MATERIAL AND METHOD

Study Setting, design, sampling and period of the study

Out-patients and in-patients of Kasturba Medical College Hospital, Attavar, Mangalore, diagnosed with polycystic ovarian syndrome were inducted into the study. The study is a descriptive, cross sectional type. Convenient sampling of subjects was done for the study. The project began in December 2008 and ended in December 2011.

Ethical approval

Institute’s Research ethical committee approval was obtained for the study. After obtaining informed consent from each participant, hundred and three (103) patients with a clinical diagnosis of PCOS (where large family trees was known) were included in the study. All patients received a long, careful and simple explanation of the purposes of the study and its pathophysiological basis.

Criteria for the definition of PCOS

The diagnosis of PCOS was made according to the ESHRE/ASRM criteria for the PCOS diagnosis (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004)\(^8\) based on the presence of two of the three following criteria: oligo- and/or anovulation (menstrual dysfunction), clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries (PCO) at ultrasonogram\(^8\).

Menstrual dysfunction was considered when the women had oligomenorrhea, defined by six or fewer cycles per year, each cycle with a length of more than 35
days, and/or when the patient had not had any menstrual bleeding for 3 consecutive
months during the last year. Clinical hyperandrogenism was defined by the presence
of hirsutism, represented by a hirsutism score of 8 or more. Hyperandrogenism could
be clinical (hirsutism, alopecia and/or acne) or subclinical, with only an increase in
serum testosterone and/or dehydroepiandrosterone sulfate. Polycystic ovaries were
diagnosed by pelvic sonography according to the Rotterdam conference criteria.

Methods

Patients and Participants:
103 female patients with a clinical diagnosis of PCOS, their 291 first and second-
dergree relatives and 50 age matched controls were included in the study after obtaining
informed consent.

Inclusion Criteria for patients:
1. Post pubertal females aged up to 35 years.
2. Irregular periods (Must have six or fewer menses/year)
3. Have clinical or laboratory evidence of hyperandrogenism (hirsutism or
elevated testosterone) and PCO on ultrasound, more than 9 follicles;
   [Rotterdam criteria].
4. Who have signed informed consent.

Exclusion criteria for patients:
1. Age > 35 years.
2. Diabetes Mellitus > 5 years
3. Confirmed malignancy.
Inclusion Criteria for first and second-degree relatives:

1. Post pubertal first and second degree relatives of patients (Patient's siblings, parents, grandparents, parent's brothers or sisters, parent's nephews or nieces).

Exclusion criteria for first and second-degree relatives:

2. Participants who do not consent for genetic study.

3. Participants with known malignancy

Inclusion and exclusion criteria for controls:

**Inclusion Criteria**

- Postpubertal females <35 years
- Age matched for cases
- No history of PCOD, diabetes, hypertension or dyslipidemia
- No family history of Diabetes hypertension or dyslipidemia
- Signed informed consent form

**Exclusion criteria**

Malignancy
Steroid therapy

Sample size:

103 samples from PCOS patients were collected for genetic study, 50 age matched controls were also taken for genetic study.

Sample size was calculated by using the following equation:

\[ n = \frac{Z_{\alpha}^2 pq}{L^2} \]
291 samples were collected from first or second-degree family members, and preference was given to members of large families. PCOS subjects were taken as cases; and their first and second-degree relatives are classified into three groups such as;

- Phenotypically affected.
- Phenotypically Partially affected (which could have one or more features related to PCOS).
- Phenotypically unaffected.

Total sample size: \((103 + 291) = 394\)

103 female patients with a clinical diagnosis of PCOS by Rotterdam criteria attended KMC, hospital, Attavar; medicine outpatient department or obstetrics and gynecology department and their first and second-degree relatives; were included in the study after obtaining informed consent.

All the subjects including patients and their family members were interviewed in detail and examined for anthropometry such as BMI, Hirsutism / excess hair, Acne, Baldism, Acanthosis nigricans, Skin tags, Buffalo humps, Moonface, Double chin.

1. Biochemical assay such as; Serum fasting insulin, Cortisol, Testosterone, Dehydroxyepiandostenedione, LH, FSH, TSH were done in all cases, along with fasting lipid profile.

2. Blood pressure was measured for all, an oral 2 hr GTT was performed after 75 gm of glucose for all patients.

**Genetic analysis:**

Study of genotype and allelic frequencies were done by means of PCR- RFLP. DNA was extracted from heparinised or EDTA blood.
Ethical clearance was obtained from Manipal university institutional Ethical Committee and the study was performed.

Collection of blood samples: The blood samples were collected from the patients with PCOS from coastal districts of Karnataka state and Kerala state. Patient’s history was collected and pedigree was drawn. Clinical documentation was undertaken with the help of a physician. DNA was extracted from both test and control samples following the standard phenol-chloroform method. PCOS patients were considered as test and normal individuals of same family were used as control. Though we recruited 50 female individual devoiding of all phenotypical feature of PCOS and family history of diabetes.

**Isolation of genomic DNA**

**Reagents employed**

<table>
<thead>
<tr>
<th>WBC lysis buffer</th>
<th>RBC lysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>Ammonium chloride</td>
</tr>
<tr>
<td>: 10 mM</td>
<td>: 138 mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Tris</td>
</tr>
<tr>
<td>: 400 mM</td>
<td>: 17 mM</td>
</tr>
<tr>
<td>Sodium EDTA</td>
<td></td>
</tr>
<tr>
<td>: 2 mM</td>
<td></td>
</tr>
</tbody>
</table>

The chemicals for WBC and RBC lysuis buffer were purchased from Sisco Research Laboratories, India.

**Procedure**

5 mL of peripheral blood was collected in a 15 mL centrifuge tube containing sodium citrate EDTA as anticoagulant. It was spun down at 5,500 rpm for 10 minutes. The buffy coat was separated and 5 mL of RBC lysis buffer was added, allowed to stand at 37°C for 10 minutes. It was spun at 3,000 rpm for 10 minutes, and supernatant was discarded. Above step was repeated, if necessary. 1 mL of WBC lysis buffer was
added and mixed to disperse the pellet. 3µL proteinase K (10 mg/mL), and 15µL of 
20% SDS were added, mixed and incubated overnight at 37°C. Equal volumes of 
buffer saturated phenol was added and mixed for 20 minutes. It was spun down at 
12,000 rpm for 15 minutes at 4°C. The upper layer was collected and transferred to a 
fresh tube. Equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed 
for 10 minutes. It was spun down at 12,000 rpm for 15 minutes at 4°C and the upper 
layer was transferred to another tube. One tenth of 3 M sodium acetate pH 5.2 and 2.5 
volumes of 100% ethanol were added, mixed gently and kept at -80°C for 2 hours. It 
was spun down at 12,000 rpm for 15 minutes at 4°C and supernatant was discarded. 1 
mL of 70% ethanol was added to the pellet and was gently dislodged. It was spun 
down at 12,000 rpm for 15 minutes at 4°C, and the supernatant was discarded. The 
pellet was semi dried on bench top and dissolved in appropriate amount of Milli Q 
water and kept at 37°C (Miller et al, 1988).

**Polymerase Chain Reaction (PCR)**

**Reagents employed**

10X PCR buffer: 50 mM KC1, 100 mM Tris HCl pH 8.3 (Merck, India), 15 mM 
MgCl2 (Merck, India), Triton X-100 (1%) (Sigma, USA).

dNTP mix (4mM): 4 uL of each of dATP (100 mM), dGTP (100mM), dCTP (100 
mM), dTTP (100 mM) - (Fermentas, USA), MilliQ water- 84 uL, Total -100 uL.

**Procedure**

The PCR reagents (Table 3) were purchased from Sigma-Aldrich, USA and Taq 
polymerase from Fermentas, USA.
Table 3. Composition of PCR reaction mixture.

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>VOLUME (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (100-150 ng)</td>
<td>1.5</td>
</tr>
<tr>
<td>MQ water</td>
<td>16</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP 4 mM</td>
<td>2.5</td>
</tr>
<tr>
<td>Forward Primer (100 ng)</td>
<td>0.75</td>
</tr>
<tr>
<td>Reverse Primer (100 ng)</td>
<td>0.75</td>
</tr>
<tr>
<td>Taq Polymerase (1U)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

PCR amplification was carried out using MasterCycler Thermocycler (Eppendorf, USA) in a reaction volume of 25µL containing 100-150 ng of genomic DNA, 100 ng of each primer (Table 4), 1.0 U Taq polymerase, 200µM dNTP mix and 10X PCR buffer with a final concentration of IX. After an initial denaturation at 94°C for 4 minutes, amplification was performed for 35 cycles of denaturation at 94°C for 45 seconds, annealing for 45 seconds with respective primer annealing temperature and extension was at 72°C for 45 seconds, followed by a final extension for 5 minutes at 72°C. PCR products were separated by agarose gel electrophoresis at 100V for 30 minutes, and documented in a Gel Documentation system (Uvitec, UK).

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Gene</th>
<th>Primer sequence 5’-&gt;3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>$11-B$-HSD1</td>
<td>CTGTTTCTCTTACCTCCTCC</td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td>TGAGCATGTCTAGTCTTCCTCG</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>$CYP$-11A</td>
<td>GAGCTATCTTGCCAGCTTG</td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td>GTGTCTCTGAGTCAGCAGCTGACTG</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>$CYP$-17A</td>
<td>CAAAAGTCAGGTCAGATCGAG</td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td>TAGGGTAAAGCAGCAAGAGAG</td>
</tr>
</tbody>
</table>
**Material and Method**

**Agarose gel electrophoresis**

To prepare a 50 mL agarose gel, 0.5 gm of agarose powder (Sigma, USA) was weighed and taken in a clean conical flask. To this 50 mL of 0.5X TBE was added and the flask was heated in a microwave oven to melt the agarose by continuous swirling. The flask was heated until boiling (until a clear solution was obtained). At this point, 2.5 uL of ethidium bromide was added and mixed by gentle swirling. After it cooled down to 50°C, the agarose solution was poured into the gel tray and allowed to solidify. The tray was placed in a gel tank filled with 0.5X TBE buffer. The comb was then carefully removed by pulling it upwards at once. 1uL of 30% glycerol was mixed with 2.5 uL of PCR product and carefully loaded into the gel. The gel was run at 100 V for specific time depending on the product size (30 minutes). The bands were visualized under UV Gel Documentation system (Uvitec, UK).

**Restriction Fragment Length Polymorphism (RFLP)**

RFLP is the technique used to detect the known mutations. The PCR product is cut into restriction fragments using suitable endonucleases, which only cut the DNA molecule where there are specific DNA sequences, termed recognition sequences or restriction sites that are recognized by the enzymes. These sequences are specific to each enzyme, and may be either four base pairs or six base pairs in length. Generally, greater the frequency of recognition sequence, the greater the number of fragments generated. If molecules differ in nucleotide in their recognition sequence, fragments of different sizes may be generated. The restriction fragments are then separated according to their size by agarose gel electrophoresis. The table below (Table 5) shows the list of restriction enzymes used in the present study.
Table 5. List of restriction enzymes used in RFLP for genotyping. The composition specific to each enzyme is given with the reaction conditions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognition site</th>
<th>Buffer</th>
<th>BSA</th>
<th>Temp</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>MspA1</td>
<td>5’..CMG\CKG..3’</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>NEB</td>
</tr>
<tr>
<td></td>
<td>3’..GKC\GMC..5’</td>
<td>2</td>
<td>4</td>
<td>37</td>
<td>NEB</td>
</tr>
<tr>
<td>Xcm1</td>
<td>5’..CCANNNNN\NNNNTGG..3’</td>
<td>37</td>
<td>NEB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’..GGTNNNN\NNNNNACC..5’</td>
<td>37</td>
<td>NEB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Single letter code: R = G or A; K = G or T; B = C, G or T; Y = C or T; S = C or G; D = A, G or T; W = A or T; H = A, C or T; N = G, A, T or C M = A or C; V = A, CorG;

Reaction mixture was prepared to 10 final volume. All the reagents were thawed before use. 500ng of the PCR product was digested with 1 U of restriction enzyme at appropriate incubation temperature for 16 hours. A master mix of MQ water, buffer and enzyme was prepared, spun down and then distributed into the individual labeled tubes. Then the digested products were loaded on agarose gel of appropriate percentage depending on the fragment size. The gel was run for 30-45 minutes and checked for the digestion.

Statistical analysis

The results are expressed as the mean ± SD in the text and Tables. Univariate analysis was done by using one-way ANOVA and Kruskal-Wallis test (non-parametric). A statistical software package was used to perform the analyses (SPSS version 17).