5.0. DISCUSSION

The present hospital-based, observational case–control study was carried on 169 PCOS and 169 control women in the southern region of India. Foremost, to comprehend the clinical presentations in women with polycystic ovarian syndrome compared with normal women in the selected population. Then to find the single nucleotide polymorphisms of genes involved in the pathogenesis of PCOS with particular interest on genes involved in the insulin secretion and action and/or its signaling pathways also, to find the implications of genetic factors contributing to insulin resistance in the pathogenesis of polycystic ovarian syndrome.

The assessment of the clinical and hormonal profiles revealed that the biochemical and hormonal variables play a very crucial role in women and any alterations of those variables disturb several biological pathways and in turn disrupt the biological system. The mean age of the subjects in the study group was 27. An increase in body mass index was seen in the PCOS group compared to the controls. Obesity was observed in 15% of PCOS women and 7% existed in the lean category. PCOS women who fall in the overweight category was 32% which was slightly lesser compared to the controls where 34% of the normal women were overweight. About 73% of obese women with PCOS presented irregular menstrual cycles, 19% showed elevated levels of insulin compared to normal controls and 58% displayed insulin resistance calculated by homeostatic model assessment (HOMA). An increase in the body mass index may contribute towards alterations of the biochemical and hormonal systems. Along with BMI, waist circumference and waist/hip ratio was calculated which are the measures of visceral or abdominal fat.
mass. An increase in the waist circumference was observed in 46.74% PCOS women and an increased waist/hip ratio was observed in 56.80% women with PCOS. Out of 96 women with increased waist/hip ratio, insulin resistance and irregular cycles was observed in 70.83% and 85.41% women with PCOS. All the control women in the study satisfied the criteria of having regular menstrual cycles and the same was also observed among 15.97% PCOS women. In women with PCOS, menstrual cycle irregularity is a common difficulty. Almost 84% women with PCOS exhibited irregular cycles. 73.37% showed oligomenorrhea and 5% displayed secondary amenorrhea. 24.85% of women with PCOS established heavy menstrual flow pattern whereas 10.65% reported a pattern of scanty flow. Dysmenorrhea was observed in 46.74% of PCOS women. Any disturbances in the menstrual cycle in turn disturb the hormonal mechanisms and add to female infertility issues. Studies across have reported women with oligomenorrhea to be 64.3 %, 10.2%, 71% and 93% (Alili & Idrizi, 2014; Najem, Elmehdawi, & Swalem, 2008; Taponen et al., 2003; Yousouf, Khan, Kounsar, Ahangar, & Lone, 2012).

In the present study LH was found to be elevated compared to FSH in PCOS women. It was twice as high compared to the control group which may be due to the increase in GnRH pulse frequency in PCOS women (Najem et al., 2008). A decrease in FSH levels could disrupt follicular growth. The increase in LH levels could be due to high and static concentrations of estrogens that alter the control of this hormone by the hypothalamus-pituitary axis (A. E. Taylor et al., 1997). Increased GnRH secretion drives the pre-ovulatory LH surge in a dose-dependent fashion. During the follicular phase in parallel with increasing estradiol concentrations LH responses to identical GnRH stimuli. Increased estradiol also may act to augment the
gonadotrope’s responsiveness to GnRH (Lasley, Wang, & Yen, 1975). During the normal menstrual cycle, central and pituitary sites respond to the estradiol positive feedback loop signal to ensure a timely gonadotropin surge. The LH: FSH ratio in the present study in PCOS women was reported to be 31%.

Also, a significant difference was observed in the testosterone levels of PCOS women compared to that of controls, corroborating with the hypothesis that an increase in testosterone levels leads to suppression of normal menstruation and ovulation confirming with our results in the multivariate logistic regression that it withholds as one of the risk variable in women with PCOS. The augmented GnRH pulse frequency is hugely associated with hyperandrogenism and increased ovarian volume (Burt Solorzano, McCartney, Blank, Knudsen, & Marshall, 2009). In the present study hyperandrogenism was reported to be 45.56% in women with PCOS. An increase in testosterone levels above a normal range leads to an unusual increase in hair growth (hirsutism). Hirsutism in PCOS women was 22.48% and 8.28% women reported to have acne. An increased conversion of testosterone to dihydrotestosterone via the enzyme 5 alpha reductase within the pilosebaceous unit may be one of the root causes for hirsutism (Burt Solorzano et al., 2009). 5 alpha reductase acts as an amplifier of the androgen signal (Mauvais-Jarvis, 1986). Studies on molecular cloning characterized two isoenzymes of 5 alpha reductase which is type 1 and type 2 (5α-R type 1 and 5α-R type 2) (Andersson, Berman, Jenkins, & Russell, 1991; Andersson, Bischop, & Russell, 1989). The predominant being 5α-R type 1 (SDR5A1) is found majorly in skin (Harris et al., 1992). A rise in 5α-reductase activity was seen in genital and pubic skin fibroblasts from hirsute patients compared to the skin of normal women (Lobo, Goebelsmann, & Horton, 1983;
Mowszowicz et al., 1983). Women with hyperandrogenism were found to have an increased waist/hip ratio in 65% women with PCOS. Excess androgen boosts insulin resistance leading to raised insulin levels, which in turn fuel more androgen synthesis. This further, increases the symptoms of PCOS making women more susceptible to diabetes, obesity and cardiovascular ailments (Pasquali et al., 2011). About 74.03% PCOS women with hyperandrogenism were insulin resistant, 40.26% demonstrated symptoms of hirsutism, 62.34% showed polycystic ovaries; 85.71% had irregular menstrual cycles and increased fasting glucose was established in 44.16% hyper-androgenic PCOS women. Correlation analysis carried out in this study has revealed that a rise in testosterone levels raises LH/FSH ratio, antral follicular count and symptoms of hirsutism in PCOS women.

A significant difference in the glucose levels, fasting insulin levels, HOMA-IR index, iHOMA2% β (interactive homeostatic model assessment β-cell functioning), iHOMA2% S (interactive homeostatic model assessment insulin sensitivity) were found between PCOS and control women with an elevation in PCOS women. Insulin resistance was observed in 70.41% of women with PCOS. 85% of PCOS women with insulin resistance were found to have irregularities in their menstrual cycle, abdominal obesity measured by BMI and waist/hip ratio were14.28% and 57.14%, 32.77% showed an elevated LH/FSH ratio, 47.89% were hyperandrogenic and in turn showed symptoms of hirsutism which was seen in 24.47% of women, 63.02% showed defects in the fasting glucose metabolism and 45.38% reported to have polycystic ovaries. There was a significant difference in the mean β-cell functioning between PCOS and control women. The β-cell function was greater in PCOS women compared to the controls but insulin sensitivity was found
to be much lesser with mean 49.83 compared to the controls (83.04). This sensitivity towards insulin could also be one of the reasons for raised insulin levels in PCOS women. We also found that the unilateral antral follicular count and ovarian volume of the right ovary showed a marginally higher mean value than that of the left in women with PCOS. Having increased BMI and waist/hip ratio, increase in total testosterone, rise in the insulin levels, increased HOMA-IR, elevated fasting glucose, increased ovarian volume, higher antral follicular counts and symptoms of hirsutism or combination of these predisposes a higher chance and a greater risk for a woman to develop PCOS.

Several polymorphic studies have been reported linking several variants of genes involved in several mechanisms and insulin resistance has found to play a very important role in the pathogenesis of PCOS. PCOS and insulin resistance are interlinked. About 40% of women with PCOS are insulin resistant (Sawathiparnich et al., 2005; Vigouroux, 2010a; Wijeyaratne et al., 2002). In the present work we tried to study the functional single nucleotide polymorphisms of several genes which were previously studied in different populations with consideration of utmost link towards PCOS. The study on single nucleotide polymorphisms of INS (rs689), IRS1 (rs1801276), IRS2 (rs1805097), CAPN10 (rs7607759), CAPN10 (rs295766) genes towards polycystic ovary syndrome study is the first of its kind to be carried out in the Indian population.

Insulin gene has a functionally central role in insulin secretion and/or action and also in the signaling pathways. The variable number tandem repeats positioned at -23bp in the 5’ flanking region of INS gene is considered to be a susceptible loci
and acts as a surrogate marker. The frequency of class III allele (12%) reported in our population was much lower compared to the Japanese, European, Korean and Han Chinese which was 97%, 30%, 94% and 93.5% accordingly. The homozygous class I alleles in our study were higher in both PCOS and control. The variant rs689 of \textit{INS} gene was not associated with PCOS in the present study. But we found that the homozygous mutant AA genotype and the heterozygote TA genotypes influenced the clinical and biochemical variables. We observed that the mean waist/hip ratio of PCOS women with AA genotype and TA genotypes was higher than the TT genotype. At the same time, the LH levels and the insulin levels were found to be elevated and the insulin sensitivity was observed to be much lesser in PCOS women with AA genotype compared to the TT and TA genotype. An increase in the LH/FSH ratio, insulin resistance and increased fasting glucose were observed in PCOS women with heterozygous TA genotype compared to the TT and TA genotype. Studies carried out on anovulatory women with PCOS selected based on criteria proposed by National Institute of Health (NIH) in Czech women also reported a negative association with OR 1.44 (0.50-4.18; \( P = 0.59 \)) (Vankova et al., 2002). Another study with 216 PCOS and 192 non-PCOS women in Han Chinese population reported no significant difference between cases and control groups either in allele (\( P = 0.996 \)) or genotype (\( P = 0.802 \)) frequencies (Y. Xu et al., 2009b) also a family based association study reported no excess transmission of alleles of rs689 VNTR polymorphism, regardless of parent of origin (Powell, Haddad, Bennett, Gharani, Sovio, Groves, et al., 2005).
The \textit{INSR} gene contains 22 exons, of which the synonymous functional SNP rs1799817 is located in exon17 having amino acid histidine at 1058 site with the conversion of C to T. In PCOS women the frequency of T allele was found to be more (66.27\%) than the C allele (33.73\%). Similar frequency was also observed in controls. The frequencies of CC genotypes (PCOS-11.24\%, control-13.02\%) were much lower in our population. While CC genotype frequency in other population reported (PCOS-43.9\%, control-52.8\%); (PCOS-34\%, control-27.66\%) (Bagheri et al., 2015 ; Mukherjee, Shaikh, Khavale, Shinde, Meherji, Shah, et al., 2009). The TT genotype confers a 1.07 odds risk compared to the CC genotype in PCOS women than the control women. And the heterozygous CT genotype confers 1.31 odds risk compared to the CC genotype in PCOS women than the control women but statistical significance was not achieved. The rs1799817 polymorphism of \textit{INSR} gene showed a negative association towards PCOS in our population. The rs1799817 polymorphism influenced an increase in LH levels (p=0.049) in PCOS women. Lack of association of His1058His was also stated in other studies (Bagheri et al., 2015 ; Tehran, Daneshpour, Hashemi, Zarkesh, & Azizi, 2013; Urbanek et al., 2005). Two GWAS studies in 2011 and 2012 by Chen et al. and Shi et al, reported an association of rs1799817 of \textit{INSR} gene in PCOS women (Z. J. Chen et al., 2011; Shi et al., 2011). Increase in frequency of uncommon T allele in lean PCOS those with body mass index < 27 kg/m2) compared with lean controls were reported (S. Siegel et al., 2002). Yet another study reported genetic variation in exon 17 of \textit{INSR} is associated with insulin resistance and hyperandrogenemia among lean Indian women with polycystic ovary syndrome but not obese women. The difference in phenotypic expression and association of exon17 polymorphism towards PCOS in
other populations could be due to the frequency of distribution of genotypes TT, CT and CC across population where the effect of genotype influences suppression or increased expression of phenotypic change.

The Gly972Arg (rs1801278) polymorphism of IRS1 gene towards susceptibility of PCOS reveal that the heterozygous Gly/Arg genotype may increase the risk of developing PCOS by 2.30 times (p=0.002) compared to the wild homozygous GG genotype and having Arg/Arg genotype which is the mutant in our population shows a 2.88 increased odds of developing PCOS compared to the wild homozygous GG genotype. These findings remained significant even after Bonferroni correction for multiple comparisons (p=0.0002). The dominant model (Gly/Arg+Arg/Arg) confers a 2.42 times risk compared to the wild type GG genotype, Bonferroni corrected value (p=0.0001). The rs1801278 of INS gene is a functional polymorphism which replaces glycine (Gly) with an arginine (Arg). Having the A allele (which produces amino acid arginine) of rs1801278 of IRS1 gene confers a 1.94 odds risk compared to having G allele (which produces amino acid glycine) in PCOS women than the control women, Bonferroni corrected value (p=0.0001). The conversion of amino acid glycine to arginine presented insulin resistant in PCOS women with raised insulin levels, elevated fasting glucose and post-prandial glucose levels. In the presence of peripheral insulin resistance, pancreatic β-cell insulin secretion increases in a compensatory fashion, leading to a hyperinsulinaemic state. We also found that the AA genotype of rs1801278 reflected a lower insulin sensitivity in PCOS women compared to the TT and TA genotypes. rs1801278 is an important functional SNP involved in the insulin signaling by activating phosphatidylinositol-3-kinase (PI3K) a vital step in the commencement of
several effects of insulin such as glucose transport and reported to be associated with insulin resistance, type2 DM and PCOS (Dilek et al., 2005; Sir-Petermann et al., 2001). A study from the Japanese population reported significantly more IRS-1 972Arg carriers among PCOS women compared to healthy controls (10.6% vs. 4.8%, p=0.029), and had a significantly increased risk of developing PCOS (odds ratio: 3.31, 95% confidence interval: 1.49–7.35) (T. Baba et al., 2007). Also, women carrying IRS-1 Gly/Arg had an increased risk of PCOS (OR = 2.49, 95% C.I. 1.16-5.37, p = 0.019)(Lin, Huang, & Wu, 2014). Two recent meta-analysis on PCOS women reported that Gly/Arg and Gly/Gly genotype is significantly associated with risk of developing PCOS (odds ratio 3.31; 1.49-7.35) which is chiefly mediated by higher levels of fasting insulin (Ruan et al., 2012). The IRS-1 variant allele occurs significantly more frequently among PCOS patients than among healthy women (Sir-Petermann et al., 2001).

The SNP rs1801276 of IRS1 gene is also an important functional SNP located on exon1 at codon 513 which replaces Alanine with a proline. This polymorphism was found to be monomorphic in both PCOS and control women in our studied population.

The variant rs1805097 of IRS2 gene is a functional SNP (Gly1057Asp) located on chromosome 13q34 and has 1354 amino acids. When IRS1 is dysfunctional, IRS2 serves as the main docking protein for the intracellular transmission of the insulin signal (Ogihara et al., 1997). The SNP rs1805097 of IRS2, we report that A (which codes for aspartic acid, Asp) is the minor allele in our studied population and the Caucasians and the African Americans also presented

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Asp as the minor allele (Ogihara et al., 1997; J. L. San Millan et al., 2004). Nevertheless Lin et al. reported Gly as the minor allele and association of Asp/Asp genotype with higher fasting insulin and HOMA index (Lin et al., 2014). The variant rs1805097 (Gly1057Asp) of IRS2 gene was not associated with PCOS in the studied population but this polymorphism may possibly have an effect in weight gain in PCOS women in our population. The non-association was confirmed in Spanish and Greek population as well. IRS1 and IRS2 polymorphisms influence glucose homeostasis and could influence obesity, regardless of women presented with PCOS (A. E. Taylor et al., 1997). Levels of both IRS-1 and IRS-2 are increased in theca cells from women with PCOS which leads to proliferation and increased androgen synthesis (Yen, Jakimiuk, Munir, & Magoffin, 2004).

Peroxisome proliferator receptor gamma (PPAR-G) is a nuclear receptor and plays a critical role in carbohydrate, lipid metabolism and adipocyte differentiation by regulating multiple genes (Desvergne & Wahli, 1999; Gonzalez Sanchez, Serrano Rios, Fernandez Perez, Laakso, & Martinez Larrad, 2002). PPAR-G gene comprises 9 exons and the SNP Pro12Ala (rs1801282) is in exon2 and is a missense mutation from CCA to GCA. The Ala variant of rs1801282 of PPAR-G gene was found to be higher in PCOS and controls. We observed a significantly 0.77-fold lower risk of GG genotype in the polymorphism rs1801282 in women with PCOS vs the control group and a 0.65- fold lower risk of CG genotype in women with PCOS vs the control group. But the polymorphism of rs1801282 was not associated with PCOS in our studied population. Nevertheless, the Ala/Ala variant tend to decrease waist/hip ratio, lower LH levels, LH/FSH ratio, reduced insulin resistance in women with PCOS with decrease in insulin levels, fasting glucose levels, right OV and AFC in
PCOS women compared to Gly/Gly and Gly/Arg genotypes in our population. Also improved insulin sensitivity was observed with GG genotype of SNP rs1801282 in PCOS women compared to CC or CG genotypes. Earlier studies have also confirmed the PPARG Pro12Ala variant allele association with higher insulin sensitivity, high-density lipoprotein (HDL) levels (Deeb et al., 1998), decreased risk of T2DM (K. Hara et al., 2000) and decreased incidence of CVD (Doney, Fischer, Leese, Morris, & Palmer, 2004). A meta-analysis reported lower insulin levels in Ala carriers considering PCOS patients and controls as an entire group (San-Millan & Escobar-Morreale, 2010). Two additional meta-analyses determined that the PPARG Pro12Ala variant may result in lower insulin levels, but exerts no effect on HOMA-IR in PCOS patients (J. He, Wang, Liu, Liu, & Li, 2012; H. Zhang, Bi, Hu, Lu, & Zhu, 2012). One published in India found an association towards PCOS at the allelic level (Dasgupta & Reddy, 2013) but another study from Mumbai, India showed significant difference in both allelic and genotypic frequency between PCOS and controls with reduced susceptibility to PCOS (Shaikh, Mukherjee, Shah, Meherji, & Mukherjee, 2013). Therefore the Ala variant of PPAR-G is thought to be associated with reduced transcriptional activity which improves insulin action towards suppression of lipolysis in turn reducing the production of free fatty acids and enables their storage in adipocytes (A. E. Taylor et al., 1997). The homozygous GG of rs180282 signifies no risk and confers protection towards PCOS over the other two genotypes.

The variant rs3856806 located in exon 6 of PPAR-G is a functional synonymous SNP. We observed a 1.90-fold higher risk of TT genotype in the polymorphism rs3856806 compared to the CC genotype in women with PCOS vs
the control group. For rs3856806 of PPAR-G (His447His), neither the genotypes nor the alleles show any association towards PCOS. The genotypes showed a negative association towards the phenotypic trait stating the genotypes display no influence on the phenotype. The SNP rs3856806 is not a susceptible marker in the diagnosis of PCOS. Also to see the effect of genotypes of SNPs together MDR analysis was carried out. The best MDR models for the studied SNP rs1801282 and rs3856806 markers had a testing accuracy (TA) of 0.541 and cross-validation consistency (CVC) of 10/10. However, this model was not significantly associated with PCOS (p=0.755). Each cell is labelled as high risk if the ratio of the affected individual to the unaffected individual exceeds a threshold 1 and low risk if the threshold is not exceeding. The higher risk group contains combinations of (corresponding to SNP rs3856806 and rs1801282) CC-CC, CC-CG, CT-CG, CT-GG, TT-CG. The pairwise linkage disequilibrium (LD) values (D’=0.289 and r2=0.22) between rs1801282 and rs3856806 also revealed that these two SNPs are not in strong LD.

The CAPN10 gene, encoded by a ubiquitous member of the calpain-like cysteine protease family, plays a vital role in insulin secretion and action (Sreenan et al., 2001). This gene was positionally cloned within the non-insulin dependent diabetes mellitus 1 (NIDDM1) region (Horikawa et al., 2000). The presence of calpain-10 mRNA in pancreatic islets, muscle, and liver, the three most important tissues that control blood glucose levels, suggests that calpain-10 may regulate pathways that affect insulin secretion, insulin action, and hepatic glucose production, each of which is altered in patients with type 2 diabetes (Sreenan et al., 2001).
The minor allele frequency of rs7607759 of CAPN10 gene was 17.75% while Whites, Blacks, Hispanics, Asian/Pacific Islanders reported 15.5%, 6.73%, 11.5% and 9.06% respectively (X. Wu et al., 2000). Even though the frequency is high in Indian population we found a negative association of rs7607759 genotype and allele frequency of CAPN10 towards PCOS. But the GG genotype showed 1.37 odds increased risk compared to the AA genotype in PCOS women than the control women. We say that the SNP rs7607759 is not a susceptible locus linked to PCOS in our study population. When genotypes of PCOS and control were compared with the phenotypes we found that the β-cell function was higher in GG genotype compared to AA and AG genotypes in PCOS women accompanied by increased insulin levels, glucose levels decreased insulin sensitivity but it did not reach significance.

The variant rs2975766 also a functional SNP of CAPN10 gene marks out in exon 13, expresses Isoleucine instead of Valine. The genotypes of SNP rs2975766 did not show any association towards PCOS. The mutant homozygous AA genotype was observed in 2 women with PCOS and 1 woman in the control group. The genotype AA of rs2975766 shows an increased risk 2.12 (0.19-23.65); p- 0.531 when compared with the GG genotype but was not significant enough to show an association. The heterozygous genotype GA showed a 1.83 fold increased risk when compared with the GG genotype. The A allele of SNP rs2975766 showed a 1.82 odds increased risk in PCOS compared to having G allele. When the genotypes of rs2975766, compared to the biochemical and other hormonal features of PCOS it was revealed that increased levels of LH LH/FSH ratio, insulin resistant, decreased β-cell function and increased fasting glucose levels was observed in women with AA genotype compared to GG and GA genotypes. The mutant AA genotype may
possibly indicate changes at the phenotypic level in PCOS women. Studies from meta-analysis reported no association with the above studied variants of \textit{CAPN10} gene towards PCOS (Shen, Li, Hu, Liu, & Song, 2013). The SNP’s rs760759 and rs2975766 of \textit{CAPN10} gene are the first of its kind to be studied in the Indian population. The pairwise linkage disequilibrium (LD) values $D^\prime=0.211$ and $r^2=0.87$, between rs7607759 and rs2975766 of \textit{CAPN10} gene revealed no significant LD between SNPs.

Multi dimensionality reduction analysis was performed to evaluate all possible combinations of the studied SNPs. Overall the best MDR model included \textit{INSR} rs1799817, \textit{IRS1} rs1801278 and \textit{PPAR-G} rs3856806 SNPs. A strong interaction effect was found among SNP rs3856806 of \textit{PPAR-G} gene and rs1799817 of \textit{INSR} gene, which had information gain values of 1.22%. A midway point between synergy and redundancy interaction was observed between \textit{IRS1} rs1801278 –\textit{PPAR-G} rs3856806 and \textit{IRS1} rs1801278- \textit{INSR} rs1799817 SNPs with an information gain (IG) of (0.26% and -0.55%) respectively.