Original Research Article

# Genotype- phenotype correlations of TNF- α gene in polycystic ovarian disease (PCOS)

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## Abstract

**Background:** Polycystic ovarian syndrome (PCOS) is an endocrine disorder very common in women of reproductive age group. Polymorphisms in the tumor necrosis factor alpha (TNF-$\alpha$) have been associated various related diseases ranging to Obesity, PCOS and infertility. In this context, we evaluated association between PCOS and three promoter single nucleotide polymorphisms of the gene TNF-$\alpha$ viz -238G/A (rs361525), -308G/A (rs1800629) and -1031T/C (rs1799964) respectively. **Material and methods:** This is inter-disciplinary study conducted in biochemistry department of a teaching medical institute by collaborating with tertiary care hospitals, and genetic diagnostic lab. In this prospective study recruited 100 PCOS patients with 100 age matched controls, we employed 3 sets of primers and screened the known single nucleotide polymorphism TNF-$\alpha$ gene. Apart from qualitative and quantitative evaluation, linkage disequilibrium, multifactor dimensionality reduction analysis and In-silico analysis was also performed. **Results:** The percentage of AA, AG and GG genotypes in patients was 32, 44, 24 while it was 25, 45 and 30 in controls respectively. Relative risk analysis of the TNF-a -238 G/A polymorphism revealed a threefold risk for the "AG" and "GG" genotypes under the codominant model of inheritance (OR= 1.11, CI= 0.49 – 2.52, p= 0.93 and OR= 1.00, CI= 0.48 – 2.17, p= 1.0 respectively). Similarly a twofold risk was also observed for the "AG+GG" genotypes under the dominant model of inheritance (OR= 2.11, CI= 0.97 – 4.69, p=1.93). When relative risk for the alleles demonstrated a twofold risk of G allele towards disease establishment (OR= 1.00, p= 0.84). **Conclusions:** The present study failed to implicate the role of the AG and GG genotypes, and the G allele in contributing towards establishment and progression of PCOS though further studies are warranted to establish in varied races.

**Keywords:** PCOS; Rotterdam criteria; TNF-alpha; Cytokines; Infertility

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## INTRODUCTION

Tumor necrosis factor alpha (TNF-$\alpha$) is a potent immunomodulator and proinflammatory cytokine displaying varied functions. It plays an important role in the onset of inherited disorders related to cancers, by regulating cellular functions such as cellular proliferation, differentiation, apoptosis, inflammation, tumorigenesis and viral replication. It is produced by monocytes/macrophages, apart from other cell types[1]. Besides the primed components of the immune system TNF-$\alpha$ is produced by cells in the female reproductive tract[2]. TNF-$\alpha$ mediates its effects through two different receptors: receptor I (also TNF-$\alpha$ called p55 or p60) and receptor TNF-$\alpha$ II (also called p75 or p80). While TNF-alpha receptor I is expressed on all cell types in the body, recep TNF-$\alpha$ receptor II is expressed selectively on endothelial cells and on cells of the immune system: it

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binds to two receptors with comparable affinity[3]. Several polymorphisms in the promoter region of the gene TNF-$\alpha$ have been described[1]. Among the best studied the -238G /A (rs361525) and -308G /A (rs1800629) polymorphisms have been associated with an increased transcriptional activity and release TNF-$\alpha$[4]. The present study aims to identify the association between PCOS and three promoter single nucleotide polymorphisms of the gene TNF-$\alpha$ viz -238G/A (rs361525), -308G/A (rs1800629) and -1031T/C (rs1799964) respectively.

## MATERIAL AND METHODS

The study was conducted on 100 PCOS and 100 healthy control women, recruited from patients visiting tertiary care hospital. PCOS participants were selected based on observation of oligoamenorrhea/ anovulation, clinical or biochemical evidence of hyperandrogenism and/or polycystic ovaries on ultrasonography[4]. 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. Exclusion criteria were women with galactorhea, hyperthyroidism, any systemic disease that affects their reproductive physiology, or any medication which interferes with the normal function of the hypothalamic-pituitary-gonadal axis. Participant's age group was in the range from 18-35yrs. The study was approved by the Institutional Ethical Committee (011/02/2015 IEC / Saveetha University Dated12-02-2015). A written informed consent was collected from all the subjects enrolled in the study. Participant's history and other anthropometric assessments were carried out. Sample collection and requirement: Blood samples were collected from participants by venipuncture and it was processed within two hours. Then the samples were centrifuged at 3000 rpm for 10 minutes at 20$^{o}$c to isolate the serum and it was stored -20$^{o}$C until used. Molecular studies Genotyping study was carried out for modifier genes by Allele Specific-PCR and PCR based Restriction fragment length polymorphism analyses (RFLP).

### Genomic DNA Isolation

5 ml of whole blood from controls and subjects was obtained for genomic DNA isolation. In cases/subjects with insufficient amount of blood sample drawn, DNA isolation by Rapid genomic DNA extraction (RGDE) was also carried out. The isolated DNA was considered for mutational screening by PCR based SSCP and for genotyping studies, PCR based RFLP analyses was adopted for the following gene/s. DNA concentration and purity was checked on 0.8% agarose gel or by spectrophotometer. The samples were then stored at -20$^{0}$C for subsequent analysis. Isolation of DNA following Rapid genomic DNA extraction Genomic DNA of high quality and quantity can be obtained in the shortest time and with just 500µl of blood sample by following this protocol.

### Quantification of DNA

DNA was quantified by measuring the absorbance values at 260 and 280nm in a NANOVIEW (GE Healthcare). The ratio of 260/280 nm was observed and the purity of DNA was checked. The isolated genomic DNA was later used for PCR-SSCP and PCR-RFLP analyses. In-vitro amplification of gene of interest by Polymerase chain reaction (PCR) Amplifying a gene of interest was done by PCR using specific primers obtained from published reports under appropriate cycling conditions of denaturation, annealing and extension in a Thermal cycler (Eppendorf, Germany).

### PCR-RFLP

PCR-restriction fragment length polymorphism (PCR-RFLP) is one among the various popular techniques utilized for genotyping single nucleotide polymorphisms. The essence of this technique is the exploitation of the fact that SNPs and micro-indels often end up creating or abolishing restriction enzyme recognition sites. The technique involves the amplification of the target sequence containing the variation. The amplified fragment is then treated with an appropriate restriction enzyme. The presence or absence of the restriction site would result in variant fragment sizes which later can be resolved using electrophoresis.

### Molecular Analysis:

PCR conditions followed for -308G/A, -1031T/C, -238G/A were, initial denaturation at 95$^{0}$C for 5 min, denaturation step at 95$^{0}$C for 30 sec, annealing at 58$^{0}$C for 30 sec, extension at 72$^{0}$C for 1 min for 30 cycles. Final extension was carried out at 72$^{0}$C for 7 min. The PCR products were subjected to RFLP with MspI enzyme (New England Biolabs) by incubating overnight at 37$^{0}$C which were later checked on 3% Agarose gel (Fig 1, Fig 2, and Fig 3).

Primer sequences of Tumor Necrosis Factor alpha (*TNF-$\alpha$*) gene polymorphisms

| Polymorphism | Primers | Amplicon (bp) | RFLP |
|---|---|---|---|
| **-308G/A** | F.P - TCT CGG TTT CTT CTC CAT CG<br>-308 'G' R.P - ATA GGT TTT GAG GGG CAT GG<br>-308 'A' R.P - ATA GGT TTT GAG GGG CAT GA | 183 | ARMS PCR |
| **-1031T/C** | FP - ACAAGGCTGACCAAGAGAGAA<br>RP - GTCCCCATACTCGACTTTCAT | 521 | BbsI<br>521 - 1031T |

| | | | |
|---|---|---|---|
| **-238G/A** | FP - AAACAGACCACAGACCTGGTC<br>RP - CTCACACTCCCCATCCTCCCGGATC | 155 | 360+161<br>-1031C<br>BamHI<br>155 - 238A<br>135+20<br>-238G |

**Statistical Analysis:** SPSS 20.0 version was utilized. Following methods were employed.

1. Qualitative and Quantitative variables were described by computation of frequency, mean, Standard Deviation (SD) and Chi square test of association.
2. Hardy-Weinberg equilibrium: Deviations from the Hardy-Weinberg equilibrium were tested for all polymorphisms in cases and controls by comparing observed and expected genotype frequencies and goodness of fit test was adopted (http://www.socscistatistics.com/tests/chisquare/).
3. SNPSTAT software was adopted for the calculation of the Odds ratios, as the estimates of relative risk of disease, with 95% confidence intervals and and ≤0.05 probabilty to determine dominant/ codominant/ recessive inheritance model for the polymorphisms studied (http://bioinfo.iconcologia.net/snpstats/custom.php).
4. Linkage Disequillibrium test was carried out by Haploview version 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php).
5. Multifactor Dimensionality Reduction MDR analysis was performed by MDR2.0 beta 8.0 software. 6) Online tools for Insilico analysis Secondary RNA structure predictions were carried out by RNA Vienna server – http://rna.tbi.univie.ac.at/cgibin/RNAfold.cgi.

## RESULTS

The present study was aimed to identify the association between PCOS and three promoter single nucleotide polymorphisms of the *TNF-α* gene viz **-238G/A (rs361525), -308G/A (rs1800629)** and **-1031T/C (rs1799964)** The percentage of GG, GA and AA genotypes in patients was 24, 44, 32 while it was 25, 45 and 30 in controls respectively. The allele frequencies of G and A allele are 0.46 and 0.54 for patients while it was 0.48 and 0.52 for controls correspondingly. The genotype and allele frequencies did not differ significantly between the groups. The patients as well as the controls were following Hardy Weinberg Equilibrium. The genotype and the allele frequencies of the patients and controls are given in **Table 7.1.** Risk analysis in order to identify the role of the individual genotype, did not reveal any protective or predisposing role towards PCOS. Mendelian effect modelling also did not yield any significant outcome. The risk estimates of *TNF*-□-238G/A genotypes and alleles among the study group are given in **Table 7.2.**Secondary structure analysis of the pre-mRNA revealed distinct changes with the entropy being high in the variant allele (-48.80 kcal/mol) compared to wild type allele (-49.80 kcal/mol) (**Figure 5**). The percentage of GG, GA and AA genotypes in patients was 25, 50 and 25 while it was 23, 47 and 30 in controls respectively. The allele frequencies of G and A allele are 0.5 and 0.5 for patients whereas it was 0.47 and 0.53 for controls correspondingly. The genotype and allele frequencies did not vary between the groups. The patients as well as the controls were following Hardy Weinberg Equilibrium. The genotype and the allele frequencies of the patients and controls is given in **Table 3**. Risk analysis in order to identify the role of the individual genotype, did not reveal any protective or predisposing role towards PCOS. Mendelian effect modeling also did not yield any significant outcome. The risk estimates of *TNF*-□-308G/A genotypes and alleles among the study group are given in **Table4** Secondary pre-mRNA structures built were showing distinct energy changes for the respective alleles of SNP with the entropy being high for the variant allele (-95.10 kcal/mol) compared to wild type allele (-95.60 kcal/mol) **Figure 6.** The percentage of TT, TC and CC genotypes in patients was 24, 38, 38 while it was 25, 40 and 35 in controls respectively. The allele frequencies of T and A allele are 0.43 and 0.57 for patients while it was 0.45 and 0.55 for controls correspondingly. The genotype and allele frequencies did not differ significantly between the groups. The controls were following Hardy Weinberg Equilibrium while the patients deviated from HWE. The genotype and the allele frequencies of the patients and controls are given in **Table 5.** Risk analysis in order to identify the role of the individual genotype, did not reveal any protective or predisposing role towards PCOS. Mendelian effect modelling also did not yield any significant outcome. The risk estimates of *TNF-α*-1031T/C genotypes and alleles among the study group are given in **Table 6.**

The pre-mRNA secondary structure demonstrated a distinct change that could be observed contributing to the less stability and high entropy of the variant "C" allele (-164.10 kcal/mol) when compared to the wild type allele (-164.20 kcal/mol) **Figure 7.**

### Linkage Disequilibrium

Linkage disequilibrium (LD) plot for the four variations was constructed with the help of Haploview 4.2 and D' values are given in the **Figure 8.** Haploview is an application for haplotype and statistical genomic analysis for visualization of family/case-control based analyses and the program uses a two marker expectation – maximization (EM) ignoring missing data to estimate the maximum likelihood values of the four gamete frequencies, from which the D', LOD and $r^2$ calculations can be derived. Each square displays the amount of LD between a pair of markers. LD is the non-random association of alleles at two or more loci; not necessary on the same chromosome. The strength of the LD between two markers is given by the intensity of color of the box. In crossing areas multi - allelic D' value corresponds to the level of recombination between two blocks. And D' value 0 indicates maximum amount of recombination between two blocks. The two most common pair-wise measures of LD are D' and $r^2$,D' is defined to be 1 in the absence of obligate recombination, declining only due to recombination or recurrent mutation. The $r^2$ is squared correlation coefficient between the two markers, thus $r^2$ is 1 when two markers remain undisrupted by recombination, but has a value less than 1 when an initially strong correlation has been disrupted by crossing over. The LD plots generated are listed below. The **Figure 8** shows the LD structure and the LD coefficients (D′) between five polymorphisms studied in PCOS and control subjects. In the D' plot each diagonal represents a different gene polymorphisms with each square representing a pair wise comparison between two genes. Red squares indicate statistically significant linkage disequilibrium (LOD>2) between the pair of genes, as measured by the D′ statistic; darker colors of red indicate higher values of D′, upto a maximum of 1. The white squares and blue squares indicate no statistically significant evidence of LD with D′ values of <1 and 1 respectively. Linkage was higher between rs1799964 and rs361525 could be observed in both the controls and PCOS group pointing out the fact that both the variations are inherited as a single entity in an individual. No significant D' values could be observed in any other combinations indicating the entities are being inherited as a single unit. The possibility of association between different haplotype combinations of the gene polymorphisms undertaken in the present study in PCOS was tested by performing haplotype analysis. Seventeen haplotypes were identified, among them three combinations of haplotypes showed significant results. The haplotype combination "A-A-C-A-C" and "A-G-T-G-C" conferred protection towards PCOS (OR= 0.321, CI= 0.15 – 0.67, p=0.002 and OR= 0.28, CI= 0.11 – 0.73, p=0.005 respectively) while the haplotype combination "A-G-C-A-C" conferred fourfold risk (OR= 3.38, CI= 1.52 – 7.53, p= 0.0018) towards PCOS susceptibility. The individual SNP involved in the haplotype combinations are: rs1137101 – rs1800629 – rs1799964 – rs361525 – rs1800795. The estimated Chi square values and relative risk estimates at p values ≤0.05 for the 17 haplotypes identified in the present study, are given in **Table 7.** Haplotypes with a frequency of p<0.01 were excluded from the analysis.

### Multifactor Dimensionality Reduction (MDR) Analysis

Sequence variants in human genes are being described in remarkable numbers. Determining which variants and which environmental factors are associated with common, complex diseases has become a daunting task. This is partly because the effect of any single genetic variation will likely be dependent on other genetic variations (gene-gene interaction or epistasis) and environmental factors (gene-environment interaction). Detecting and characterizing interactions among multiple factors is both a statistical and a computational challenge. To address this problem, multifactor dimensionality reduction (MDR) method for collapsing high dimensional genetic data into a single dimension has been developed, thus permitting interactions to be detected in relatively small sample sizes. In the present study, the MDR method using a software package for implementing MDR in a case-control design was used to study gene-gene interactions in PCOS. **Table 8** represents the results obtained for the number of loci evaluated by MDR software (Version 2). The multi locus model with the best/maximum cross-validation consistency at p≤0.05 is considered to be the best model for disease manifestation and progression. The MDR analysis revealed the four locus model involving A668G, -308G/A, -1031T/C and -174G/C of *IL-6* with a testing accuracy of 48% and CV consistency of 9/10 to be the best model in case of PCOS. Even though the table displays loci with a CV consistency of 10/10 for five locus, they were not considered since the testing accuracy was observed to be below the four locus model. As observed from the above entropy dendrogram, a synergistic interaction between -238G/A and -308G/A polymorphisms of the TNF-exists and both together express and appear to protect individuals from PCOS, while the leptin receptor polymorphism (rs1137101) exert its risk in an independent (redundant) manner conferring risk towards PCOS susceptibility **(Figure 8)**

**Table 1:** Genotypic and allelic frequency distribution of the *TNF-α*–238G/A polymorphism in controls and PCOS

| SNP | Genotype | Controls n (%) | PCOS n (%) | $\chi^2$ (p value) |
|---|---|---|---|---|
| **-238G/A** | GG | 25 (25) | 24 (24) | 0.19 (0.91) |
| | GA | 45 (45) | 44 (44) | |
| | AA | 30 (30) | 32 (32) | |
| | **Allele** | **Controls n (f)** | **PCOS n (f)** | **$\chi^2$ (p value)** |
| | G | 95 (0.48) | 92 (0.46) | 0.18 (0.67) |
| | A | 105 (0.52) | 108 (0.54) | |
| **HWE** | | **Controls** | 9.55 (0.32) | |
| **χ2 (p value)** | | **Patients** | 1.30 (0.25) | |

**HWE**: Hardy Weinberg Equilibrium; **$\chi^2$**: Chi square

**Table 2:** Odds risk estimates of genotypes and alleles of PCOS compared to controls of the *TNF-α* -238G/A polymorphism

| Model | Genotype | Controls | PCOS | OR (95% CI) | p value |
|---|---|---|---|---|---|
| **Codominant** | G/G | 25 | 24 | 1.00 | |
| | G/A | 45 | 44 | 1.02 (0.48 – 2.17) | 1.00 |
| | A/A | 30 | 32 | 1.11 (0.49 – 2.52) | 0.93 |
| **Dominant** | G/G | 25 | 24 | 1.00 | |
| | G/A-A/A | 75 | 76 | 1.06 (0.53 – 2.11) | 1.00 |
| **Recessive** | G/G-G/A | 70 | 68 | 1.00 | |
| | A/A | 30 | 32 | 1.09 (0.58 – 2.09) | 0.88 |
| **Over dominant** | G/G-A/A | 55 | 56 | 1.00 | |
| | G/A | 45 | 44 | 0.96 (0.53 – 1.74) | 1.00 |
| | G | 95 | 92 | 1.00 | |
| | A | 105 | 108 | 1.06 (0.7 – 1.6) | 0.84 |

**Table 3:** Genotypic and allelic frequency distribution of the TNF-α 308G/A polymorphism in controls and PCOS

| SNP | Genotype | Controls N (%) | PCOS N (%) | $\chi^2$ (p value) |
|---|---|---|---|---|
| **-308G/A** | GG | 23 (23) | 25 (25) | |
| | GA | 47 (47) | 50 (50) | 1.34 (0.512) |
| | AA | 30 (30) | 25 (25) | |
| | **Allele** | **Controls N (f)** | **PCOS N (f)** | **$\chi^2$ (p value)** |
| | G | 93 (0.47) | 100 (0.5) | |
| | A | 107 (0.53) | 100 (0.5) | 0.98 (0.322) |
| **HWE** | | **Controls** | 0.31 (0.57) | |
| **χ2 (p value)** | | **Patients** | 0 (1.0) | |

**HWE**: Hardy Weinberg Equilibrium; **$\chi^2$**: Chi square

**Table 4:** Odds risk estimates of genotypes and alleles of PCOS compared to controls of the *TNF-α* -308G/A polymorphism

| Model | Genotype | Controls | PCOS | OR (95% CI) | p value |
|---|---|---|---|---|---|
| **Codominant** | G/G | 23 | 25 | 1.00 | |
| | G/A | 47 | 50 | 0.51 (0.26 – 1.01) | 0.054 |
| | A/A | 30 | 25 | 0.4 (0.18 – 0.88)* | 0.021* |
| **Dominant** | G/G | 23 | 25 | 1.00 | |
| | G/A-A/A | 77 | 75 | 0.47 (0.25 – 0.88)* | 0.016* |
| **Recessive** | G/G-G/A | 70 | 75 | 1.00 | |
| | A/A | 30 | 25 | 0.6 (0.31 – 1.15) | 0.13 |
| **Over dominant** | G/G-A/A | 53 | 50 | 1.00 | |
| | G/A | 47 | 50 | 0.77 (0.44 – 1.36) | 0.42 |
| | G | 93 | 100 | 1.00 | |
| | A | 107 | 100 | 1.15 (0.76 – 1.74) | 0.55 |

**Table 5:** Genotypic and allelic frequency distribution of the *TNF-α*-1031T/C polymorphism in controls and PCOS

| SNP | Genotype | Controls n (%) | PCOS n (%) | $\chi^2$ (p value) |
|---|---|---|---|---|
| -1031T/C | TT | 25 (25) | 24 (24) | |
| | TC | 40 (40) | 38 (38) | 0.384 (0.83) |
| | CC | 35 (35) | 38 (38) | |

| | Allele | Controls n (f) | PCOS n (f) | $\chi^2$ (p value) |
|---|---|---|---|---|
| | T | 90 (0.45) | 86 (0.43) | 0.33 (0.57) |
| | C | 110 (0.55) | 114 (0.57) | |
| **HWE** | **Controls** | 3.68 (0.05) | | |
| **χ2 (p value)** | **Patients** | 5.05 (0.02) | | |

**HWE** : Hardy Weinberg Equilibrium; **$\chi^2$**: Chi square; p≤0.05

**Table 6 :** Odds risk estimates of genotypes and alleles of PCOS compared controls of the *TNF-α* -1031T/C polymorphism **(p≤0.05)**

| Model | Genotype | Controls | PCOS | OR (95% CI) | p value |
|---|---|---|---|---|---|
| **Codominant** | TT | 25 | 24 | 1.00 | |
| | TC | 40 | 38 | 0.99 (0.46 – 2.15) | 1.00 |
| | CC | 35 | 38 | 1.13 (0.51 – 2.49) | 0.88 |
| **Dominant** | TT | 25 | 27 | 1.00 | |
| | TC+CC | 75 | 76 | 1.06 (0.53 – 2.11) | 1.00 |
| **Recessive** | TT+TC | 65 | 62 | 1.00 | |
| | CC | 35 | 38 | 1.14 (0.61 – 2.11) | 0.77 |
| **Over dominant** | TT+CC | 60 | 62 | 1.00 | |
| | TC | 40 | 38 | 0.92 (0.5 – 1.69) | 0.89 |
| | T | 90 | 86 | 1.00 | |
| | C | 110 | 114 | 1.09 (0.72 – 1.64) | 0.76 |

**Table 7:** Haplotype Analysis

| Haplotype | $\chi^2$ | Odds Ratio [95%CI] | p-value |
|---|---|---|---|
| A A C A C | 9.782 | 0.321 [0.153~0.674] | **0.002** |
| A A C A G | 0.973 | 1.836 [0.539~6.250] | 0.324044 |
| A A C G C | 0.327 | 0.530 [0.058~4.853] | 0.567683 |
| A A T G C | 1.658 | 2.347 [0.617~8.924] | 0.197911 |
| A A T G G | 0.890 | 1.450 [0.668~3.150] | 0.345482 |
| A G C A C | 9.798 | 3.380 [1.518~7.527] | **0.001757** |
| A G C A G | 0.020 | 0.938 [0.386~2.283] | 0.888094 |
| A G T G C | 7.678 | 0.280 [0.108~0.726] | **0.005609** |
| A G T G G | 1.114 | 0.660 [0.304~1.434] | 0.291240 |
| G A C A C | 1.817 | 2.216 [0.678~7.246] | 0.177705 |
| G A C A G | 0.564 | 0.786 [0.418~1.476] | 0.452801 |
| G A T G C | 0.080 | 0.896 [0.420~1.915] | 0.777549 |
| G A T G G | 0.051 | 0.909 [0.398~2.079] | 0.821932 |
| G G C A C | 9.946 | 0.159 [0.044~0.580] | **0.001621** |
| G G C A G | 7.370 | 2.961 [1.310~6.689] | **0.006655** |
| G G T G C | 2.920 | 2.062 [0.886~4.800] | 0.087577 |
| G G T G G | 0.001 | 1.020 [0.194~5.360] | 0.981362 |

**Table 8:** Gene combinations leading to disease manifestation

| Model | Training Bal. Acc. | Testing Bal. Acc. | CV Consistency |
|---|---|---|---|
| **-174G/C** | 0.55 | 0.46 | 6/10 |
| **-308, -238** | 0.61 | 0.51 | 5/10 |
| **A668G, -308, -174G/C** | 0.66 | 0.48 | 5/10 |
| **A668G, -308, -1031, -174G/C** | 0.74 | 0.48 | 9/10 |
| **A668G, -308, -1031, -238, -174G/C** | 0.74 | 0.47 | 10/10 |

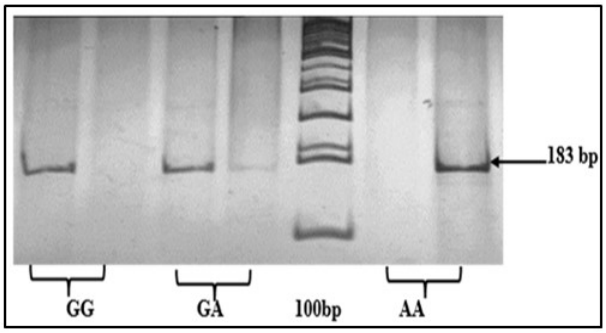


**Figure 1:** ARMS PCR showing genotypes of TNF-α -308G/A Polymorphism

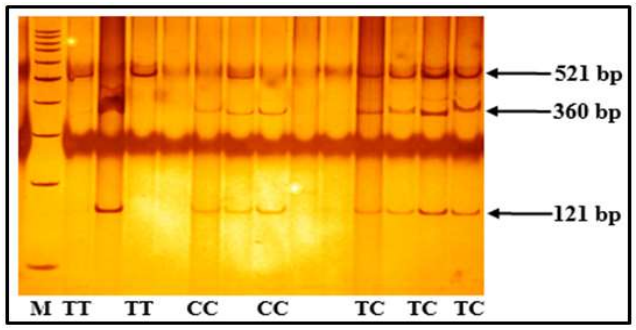


**Figure 2:** Genotypes of TNF-α -1031T/C polymorphism based on PCR-RFLP TT: 521 bp, TC: 521+360+121 bp, CC: 360+121 bp, Marker (M) = 100bp

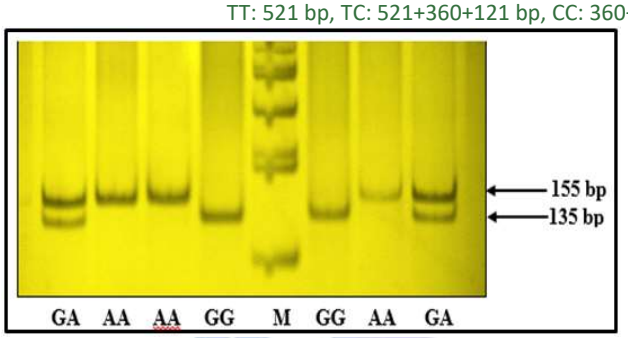


**Figure 3:** Depicting genotypes of TNF-α -238G/Apolymorphism GG: 135+20 bp, GA: 155+135+20 bp, AA: 155 bp, Marker (M) = 100bp

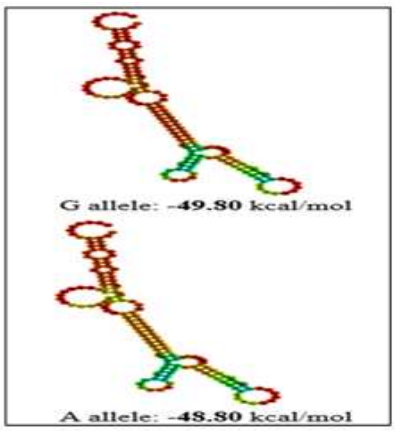


**Figure 4**

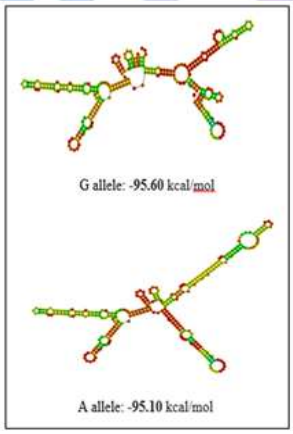


**Figure 5**

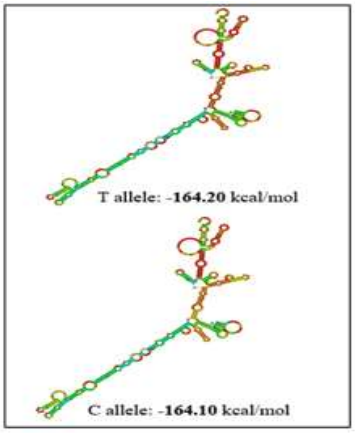


**Figure 6**

**Figure 4:** Secondary pre-mRNA structures of the TNF-α –238G/A polymorphism; **Figure 5:** Secondary pre-mRNA structures of the TNF-α -308G/A polymorphism; **Figure 6:** Secondary pre-mRNA structures of the TNF-α -1031T/C polymorphism

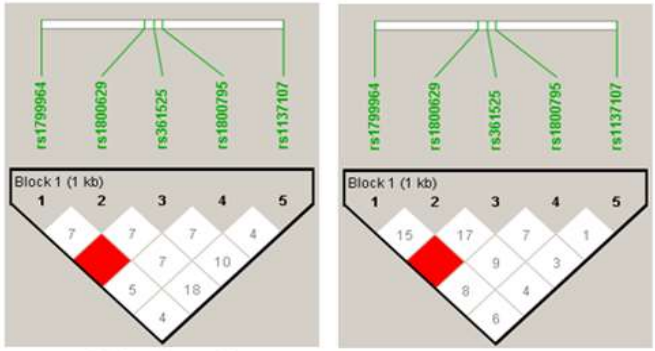


**Figure 7:** Linkage disequilibrium plot of the variations in PCOS and controls in the present study (D' values were indicated in the respective squares)

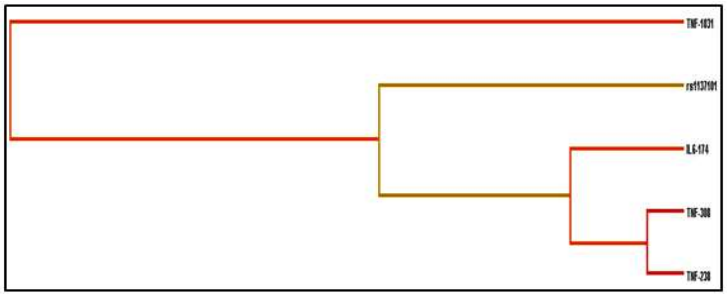


**Figure 8:** Dendogram showing the interaction between various genes involved in the study

## DISCUSSION

PCOS represents a common endocrine disorder seen in women of reproductive age and is often associated with inflammation[6]. The persistent chronic inflammation observed in PCOS could lead to ovarian dysfunction and other metabolic abnormalities[7]. TNF α is a proinflammatory cytokine present in follicular fluid of human ovary, granulosa cells and oocytes[8], plays an important role in a wide range of diseases such as type 2 diabetes, coronary artery disease and dyslipidemia[9]. It elicits inflammatory responses as a normal physiological function for ovulation and influence follicular atresia, adiposity, insulin resistance, ovarian apoptosis, increased ovarian steroid secretion, anovulation and hyperandrogenism[10]. The present study was investigated to identify the association between three promoter polymorphisms (-238G/A, -308G/A and -1031T/C) and PCOS, a condition associated with inflammation. The genotypic and allele frequencies of -238G/A did not differ significantly between the patients and the controls indicating no role of this SNP in PCOS susceptibility. The minor allele 'A' of the −238G/A polymorphism and the rare allele 'C' of the −1031T/C polymorphism were reported to be associated with higher *TNF-* production[11].The *TNF-α* -238G/A promoter polymorphism leads to addition of transcription factors T3R and Sp1 which play an important role in controlling cell growth and apoptosis The distribution of genotypes and did not vary between the groups; suggesting no association of -308G/A polymorphism with PCOS. Our results were in accordance with the studies in Australian, Caucasian and Indian population[12]. The rare 'A' allele of −308G/A polymorphism reportedly influences gene expression, resulting in higher *TNF-α* production *in vitro*[13]. The *TNF-α* -308G/A promoter polymorphism leads to deletion of binding sites of two transcription factors Sp1 and Usf (Upstream Stimulatory Factor) which may result in dysregulation of transcription and translation hence regulatory in nature. Our study also demonstrated no association of *TNF-α -1031 T/C* with PCOS and these findings suggested a strong influence on PCOS, similar to the findings of others in Asian population[8,14] however contradictory to our study. The *TNF-α* -1031T/C promoter polymorphism leads to addition of GCN4 transcription factor containing leucine zipper or zinc finger motifs. It contributes to the ribonuclease activities controlling gene expression and RNA biogenesis in the nucleus, the major site of RNA turnover. A potential physiological role of TNF-α -308 G/A polymorphism has been identified in several studies[15,16] and much recent reporter gene studies demonstrated a significant effect on its transcriptional activity (8, 17). Variation in TNF-α gene was reported to be linked with PCOS susceptibility in Korean population. Studies on TNF-α gene in PCOS patients from Australian, Caucasian and Indian population revealed lack of association of TNF-α -308 G/A polymorphism[8,18,19]. Li S *et al.* proposed that elevated TNF-α have been linked to obesity and insulin resistance in the Chinese population[20]. Literature suggest PCOS is a complex disorder that might result from the interaction of susceptible and protective gene variants under the influence of environmental factors [21] suggesting that the clinical phenotype of affected individuals is also influenced by gene-environment interaction.

## CONCLUSION

The present study could not provide a concrete association between the three *TNF α* polymorphisms and PCOS, further extensive studies on the influence of TNF-α polymorphism towards PCOS in different ethnic groups may identify the potentiality of these polymorphisms as markers of inflammation and in turn may help the clinicians for the better management of the condition. Functional studies on the reporter gene assays of this gene would be required further to gain meaningful insights on the aetiology of TNF-α towards PCOS phenotype.

## REFERENCES

1.Lakshmi, K. V., Shetty, P., Vottam, K., Govindhan, S., Ahmad, S. N., and Hasan, Q. (2010). Tumor necrosis factor alpha -C850T polymorphism is significantly associated with endometriosis in Asian Indian women. *Fertility and Sterility*, *94*(2), 453–456.

2.Kurachi, O., Matsuo, H., Samoto, T., and Maruo, T. (2001). Tumor necrosis factor-alpha expression in human uterine

leiomyoma and its down-regulation by progesterone. *The Journal of Clinical Endocrinology and Metabolism*, *86*(5), 2275–2280.

3.Sethi, G., Sung, B., and Aggarwal, B. B. (2008). TNF: a master switch for inflammation to cancer.*Frontiers in Bioscience : A Journal and Virtual Library*, *13*, 5094–5107.

4.Fargion, S., Valenti, L., Dongiovanni, P., and Fracanzani, A. L. (2004). TNFalpha promoter polymorphisms. Methods in Molecular Medicine, 98, 47–58.

5.The Rotterdam Citeria, 2003 *et al.*, Rotterdam Criteria for diagnosing Polycystic Ovarian Syndrome (PCOS), Fertil Steril, 2004 Jan;81(1):19-25.

6.Sathyapalan, T. and Akin, S. L. (2010). Mediators of Inflammation in Polycystic Ovary Syndrome in Relation to Adiposity. Mediators of Inflammation.

7.Ebejer, K. and Calleja-Agius, J. (2013). The role of cytokines in polycystic ovarian syndrome. Gynecol Endocrinol, 2013; 29(6): 536–540.

8.Yun JH, Choi JW, Lee KJ, Shin JS and Baek KH (2011). The promoter -1031(T/C) polymorphism in tumor necrosis factor-alpha associated with polycystic ovary syndrome. Reproductive Biology and Endocrinology. 9(1):131.

9.Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A (1993). Tumor necrosis factor-alpha supresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem. 268:26055–8.

10. LavenJS, Imani B, Eijkemans MJC and Fauser BCJM (2002). New approach to Polycystic Ovary syndrome and other forms of Anovulatory infertility. Obstetrical and Gynecological Survey. 57(11):755–767.

11. Higuchi T, Seki N, Kamizono S, Yamada A and Kimura A (1998). Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens. 51(6):605–612.

12. Milner CR (1999b). No association between the 308 polymorphism in the tumour necrosis factor alpha (TNFalpha) promoter region and polycystic ovaries. Molecular Human Reproduction. 5(1):5–9.

13. Bouma G, Xia B, Crusius JBA, Bioque G, Koutroubakis I, Blomberg BM and Peña AS (1996). Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). Clinical and Experimental Immunology. 103(3): 391–396.

14. Choi SW, Gu BH, Ramakrishna S, Park JM and Baek KH (2009). Association between a single nucleotide polymorphism in MTHFR gene and polycystic ovary syndrome.European Journal of Obstetrics and Gynecology and Reproductive Biology. 145(1):85–88.

15. Escobar-Morreale HF (2001a). TNF-α and Hyperandrogenism: A clinical, biochemical, and molecular genetic study. Journal of Clinical Endocrinology and Metabolism. 86(8):3761–3767.

16. Legato MJ (1997).Gender-specific aspects of obesity. Int J Fertil Womens Med. 42: 184-197.

17. Ehrmann D.A. 2005. Polycystic ovary syndrome.*N. Engl. J. Med.*352: 12.

18. Kurachi O, Matsuo H, Samoto T and Maruo T (2001). Tumor necrosis factor-alpha expression in human uterine leiomyoma and its down-regulation by progesterone. The Journal of Clinical Endocrinology and Metabolism. 86(5):2275–2280.

19. Bhattacharya SM and Ghosh M (2010). Insulin resistance and adolescent girls with polycystic ovary syndrome. J Pediatr Adolesc Gynecol. 23:158–161.

20. Lee S, Yoo KJ, Kim SJ, Lee SH, Cha KY and Baek KH (2008). Single nucleotide polymorphism in exon 17 of the insulin receptor gene is not associated with polycystic ovary syndrome in a Korean population. Fertility and Sterility. 86(2): 380–384.

21. Diamanti-Kandarakis E, Alexandraki K, Piperi C, Protogerou A, Katsikis I, Paterakis T *et al.* (2006). Inflammatory and endothelial markers in women with polycystic ovary syndrome. Eur J Clin Invest. 36:691–7.

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