3.0. MATERIALS AND METHODS

3.1. Study design

The present work is an observational hospital based case-control study. A total of 338 subjects were recruited from patients visiting Sri Ramachandra Medical Centre and Assisted Reproductive Technology and Obstetrics and Gynecology department of Sri Ramachandra Hospital, Chennai from April 2012 to April 2014. Initially 175 cases and 177 controls were recruited. Of which 6 cases and 8 control subjects decided to drop out which brought the number of cases and controls to 169 each. Finally, 169 cases who fulfilled the criteria for PCOS and 169 unaffected, age matched control with regular menstrual cycles (28-35 days) and normal ovaries of the same geographical area were included in the study. Subject’s history such as age, menarche onset, menstrual cycle regularity, marital status, consanguinity, family history with PCOS were collected after obtaining a written informed consent from all the women enrolled in the study [Figure 3.1].

3.2. Inclusion/Exclusion Criteria

Cases were selected based on Rotterdam criteria which is based on diagnosis of two of the three criteria such as; observation of oligo/ anovulation, clinical or biochemical evidence of hyperandrogenism and polycystic ovaries on ultrasonography (The Rotterdam ESHRE/ASRM—Sponsored PCOS Consensus Workshop Group, 2004). Unaffected age matched fertile women with regular menstrual cycles (interval of <35 days) with normal ovaries from the same geographical region was included in the study as controls. This case-control study
includes women in the age group of \( \geq 20 \text{yrs} \) to \( \leq 40 \text{yrs} \). Besides, women with galactorrhea, thyroid issues, women with any systemic disease that affect their reproductive physiology and women under medication which interfere with the normal function of the hypothalamic-pituitary-gonadal axis were excluded from the study group.

3. 3. Sample Size Calculation

Power and sample size calculation software (version 2.1.31) was used to calculate the sample sizes in the study. We used continuity corrected chi-squared statistic to evaluate this null hypothesis. Based on power analysis, a study with 150 case patients and 150 controls was large enough to detect a significant odds ratio (OR) of 0.5 with a power of 80% and an alpha of 5%.

**Figure 3.1. Flowchart representing recruitment of subjects in the study**
3. 4. Sample and data collection procedure

The study protocols were approved by the Institutional Ethics Committee of Sri Ramachandra University, Chennai, India. A written informed consent was collected from the study participants before collecting the blood samples. Blood Samples were taken after an eight hour fast through vein puncture from the subjects for carrying out biochemical and hormonal analysis on day 2 (D2) or day 3 (D3) of their follicular phase. Thyroid profile (free T4, thyroid stimulating hormone), hemoglobin (Hb), packed cell volume (PCV), pre-prandial and postprandial glucose, serum prolactin, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), total testosterone (T.T), fasting insulin (F.I) were analyzed using approved test kits, Siemens-ADVIA Centaur Automated System and by Chemiluminescent immuno assay (CLIA). From all the study subjects, peripheral blood was collected and DNA was isolated using modified salting-out method. Genotyping of single nucleotide polymorphisms (SNP’s) was carried out with Real-time polymerase chain reaction technology.

3. 5. SNP selection

Single nucleotide polymorphisms of multiple selected genes are considered with main importance to genes involved in the insulin secretion and action were chosen (INS, INSR, IRS1, IRS2, PPAR-G, and CAPN10). Polymorphism of the above said genes has shown to have greatest link with PCOS and has been reported by several studies in different populations, but very few studies have been reported in the Indian population. The selected genes are studied for the first time in the Tamil Nadu region. Name of the gene, SNP and genotyping method used are given in Table 3.1.
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### 3.6. Anthropometric assessment

Anthropometric measurements like body height (in cm), weight (in Kg), hip circumference (HC) and waist-circumference (WC) (in cm) was assessed using a standardized instrument. In accordance to WHO classification, body-mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m²) and the subjects were categorized as underweight (<18.5), Normal (18.5-24.9), overweight (25-29.9) and obese (≥30). Waist circumference (WC) was measured in the standing position, halfway between the lower ribs and the superior anterior iliac spine of the pelvis. Hip circumference (HC) was measured at the level of the pubic symphysis. The participants with waist/hip ratio (WHR) 0.80-0.84 were classified as overweight and with WHR > 0.85 were classified as obese.

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>SNP</th>
<th>Gene location</th>
<th>Functional</th>
<th>Genotyping method</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS</td>
<td>rs689</td>
<td>chromosome 11</td>
<td>Intron</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>INSR</td>
<td>rs1799817</td>
<td>chromosome 19</td>
<td>His-His</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>IRS1</td>
<td>rs1801276</td>
<td>chromosome 2</td>
<td>Ala-Pro</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>IRS1</td>
<td>rs1801278</td>
<td>chromosome 2</td>
<td>Gly-Arg</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>IRS2</td>
<td>rs1805097</td>
<td>chromosome 13</td>
<td>Gly-Asp</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>PPAR-G</td>
<td>rs1801282</td>
<td>chromosome 3</td>
<td>Pro-Ala</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>PPAR-G</td>
<td>rs3856806</td>
<td>chromosome 3</td>
<td>His-His</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>CAPN10</td>
<td>rs7607759</td>
<td>chromosome2</td>
<td>Thr-Ala</td>
<td>Real-Time(TaqMan)</td>
</tr>
<tr>
<td>CAPN10</td>
<td>rs2975766</td>
<td>chromosome 2</td>
<td>Val-Ileu</td>
<td>Real-Time(TaqMan)</td>
</tr>
</tbody>
</table>

### Table 3.1. List of the genes, SNP and genotyping method used in the study
3.7. Clinical, biochemical and ultrasound assessment

Indication of hyperandrogenism (HA) was determined by the presence of clinical hyperandrogenism (CH) and/or biochemical hyperandrogenemia (BH). CH was described by the presence of hirsutism (FG ≥8), acne, or the presence of androgenic alopecia. Hirsutism was evaluated using the Ferriman-Gallwey (FG) scoring method. It enumerates the presence of terminal hairs over nine body areas (upper lip, chin, chest, upper and lower abdomen, upper and lower back, upper arms and thighs). Acne was recorded by the presence of comedones on the face, neck, upper chest, upper back, or upper arms. Biochemical HA was estimated by checking the testosterone levels in the body [total testosterone (>76ng/dl)] according to the normal upper limit reference value followed in the laboratory.

Oligomenorrhea is defined as more than 36 days between menstrual cycles or fewer than eight cycles per year. Insulin resistance was assessed using the homeostatic model assessment (HOMA-IR), calculated as (fasting insulin × fasting glucose)/22.5 and >3 (Geloneze, Repetto, Geloneze, Tambascia, & Ermetice, 2006; Summer & Cowie, 2008) was selected as the cut-off point. Interactive homeostatic model assessment β- cell functioning (iHOMA2 % β) and interactive homeostatic model assessment of insulin sensitivity (iHOMA2 % S) was calculated using iHOMA2 (iHOMA2 version 8.8.2.R2), an interactive, 23-variable, Homeostatic Model of Assessment of the relationship between fasting glucose and insulin in man, and insulin resistance and beta-cell function. PCO was confirmed by the presence of 12 or more follicles in each ovary, measuring 2-9 mm in diameter and/or increased ovarian volume (10 cm³). PCO was diagnosed by means of transvaginal
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3.8. Statistical analysis

Descriptive statistics was carried out. The characteristics between cases and controls were assessed and their percentages were calculated and represented graphically. The categorical variables estimates were analyzed using Chi-square test ($\chi^2$). The continuous variables were expressed as mean ± standard deviation. The significant difference obtained in PCOS subjects and controls were calculated and compared using ANOVA. Correlation analysis was carried out using Pearson’s correlation coefficient test. Multivariate logistic regression analysis was carried out using software R. Hardy–Weinberg equilibrium (HWE) tests were performed by comparison of observed and expected genotype frequencies using chi-squared goodness-of-fit test. The genotype and allele frequencies of each polymorphism were compared between subjects with PCOS and the controls by the chi-square test. The odds ratio and 95% confidence interval were calculated using wild type genotypes or alleles as the reference group. A p-value < 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 was computed as both $D'$ and $r^2$ using Haploview v. 4.1. SNP-SNP interactions were assessed by non-parametric multifactor dimensionality reduction (MDR) analysis using MDR software version 3.0.2. All statistical analysis
was performed using the SPSS statistical software version 9.0. A p-value < 0.05 was considered to be statistically significant.

3. 9. Chemicals and Reagents used

3. 9. 1. 1M Tris-HCl (pH 7.6)

121.1 g Tris was dissolved in 700 ml of dH2O. pH was adjusted to 7.6 with concentrated HCl and volume was adjusted to 1000 ml. Solution was autoclaved and stored at 4°C.

3. 9. 2. 0.5M EDTA (pH 8.0)

186.1 g EDTA (disodium, dihydrate) was weighed and dissolved in 700 ml dH2O. Dissolution of EDTA was achieved by adjusting the pH to 8.0 with NaOH (about 20g). Volume was adjusted to 1000 ml. Solution was autoclaved and stored at 4°C.

3. 9. 3. TE buffer (pH 7.6)

10ml of 1M trisHCl (pH 8.0) was added with 2ml of 0.5M EDTA. pH was adjusted to 7.6. Volume was adjusted to 1000ml with distilled water. Prepared reagent was autoclaved before using.

3. 9. 4. 10% N-Lauryl sarcosine

10 gram of N-lauryl Sarcosine was added in autoclaved distilled water and made up to 100 ml.
3.9.5. 10% SDS

10g of SDS was dissolved in 80ml double distilled water (DDW) at 65°C and the volume was then adjusted to 100ml with distilled water.

3.9.6. DNA Extraction Buffer (DEB)

5 ml of 1M tris, 1 ml of 0.5 M EDTA, 5 ml of 10% SDS, 25 ml of 10% N-Lauryl sarcosine were mixed in 464 ml of autoclaved distilled water and the final volume was adjusted to 500 ml.

3.9.7. Proteinase K

100 mg of proteinase K was dissolved in 10 ml of distilled water and was aliquoted into 1.5 ml Eppendorf tubes before storing at -20°C.

3.9.8. 5 M NaCl

29.22 g of NaCl was dissolved in 100 ml of distilled water and autoclaved.

3.9.9. 20% SDS

20g of SDS was dissolved in 80ml double distilled water (DDW) at 65°C and the volume was then adjusted to 100ml with distilled water.

3.9.10. Absolute ethanol

Absolute ethanol was commercially procured.
3. 9. 11. 80% ethanol

20ml of distilled water was mixed with 80ml absolute ethanol and stored in refrigerated conditions.

3. 9. 12. Agarose

Fine grade agarose was commercially procured.

3. 9. 13. 10X TAE buffer

48.4 gm of Tris Base, 20ml 0.5M EDTA (pH 8.0) and 11.40 ml glacial acetic acid was added and the volume was adjusted to 1000ml with distilled water and autoclaved.

3. 9. 14. DNA Loading Dye – 6X

It was prepared by adding 10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol and 60mM EDTA.

3. 9. 15. Ethidium bromide

To 10ml of sterile double distilled water, 10mg of Ethidium bromide was added and stirred well with a magnetic stirrer for complete dissolution. The solution was subsequently stored in an airtight container wrapped with aluminum foil.

3. 9. 16. 100 bp DNA marker (0.1 μg/μl)

The 100 bp DNA marker at a concentration 0.1 μg/μl, pre-mixed with 6X loading dye was commercially procured from RBC Bioscience Corporation. The
100 bp Sharp DNA Ladder contained the following discrete fragments (in base pairs): 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100.

3. 10. Genomic DNA Isolation

DNA was isolated from all samples using the modified salting out method. Blood samples were re-suspended in 2 to 3 volumes of TE buffer and mixed gently and centrifuged at 3000rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in TE buffer and Centrifuged for 10mins at 2500rpm. This procedure was repeated thrice until a white pellet is obtained. The pellet was again re-suspended in 3-5ml of DNA Extraction Buffer (DEB). To the solution 30μL of proteinase K was added and incubated at 37°C overnight. After a clear viscous solution is obtained, 300μL of 5M NaCl was added and mixed well. Finally 6ml of ethanol was added to precipitate DNA. The supernatant was discarded immediately after centrifugation, and the pellet was washed with ice-cold 80% ethanol and air-dried until no drops of ethanol remained in the tube. The pellet was re-suspended in TE (Tris-EDTA) buffer and stored at -20°C for long-term storage.

3. 11. Estimation of Genomic DNA concentration (Spectrophotometric method)

The extracted DNA was quantified by the spectrophotometer method. Absorption spectrum of DNA is between 260 and 280nm. At 260nm, an absorbance of 1.00 OD, measured in a cuvette with 1 cm path length, is indicative that concentration of DNA is approximately 50µg/ml.

Concentration of DNA (µg/µl) = OD at 260nm x dilution factor x 50µg/µl
The ratio of absorbance at 260 to 280nm indicates the purity of the sample. This ratio of DNA solutions should range from 1.7 to 1.8. The presence of impurities like proteins or phenol tends to decrease this ratio. 5µl of the DNA solution was taken and diluted 100 times by adding 995µl of DDW and mixed well. OD readings were taken in a preset spectrophotometer at 260nm and 280nm using DDW as blank. The concentration of DNA was then accordingly calculated. With the help of concentration of DNA (ng/µl), the respective volume of DNA was taken and diluted with TE solution, so that the conc. becomes 10ng/µl DNA. The instrument used for quantification and screenshot of estimation of genomic DNA concentration of a sample are shown [Figure 3.2 and 3.3].

**Figure 3.2. NanoDrop instrument used for quantification of DNA**
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Figure 3.3. Estimation of Genomic DNA concentration using NanoDrop

3.12. Genomic DNA Quality check by Agarose gel electrophoresis

Agarose gel electrophoresis is an efficient technique to separate DNA molecule according to their molecular weights in the same manner as a sieve. 0.8 gm of agarose was dissolved in 100 ml 0.5X TAE buffer in a 250ml conical flask and was boiled to dissolve agarose completely. 0.7μl Ethidium bromide was added from stock solution to make a final concentration of 0.5µg/ml. Gel was cooled down to 60°C, poured onto a gel tray and was allowed to set. A standard DNA sample of known concentration was also loaded along with the samples to quantify DNA and electrophoresis was carried out at a constant voltage of 80V. After 30 Min. of run halfway the gel was observed under ultraviolet light of UV trans-illuminator and photographed [Figure 3.4].
Figure 3.4. Horizontal electrophoresis unit used to determine the intactness of isolated genomic DNA

3. 13. Protocol for genotyping

TaqMan allelic discrimination is a method combining PCR and mutation detection in a single step, by measuring the increase in fluorescence of dye-labeled DNA probes. It provides a rapid and sensitive method for detecting polymorphisms. This method employs a probe technology that exploits the 5'-3' nuclease activity of Taq Gold DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR. In this procedure, two TaqMan probes corresponding to two target alleles are used. Each probe consists of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. Carboxy fluorescein FAM and VIC are used as 5'-reporter dye for detecting different allele; the 3'-quencher dye used tetramethylrhodamine (TAMRA). The probe hybridizes to a
smaller 20- to 24mer sequence within the PCR product, which includes the SNP. The fluorescent signal given from the reporter is suppressed by the quencher when the probe is intact.

Taq DNA polymerase carries out the extension of the primer and replicates the template till the verge of the probe, and the enzyme cleaves the probe with its 5’-exonuclease activity. When the probe is cleaved the reporter dye is separated away from the vicinity of the quencher, resulting in increased fluorescence intensity of the reporter. This process occurs in every cycle and does not interfere with the accumulation of PCR product. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR. At last, the allelic discrimination plate will be scanned by laser light of Applied Biosystem 7900-HT instrument for detecting the fluorescence signals, with simple FAM/VIC/TET signals corresponding to different homozygous, with mixed signals meaning heterozygous. All the reagents for SNP genotyping was purchased from Applied Biosystems, Foster City, CA, USA. Before analyzing the DNA, a preliminary test was conducted to confirm the accuracy of the assays and the reaction conditions for the real-time PCR were optimal. After a successful preliminary test, sample analysis was carried out on a 384-well optical reaction plate (Axygen Scientific).

Real-time polymerase chain reaction technology (Taqman® SNP Genotyping Assay; Applied Biosystems, Carlsbad, California, USA) each reaction mixture had a final volume of 5μl [2X Taqman Genotyping Master Mix -2.50μl, 20X Taqman Drug Metabolism Genotyping assay mix – 0.25μl, Genomic DNA diluted in distilled water – 2.25μl (3-20ng of genomic DNA)] [Table 3.2]. Thermal Cycle reaction with initial denaturation of 95° for 10mins followed by 40 cycles of
denaturation 92\(^\circ\) for 15 secs, Annealing/Extension 60\(^\circ\) for 1 min was carried out in a 384 well optical plate on a 7900- HT Fast Real Time PCR machine [Table 3.3]. For every SNP, a positive control for wild type, heterozygote and/or variant genotype was used. The plate also had two negative water controls without any DNA. The Taqman Drug Metabolism Genotyping assay mix contains the primers and fluorescent probes. The alleles were labeled with VIC\(^\text{®}\)-dye and FAM\(^\text{TM}\) dye [Figure 3.5 and 3.6]. The overall study design is presented as a flowchart [Figure 3.7].
Table 3.2. Allelic Discrimination PCR Reaction

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>384-Well</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X TaqMan® Genotypin Master Mix</td>
<td>2.50 µL</td>
<td>1X</td>
</tr>
<tr>
<td>20X TaqMan® Drug Metabolism Genotyping Assay Mix</td>
<td>0.25 µL</td>
<td>1X</td>
</tr>
<tr>
<td>Genomic DNA diluted in dH₂O</td>
<td>2.25 µL</td>
<td>-</td>
</tr>
<tr>
<td>Total Volume per Well</td>
<td>5µL</td>
<td>-</td>
</tr>
</tbody>
</table>

3-20 ng of genomic DNA per well. All wells on a plate should have equivalent amounts of genomic DNA.

Table 3.3. Thermal cycler conditions

<table>
<thead>
<tr>
<th>Steps</th>
<th>Stage</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial steps</td>
<td>Hold</td>
<td>10 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>Denature</td>
<td>40 Cycles</td>
<td>15 seconds</td>
<td>92°C</td>
</tr>
<tr>
<td>Anneal/Extend</td>
<td>40 Cycles</td>
<td>1 minute</td>
<td>60°C</td>
</tr>
</tbody>
</table>
Figure 3.5. Real Time Polymerase chain reaction-TaqMan allelic discrimination method
Figure 3.6. Example of an allelic discrimination plot (AD plot)

An allelic discrimination plot is shown in the above Figure. Ideally, an AD plot shows one, two, or three clusters and, the no template controls (NTCs). The points in each cluster are grouped closely together and each cluster is located well away from the other clusters.
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Figure 3.7. Flowchart demonstrating overall study design carried out

- **Ethical approval**
- **Written informed consent**
- **Peripheral blood (vein puncture)**
  - **DNA isolation** *(Modified salting-out method)*
  - **DNA quantification** *(Nanodrop)*
  - **DNA quality check** *(Agarose gel electrophoresis)*
  - **Genotyping** *(Real-time polymerase chain reaction)*
  - **Statistical analysis** *(SPSS, $\chi^2$, ANOVA, MDR, …)*
  - **Day2/Day3 of follicular phase**
  - **Biochemical analysis**