The 312N variant of luteinizing hormone/choriogonadotropin receptor gene (LHCGR) confers up to 2.7-fold increase of risk of polycystic ovary syndrome in a Sardinian population

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The 312N variant of luteinizing hormone/choriogonadotropin receptor gene (LHCGR) confers up to 2.7-fold increase of risk of polycystic ovary syndrome in a Sardinian population.

short title: LHCGR gene and PCOS in Sardinian women

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Nothing to declare.
Abstract

Objective. Polycystic ovary syndrome (PCOS) is a frequent condition, affecting about 15% of women in reproductive age. Due to familial recurrence, a multifactorial model of susceptibility, including both genetic and environmental factors, has been proposed. However, the identification of genetic factors has been elusive.

Design. Case-control study, aimed at evaluating possible associations between functionally relevant variants of luteinizing hormone/choriogonadotropin receptor gene (LHCGR) and PCOS phenotype.

Patients. 198 PCOS and 187 non-PCOS women, aged 14-35 years, of Sardinian origin, referred to the outpatient clinic of the Department of Obstetrics and Gynecology of the University of Cagliari (Sardinia). PCOS diagnosis was based on the Rotterdam criteria.

Measurements. We have determined the genotype of ins18LQ, S291N, and S312N variants at the LHCGR locus. Genotype was related to the presence or absence of PCOS and to several clinical and biochemical characteristics.

Results. The presence of at least one 312N allele was strongly associated with PCOS risk (O.R.:2.003, 95% C.I.: 1.32-3.15, \( \chi^2 \):10.47, p:0.001). 312N homozygosity was associated with a further risk increase (O.R.:2.72, 95% C.I.:1.24-5.95, \( \chi^2 \):6.65, p:0.005). The number of ins18LQ alleles was associated with LH serum levels in controls (\( \chi^2 \):8.04, p:0.017).

Conclusions. For the first time, we have identified a genetic variant strongly associated with PCOS in an isolated population. These results, if confirmed in other cohorts, may provide the opportunity to test the S312N genotype at the LHCGR locus in fertile women to assess the risk of PCOS. The avoidance of triggering factors like weight increase may improve the reproductive outcome of potentially at-risk subjects.
Introduction

Polycystic ovary syndrome (PCOS) is a very common endocrine disorder, affecting about 15% of women in reproductive age. The clinical picture of the condition is highly variable, resulting in several revisions of the diagnostic criteria. The most recent consensus on clinical criteria for the diagnosis of PCOS was reached in 2003 in the context of the Rotterdam workshop, sponsored by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine. Based on these criteria, the diagnosis of PCOS is made when two of the following signs are present: oligo-amenorrhea/anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovary (PCO) at ultrasound examination. However, that of PCOS is an exclusion diagnosis since other conditions determining hyperandrogenism should be ruled out, including non-classical adrenogenital syndrome, Cushing syndrome and disease, ovarian and suprarenal neoplasms, etc.

The etiology of the condition is largely unknown; the existence of genetic factors responsible for, or predisposing to the syndrome has been postulated on the basis of familial aggregation of PCOS and/or related conditions, such as hyperandrogenism or hyperinsulinism. Some Authors proposed an autosomal dominant pattern of inheritance with incomplete penetrance and variable expressivity. At present, the most commonly accepted model is that of a multifactorial etiology with a strong genetic component but also with a relevant role of environmental factors. Among the latter, the role of diet, physical exercise and life styles has been evoked. Obesity may induce the switch from a subclinical form to the full clinical picture of PCOS.

The identification of genetic factors predisposing to PCOS has been frustrated so far by several complicating elements, including the clinical variability, the absence of univocal
diagnostic criteria and of a confirmatory laboratory test, the reduced fertility of affected
subjects; on the other hand, the recent identification of metabolic abnormalities, similar to
those found in PCOS, also in male first degree relatives of affected women\textsuperscript{11, 12}, might
improve the yield of genetic association studies in the next future. The high prevalence of the
condition allows hypothesizing a wide genetic heterogeneity with several loci involved, rather
than a major locus with a prevalent role in the predisposition to PCOS. Several case-control
and linkage studies have been performed so far, aimed at identifying genetic susceptibility
factors\textsuperscript{13, 14}. The majority of genes investigated, potential candidates for PCOS susceptibility,
belong to specific pathways mediating sex hormone response, peripheral sensitivity to insulin,
inflammatory response, cardiovascular risk, and steroid biogenesis\textsuperscript{15, 16, 17}. However, these
studies have not led to the identification of gene variants determining a high risk of
developing PCOS, and/or most studies were not confirmed in replicative populations. Very
recently, a genome-wide association study has been performed in Han Chinese population\textsuperscript{18}:
the authors identified three different single nucleotide polymorphisms associated with PCOS,
two with protective effect (rs13405728 and rs13429458, in 2p16.3 and 2p21, respectively)
and one with predisposing effect (rs2479106 in 9q33.3). The functional effect of these
variants has not been defined.

In the present study, we have evaluated the potential role of three functionally relevant
variants of the luteinizing hormone/choriogonadotropin receptor (\textit{LHCGR}) gene, located in
2p21: 1) rs4539842 (c.54insCTCCAG, ins18LQ) in exon 1, 2) rs12470652 (c.A872G,
N291S), and 3) rs2293275 (c.G935A, S312N), in exon 10. The rationale behind the choice of
\textit{LHGCR} as a candidate gene is based on several reasons: it is expressed in thecal cells of the
ovary and in Leydig cells of the testicle (as well as in adipose tissue\textsuperscript{19}), and it encodes for the
receptor mediating the action of both luteinizing hormone (LH) and choriogonadotropin
(hCG) on steroid biosynthesis\textsuperscript{20, 21}. Mitogen-Activated Protein Kinases (MAPK), mediating the signal transduction of LH, are downregulated in thecal cells of PCOS individuals\textsuperscript{22, 23}; mutations in \textit{LHCGR} have been reported in different disorders of sexual differentiation and maturation, both in human and mouse\textsuperscript{24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35}. \textit{LHCGR} encodes for a trans-membrane integral protein, belonging to the G-protein coupled receptor super-family (GPCR), is composed of 11 exons spanning about 70 kbp. Over 300 SNPs have been identified in the \textit{LHCGR} gene (\url{http://SNPper.chip.org}) with an average spacing of 306 bp, the majority being located in intronic sequences. We have selected, among the most frequent SNPs located in the coding region, those which may modulate the receptorial activity of the \textit{LHCGR} gene product. In particular, ins18LQ, due to the insertion of an exanucleotide in position 54 of the mRNA, has a minor allelic frequency (MAF) of about 30\% in Caucasian population, is located in the signal peptide of the receptor and has been associated with an increase in both expression and bioactivity of the LH receptor\textsuperscript{36}; N291S (MAF: 3\%) and S312N (MAF: 42\%) are located in exon 10, are involved in the transduction of LH but not of hCG signal, and are next to the \textit{glycosylation} signals of the protein and may thus affect translocation and stability of the receptor\textsuperscript{37}. However, to our knowledge, no data are available on the functional effect of these variants on receptorial activity.

In the present study, we have analyzed a selected population of cases and controls of Sardinian origin. We have chosen this geographic area of Italy, not because of a specific ethnic origin, but rather for the relative isolation of the population\textsuperscript{38}. However, if on one side the choice of genetic isolates may increase the potential yield of genetic association studies, by reducing the noise of genetic background, on the other side the results need to be confirmed in replicative populations.
Materials and Methods

Subjects.

For all subjects, a written informed consent for the participation to the study was obtained. The present study was approved by the ethic committees of the University of Cagliari, and of the Università Cattolica del Sacro Cuore. Three-hundred-eighty-five unrelated women of Sardinian origin have been recruited in a prospective study between May 1\textsuperscript{st} 2006 and October 30\textsuperscript{th} 2009 at the outpatient clinic of the Department of Obstetrics and Gynecology of the University of Cagliari. Subjects were divided into two groups: 198 PCOS patients (age range 14-35 years, mean: 18.4) and 187 non-PCOS, included as controls (age range 15-36, mean: 19.1). Twenty-one out of 198 patients were \leq 16 years of age. In this group, since menstrual disorders and/or multicystic ovaries are frequent findings during the first years of fertile life, subjects without clinical or biochemical signs of hyperandrogenism were not included. Among patients, the diagnosis of PCOS was made according to Rotterdam criteria\textsuperscript{1, 2}

Biochemical hyperandrogenism was established for total testosterone (totT) levels \textgreater= 1.74 nmol/L. In the absence of hirsutism, isolated acne was not considered a clinical sign of hyperandrogenism, due to the high frequency of this condition. The presence of signs of PCO was evaluated in all subjects by ultrasound examination (transabdominal or, when possible, transvaginal)\textsuperscript{39}. Control subjects were women with regular menstrual cycles, absence of clinical and biochemical signs of hyperandrogenism, and of PCO signs at ultrasound examination, enrolled on a voluntary basis during regular gynecologic check-up visits, covered by the regional health system. Exclusion criteria from the study were: use of hormone preparations, including oral contraceptives, for at least 3 months preceding recruitment, hyperprolactinemia, Cushing disease, androgen-secreting neoplasms, thyroid disorders.
For healthy controls, blood sampling was performed during the morning of the third day of the menstrual cycle, while for PCOS patients, at the 5-7th day after progesterone-induced menstrual flow. DNA extraction and for the following biochemical analyses on serum were performed in all subjects: Follicle Stimulating Hormone (FSH), LH, LH/FSH ratio, estradiol (E2), totT, androstenedione (A), progesterone (P), dehydroepiandrosterone sulphate (DHEAS), 17-hydroxyprogesterone (17-OHP), and prolactin. Hormonal levels were evaluated by commercially available radio immunoassays (RIA, Radim, Pomezia, and Ares Serono, Milan, Italy). Serum samples were immediately processed in a refrigerated centrifuge upon sampling, and stored at −20°C until assay. The intra-assay and inter-assay coefficients of variation obtained were ≤9% for all variables.

**Genotype analysis**

For genomic DNA (gDNA) extraction, 5-10 ml of whole blood were collected in EDTA tubes. Red blood cells were lysed twice in hypotonic solution (NH₄Cl 150 mM, KHCO₃ 10 mM, EDTA 0.1 mM). White blood cells pellet was digested O/N in hypotonic solution (NaCl 75mM, EDTA 25 mM) containing 200 µg/ml of proteinase K and 1% SDS. Subsequently, DNA was extracted with 1 volume of chloroform, precipitated with isopropanol/ NaCl 1.5 M, washed in ethanol 70%, and resuspended in TE pH8 (Tris-HCl 10mM, EDTA 1mM) at a concentration of 100 ng/µl. Concentration and purity of DNA samples were assessed by spectrophotometer absorbance at 260 and 280 nm (GeneQuant II Pro, Pharmacia).

Genotype analysis at the three SNPs was performed by restriction fragment length polymorphisms – polymerase chain reaction analysis (RFLP-PCR). PCR reactions were performed in a final volume of 25 µl containing 100-200 ng of gDNA, 200 µM of each dNTP,
1µM of each primer, 1x reaction buffer, 1mM MgCl₂ and 1 U of Taq polymerase (Fisher scientific, Illkirch, France).

For ins18LQ, a specific fragment of exon 1 (215 bp) of LHCGR gene was amplified by using the following oligonucleotides: LHCGR-ex1 F: 5’ – GACACTGGCAAGCCGCAGAAGCCC - 3’; LHCGR-ex1 R: 5’ – GCTGTGTACTCACAGTGAGTGAG - 3’. PCR products were digested with 0.2 U of PvuII in a final volume of 20 µl. The presence of ins18LQ variant created an additional PvuII restriction site, resulting in the three fragments of 27, 65, and 128 bp, respectively. The digestion of wild type alleles resulted in two fragments of 65 and 149 bp, respectively.

For N291S and S312N SNPs, a fragment of 111 bp of exon 10 of LHCGR gene was amplified with the following primers: LHR ex10-F: 5’ – CCTCTTCTCTTTCAGACAGA - 3’; LHR-ex10 R: 5’ – CATGCAATACTTACAGTGTTTTTTGTA - 3’. When digested with Apol restriction endonuclease, 291N allele remained uncut while 291S allele generated two fragments of 19 and 92 bp, respectively. For S312N variant analysis, PCR products were digested with 0.2 U of RsaI which generated, in the presence of 312N allele, two fragments of 26 and 85 bp, respectively. In all cases, fragment size analysis was performed by 3.5% agarose gel/TAE 1x electrophoresis.

Statistical analysis

The primary endpoint was the comparison of allelic frequencies of S312N variant between PCOS and non-PCOS subjects. The statistic power of the study was established by Sample power version 2.0 (SPSS, Inc., Chicago, Ill.).Based on the expected frequencies of two of the variants analysed (ins18LQ and S312N, about 30%, http://www.ncbi.nlm.nih.gov/snp), a chi-
squared test of association at an alpha level =0.05 would guarantee a power of at least 80%, with a sample size of about 180 patients, if assuming a delta of 15% in the frequency of allelic variants in PCOS vs. control subjects.

We also analyzed the distribution of allelic frequencies of N291S, Ins18LQ and haplotype for S312N and Ins18LQ; genotype was related with phenotypic characteristics.

χ² Pearson’s tests were used to establish whether allelic and genotypic frequency of each SNP respected Hardy-Weinberg equilibrium. Odds ratio and relative risk associated to the genotype were calculated by contingency tables (Confidence Interval, CI, 95%). Continuous outcomes like hormonal levels were compared by means of Student’s t-test in case of two groups and one-way ANOVA in case of more than two groups, upon verification of normality by Kolmogorov- Smirnov test. In case of non-normality, medians were compared by Mann-Whitney or Kruskall-Wallis tests. For allelic and genotypic frequencies, the cut-off of significance was fixed at p<0.017, after Bonferroni correction, while in all other cases a p-value smaller than 0.05 was deemed as statistically significant. All statistical analyses were performed by using SPSS v18.0 software (SPSS Inc.).
Results

Hormonal and clinical characteristics of controls and patients

Characteristics of the two populations are summarized in Table I. As expected, we did not observe any difference between patients and controls for BMI, age, E2, Prl, and P levels (p>0.11); LH, totT, A, DHEAS, and 17-OHP levels were significantly higher in PCOS (p=0.001), indicating the correct sampling of the two populations. Among patients, 92/198 (46.5%) fulfilled the three Rotterdam diagnostic criteria; 71/198 (35.8%) had amenorrhea and PCO, while 30 (15.1%) had hyperandrogenism and PCO. The remaining 5 patients (2.5%) had hyperandrogenism and amenorrhea, in the absence of ultrasound signs of PCO. Biochemical hyperandrogenism was found in 64% of cases. For all biochemical characteristics, except those related to the condition, the PCOS and non-PCOS groups were homogenously distributed.

Ins18LQ variant is not associated with PCOS but is related to LH levels in controls

Genotype of ins18LQ variant was assessed in 156 patients and 174 controls. No differences were observed in allelic and genotypic frequencies in the two cohorts (Table II, OR:1.16, C.I.:0.83-1.64, p=0.38). Subsequently, we have evaluated whether ins18LQ genotype may be involved in the modulation of serum levels of hormones analyzed. While in PCOS individuals no correlations were found, in controls we observed that median LH levels were 4.39, 3.36, 1.89 UI/l in non-ins18LQ homozygous, ins18LQ heterozygous, and ins18LQ homozygous individuals, respectively (Table III, Kruskal-Wallis test, p=0.017).

N291S variant is not associated with PCOS phenotype

This SNP was analyzed in 153 patients and 180 controls. As in the case of ins 18LQ, both populations were at Hardy-Weinberg equilibrium. No differences were observed in the
distribution of genotypic and allelic frequencies in the two cohorts (Table II, OR:1.16, C.I.:0.83-1.64, p=0.27). Due to the rarity of this SNP, it was not possible to analyze possible correlation between genotype and hormonal levels.

**312N allele is associated with higher PCOS risk**

The S312N variant was analyzed in 159 patients and 180 controls. Both populations were at Hardy-Weinberg equilibrium. Genotypic and allelic frequencies were significantly different in the two groups (Table II). In particular, the 312N allele frequency was 0.46 and 0.35, in PCOS and controls, respectively. In the PCOS group, 60.4% were carriers of at least one 312N allele, compared to 42.8% of controls, conferring an increase in PCOS risk of about 2-fold (OR:2.04, C.I.:1.32-3.14, p=0.001). The frequency of individuals homozygous for 312N allele was significantly higher in the group of cases compared to controls (Table II, 62.5% vs. 37.5%; p<0.01). 312N homozygous individuals had a 2.73-fold increased risk of PCOS compared to controls (C.I.: 1.25-5.95; p=0.01). To rule out the possible confounding effect of younger age in the diagnosis of PCOS, we have analysed data excluding patients and controls of age ≤ 16 years (21/198 and 6/187, respectively). The correlation with the 312N variant remain unaltered (OR: 2.01, C.I.: 1.29-3.15; p=0.002). Also, when evaluating hyperandrogenic (n=103) and non-hyperandrogenic (n=56) women separately, the percentage of subjects bearing at least one 312N allele remained substantially unchanged (63.1% and 55.4%, respectively), as well as the risk of PCOS in hyperandrogenic women (OR:2.29, C.I.:1.39-3.76, p=0.001).

No correlations were found between the genotype at S312N variant and hormonal or clinical variables, both in patients and controls.

**Haplotype analysis of polymorphic variants**
To evaluate a possible combinatorial effect of the variants analyzed, we determined the haplotypic frequency of the ins18LQ and S312N variants in cases and controls. N291S SNP was not included in the analysis, due to the low frequency of the 291N allele. Data are summarized in Table IV. The distribution of haplotypes was very similar in the two groups. However, the statistic power of this analysis is low due to the small number of samples analyzed.
Discussion

In the present study we have evaluated the role of three polymorphic variants of the \textit{LHCGR} gene as a risk factor for PCOS, in a case control study of Sardinian women. In our cohort of PCOS subjects, the prevalence of hyperandrogenism was lower than that observed in other series of patients evaluated\textsuperscript{40, 41}; this finding might be explained by the younger mean age of our patients, compared to the previously published cohorts (19.1 vs. 25.5 years, respectively).

Of the three polymorphisms investigated, ins18LQ did not associate with the phenotype, since the two cohorts displayed a similar genotypic distribution. Interestingly, ins18LQ variant was correlated with serum LH levels in controls but not in patients. This finding may be related to the functional effect of the variant, which is located in the signal peptide of LH receptor and is associated with higher receptorial activity\textsuperscript{35}. It can be speculated that the increase in receptorial activity, linearly and inversely related to the number of ins18LQ alleles, determines the up-regulation of the hypophyseal negative feedback system, controlling LH secretion. On the other hand, since in PCOS individuals this feedback is altered, the control of LH receptor on hormonal levels is lost, and consequently also the correlation between genotype and LH levels. The lack of correlations between genotype of single variants at the \textit{LHCGR} locus and different hormonal variables is not unexpected. Indeed, if on one side it is conceivable that functionally relevant variants at the \textit{LHCGR} locus might play a pivotal role on the androgen synthesis, on the other hand the regulation of hormonal production is likely modulated at different levels and thus should be evaluated in a multigenic/multifactorial context.

N291S appeared not to be related to the PCOS phenotype, since genotypic distribution was similar in the two cohorts. However, this variant is rather rare and thus the number of patients analyzed is not sufficient to generate meaningful results.
The most relevant finding of our study is the strong correlation between PCOS phenotype and S312N variant. Differently from previous studies of genetic susceptibility factors, the presence of 312N allele correlates with an increase in relative risk of PCOS in carriers of at least one copy of this variant. The significance of these data is strengthened by the finding that in homozygous individuals the risk of PCOS increases further, raising up to 2.7 folds compared to homozygous wild type individuals. The observed correlation is not influenced by the presence or absence of hyperandrogenism, since no differences were observed when analyzing the two endophenotypes separately. The S312N variant had already been investigated in a previous study by Valkenburg et al\textsuperscript{42}. These authors did not find an increase in PCOS risk related to S312N genotype. The discrepancy between the results of the two studies is partially unexplained, since the same diagnostic criteria for PCOS were used. One hypothesis could be related to the different geographic origin of patients included or, more likely, to the fact that control cohort in the study of Valkenburg was a sample from the general population, selected independently from the PCOS phenotype. Based on the prevalence of the condition in the general population, it can be expected that about 15\% of women included as controls were affected by the condition, and this may have masked the association between the 312N variant and PCOS phenotype. This hypothesis may be supported by the finding that in our control cohort the MAF of 312N allele is slightly lower than that reported by Valkenburg et al (35\% vs. 42\%, respectively).

Chen et al.\textsuperscript{18} have very recently reported a genome-wide association study performed by SNP array. These Authors found 21 SNPs in the 2p21 region strongly associated with PCOS phenotype. The strongest association was found with 3 SNPs in the thyroid adenoma associated (\textit{THADA}) gene. The putative functional relevance of these variants (two of which are located in intronic regions) remains to be investigated. Intriguingly, \textit{THADA} is located at
a distance of 5Mb only from *LHCGR* gene, and it can be hypothesized either that S312N variant in *LHCGR* is in linkage disequilibrium with variants in other gene/genes in the same locus not yet identified, or that the prevalent PCOS-predisposing effect is played by *LHCGR* variants. The latter hypothesis is, in our opinion, more likely due to the fact that *LHCGR* is a strong functional candidate to PCOS susceptibility and that 312N allele confers the highest relative risk defined so far.

Our results need to be confirmed in an independent study, performed in a replicative population. In a context of translational medicine, if our data will be confirmed, the increased relative risk of PCOS, associated to the presence of 312N allele, suggests the opportunity of testing the genotype at *LHCGR* locus in patients affected by PCOS and their relatives. This information may be useful for PCOS families carrying the 312N variant since, in case of homozygosity for this allele, the probability to be affected with a condition belonging to PCOS clinical spectrum raise up to about 40%. Subsequently, in at-risk subjects, it may be more strongly advisable rather than in general population to avoid some detrimental behaviors, like maintain a good body weight control, in order to reduce the risk of worsening the clinical and reproductive outcome associated with PCOS phenotype.
References


Table I: Clinical and hormonal characteristics of control and PCOS women

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<td>17-OH-Progesterone (nmol/L)</td>
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<td>0.89</td>
<td>2.70-3.08</td>
<td>4.26</td>
</tr>
<tr>
<td>Progesterone (nmol/L)</td>
<td>2.61</td>
<td>0.99</td>
<td>2.39-2.80</td>
<td>2.35</td>
</tr>
</tbody>
</table>

AD: androstenedione;  
DHEAS: dehydroepiandrosterone sulfate; P: progesterone; Prl: prolactin  
Ttot: total testosterone  
a = statistically different between PCOS and controls (p<0.01)
Table II: Frequency of alleles and genotypes in PCOS and controls

<table>
<thead>
<tr>
<th>Variant</th>
<th>Genotype</th>
<th>PCOS n</th>
<th>PCOS %</th>
<th>Controls n</th>
<th>Controls %</th>
<th>OR (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ins18LQ</td>
<td>ins/ins</td>
<td>9</td>
<td>5.8</td>
<td>8</td>
<td>4.6</td>
<td>1.16</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>non ins/ins</td>
<td>74</td>
<td>47.4</td>
<td>76</td>
<td>43.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>non ins/non ins</td>
<td>73</td>
<td>46.8</td>
<td>90</td>
<td>51.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF (ins)</td>
<td>0.29</td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N291S</td>
<td>N/N</td>
<td>174</td>
<td>98.3</td>
<td>144</td>
<td>94.1</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>N/S</td>
<td>6</td>
<td>1.7</td>
<td>9</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S/S</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF (S)</td>
<td>0.03</td>
<td></td>
<td>0.02</td>
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<td>1.78</td>
<td>0.27</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>(0.63-5.08)</td>
<td></td>
</tr>
<tr>
<td>S312N</td>
<td>S/S</td>
<td>63</td>
<td>39.6</td>
<td>103</td>
<td>57.2</td>
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</tr>
<tr>
<td></td>
<td>S/N</td>
<td>76</td>
<td>47.8</td>
<td>65</td>
<td>36.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/N</td>
<td>20</td>
<td>12.6</td>
<td>12</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF (N)</td>
<td>0.46</td>
<td></td>
<td>0.35</td>
<td></td>
<td>1.75</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.26-2.43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/N and S/N vs. S/S</td>
<td>2.04</td>
<td></td>
<td></td>
<td></td>
<td>2.04</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.32-3.15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/N vs. S/S</td>
<td>2.75</td>
<td></td>
<td></td>
<td></td>
<td>2.75</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.25-5.95)</td>
<td></td>
</tr>
</tbody>
</table>
Table III: Serum LH levels (IU/l) in controls relative to ins18LQ genotype

<table>
<thead>
<tr>
<th>n</th>
<th>non ins/non ins</th>
<th>ins/non ins</th>
<th>ins/ins</th>
<th>p&lt;sub&gt;a&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>4.39</td>
<td>3.36</td>
<td>1.89</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>3.5-5.31</td>
<td>2.32-3.94</td>
<td>1.2-3.85</td>
<td></td>
</tr>
</tbody>
</table>

a. Kruskal Wallis Test
Table IV: Contingency table of haplotypes at S312N and ins18LQ variants

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Controls</th>
<th>PCOS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>homo non ins/</td>
<td>27</td>
<td>21.3</td>
<td>27</td>
</tr>
<tr>
<td>homo 312S</td>
<td>33</td>
<td>26.0</td>
<td>32</td>
</tr>
<tr>
<td>homo non ins/</td>
<td>22</td>
<td>17.3</td>
<td>30</td>
</tr>
<tr>
<td>het 312S</td>
<td>29</td>
<td>22.8</td>
<td>40</td>
</tr>
<tr>
<td>homo non ins/</td>
<td>6</td>
<td>4.7</td>
<td>6</td>
</tr>
<tr>
<td>homo 312N</td>
<td>4</td>
<td>3.1</td>
<td>6</td>
</tr>
<tr>
<td>homo ins/</td>
<td>4</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>homo 312S</td>
<td>2</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>homo non ins/</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>homo ins/</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>100</td>
<td>155</td>
</tr>
</tbody>
</table>

Pearson’s χ²=5.24, p=0.73. Table legend: homo or het non ins: homozygous or heterozygous wild type; homo or het 312S: homozygous or heterozygous wild type; homo ins: ins18LQ homozygous; homo 312N: 312N homozygous.