of this gene showed significant associations with PCOS in African-American [48], Korean [93] or Indian populations [34]. A recent meta-analysis using 11 case control studies demonstrated that the CAPN10 UCSNP-63 homozygous allele and the UCSNP-19 insert allele are protective factors for PCOS [77].

Metformin and PCOS

Several studies have postulated that the use of metformin in women with PCOS may reduce the endocrine and metabolic features of PCOS. The first and foremost study of metformin in obese women with PCOS demonstrated a restoration of normal menses and reduced hyperinsulinemia, insulin resistance, hyperandrogenemia, and systolic blood pressure [161]. Ensuing studies have reported that metformin can alter sex hormone binding globulin (SHBG) and free T levels [112]; increase the rate of ovulation [114, 160]; and improve the efficacy of ovulation induction medications including clomiphene citrate [113] and exogenous gonadotropins [37]. A meta-analysis using 13 randomized trials involving 543 women with PCOS reported that the use of metformin significantly increased ovulation frequency compared to placebo. Furthermore, this meta-analysis confirmed that the metformin in combination with clomiphene citrate showed superior ovulation when compared to the clomiphene alone [98]. Although metformin’s actions are mediated by activation of AMP-activated protein kinase (AMPK) [180], its exact molecular mode of action remains unclear.

Several lines of evidence indicate that metformin treatment in women with PCOS results in a decline of insulin as well as total bioavailable T [91], which leads to a significant reduction in hyperinsulinemia and hyperandrogenism [128]. It has been shown that luteinizing hormone (LH) and insulin reduction with metformin increases progesterone [103]; serum glycochen; and insulin-like growth factor-binding protein 1 concentrations [82] during luteal phases. Importantly, this indicates an improved endometrial milieu for the establishment and maintenance of pregnancy in PCOS women. A meta-
analysis of 8 randomized controlled studies investigating metformin in PCOS women found a significant reduction in the risk of ovarian hyperstimulation syndrome, while at the same time showing no improvement in pregnancy rates after metformin treatment [33]. Moreover, significant teratogenicity is also not evident. Despite its advantages, metformin is listed as a “Category B” drug because its safety in pregnancy has not yet been established.

Environmental factors and PCOS

Many studies have concentrated on possible environmental factors that contribute to the development or progression of PCOS. Several environmental factors are known to unveil genetically programmed susceptibility to PCOS and contribute to its phenotypic expression. These factors interact chiefly with early stages of human development and convert a predisposed genotype to the phenotypic expression of PCOS. Low-birth-weight infants show an increased incidence of precocious puberty, hyperinsulinemia, and hyperandrogenism compared to normal-weight infants [79]. The foetus or infant with retarded growth will develop PCOS when exposed to nutritional surplus later in life [3]. A nutritional surplus with the consumption of high-calorie diets leads to obesity and induces the development or progression of the clinical spectrum of PCOS [108, 122]. Furthermore, environmental determinants may influence the clinical severity of PCOS, ranging from a less-severe phenotype to the mature phenotype of classic PCOS. The exposure of pregnant non-human primates and sheep to excess androgens can cause the development of a syndrome similar to PCOS, indicating that the exposure to androgen-like chemicals absorbed by the human body can lead to PCOS [1, 129]. A retrospective study demonstrated that disposable plastic drinking cups, cooking oil flumes and indoor decorations made of plastic increased the PCOS risk, indicating that environmental endocrine-disrupting chemicals are associated with the risk of PCOS [76]. Bisphenol A (BPA), a known hormone disrupter which is present in our environment, food, and consumer products, is elevated and associated with higher levels of male hormones in the blood of women and results in a deviation from normal homeostasis or reproduction. Studies using experimental animals have demonstrated that neonatal exposure to BPA leads to PCOS development [57]. Moreover, serum BPA levels were positively associated with serum androgen levels and insulin resistance indices in both lean and obese PCOS women [87].

Conclusions

The cited genetic studies which focused on PCOS using several different approaches in different populations are limited by heterogeneity in the PCOS diagnosis as well as the relatively small number of participants in the researches. No consistent evidence emerged of a strong association between the risk of PCOS and any of the known genes related to insulin signaling and glucose homeostasis. Although an individual’s geographic location, ethnic origin, and cultural or social practices are known to alter manifestations of PCOS, earlier studies did not consider these factors. Moreover, the failure of researchers to replicate the results of more recent genome-wide association and linkage studies leaves this field with the both phenotypic variability and lack of a male phenotype as well as the associated comorbidities of PCOS.

Future directions

Future research should focus on early detection of the predisposing risk factors in PCOS development, including long-term studies with the goal of modifying environmental factors so that risk may be significantly reduced. Large genome-wide association studies devoted solely to PCOS will be necessary to identify new candidate genes and proteins that are involved in PCOS risk. Experiments related to pathophysiological perturbations and interventions which will normalize signal transduction of these pathways should be conducted in a number of cell culture and animal models to shed more light on our understanding of the pathophysiology of PCOS. The use of Systems Biology approaches in analyzing biochemical networks will enable us to better comprehend the multi-system cross-talk underlying the etiology of PCOS.

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