2.0. REVIEW OF LITERATURE

The majority of the evidence supports the finding that most women with PCOS have both insulin resistance and compensatory hyperinsulinemia. Insulin resistance in PCOS predisposes the individual to type 2 diabetes. The heritability of beta-cell dysfunction observed in families of women with PCOS demonstrated beta-cell dysfunction, a significant factor that predisposes to type 2 diabetes (Colilla, Cox, & Ehrmann, 2001). Therefore, several candidate genes involving signaling pathways (insulin secretion and action) are examined for PCOS.

2.1. Insulin gene (INS)

Insulin is composed of 2 dissimilar polypeptide chains, A and B, which are linked by 2 disulfide bonds. The gene coding for insulin is localized to 11p15.5 (Harper, Ullrich, & Saunders, 1981) and is located between the genes for tyrosine hydroxylase and the insulin-like growth factor-II (IGF-II) (Junien & van Heyningen, 1990) [Figure 2.1]. The human insulin gene contains three exons: exon 2 encodes the signal peptide, the B chain, and a fraction of the C peptide, while exon 3 encodes the balance of the C peptide and the A chain (Steiner & Oyer, 1967). Insulin hormone is not synthesized as an active protein; insulin mRNA is initially translated into a single chain precursor called preproinsulin. The preproinsulin is 110 amino acids long and made up of a signal peptide, the A, B and C chains. The preproinsulin enters the endoplasmic reticulum and loses its signal peptide and converts into proinsulin which is 86 amino acids long. Later, the proinsulin is exposed to several specific endopeptidases and further loses the C chain; it is thus left with only the A and B chains, which is considered as insulin hormone. The transcription factor Pur1
initiates transcription after binding to the promoter element that is located 596 bp upstream of the insulin gene translation initiation site. This promoter element is known to have a variable number of tandem repeat (VNTR) regions with varying repeats: 26–63 repeats (Class I); 80 repeats (Class II); and 140-200 repeats (Class III) (Bennett et al., 1995). Class I and class III alleles are common in Caucasians. While the class II alleles are very rare in Caucasians, they are common in Africans (Stead & Jeffreys, 2002) as the HphI T/A SNP at the locus −23 (rs689) polymorphism of the insulin promoter region which is in strong linkage disequilibrium and acts as a surrogate marker to INS-VNTR (Lucassen et al., 1993). Hence, the Class I and III alleles of INS-VNTR were determined by −23 HphI A and T alleles, respectively. Class III alleles are associated with reduced expression of INS and IGF2 in the pancreas and placenta (Paquette, Giannoukakis, Polychronakos, Vafiadis, & Deal, 1998).

**Figure 2.1. Location of INS gene in Chromosome 11**

Chromosome 11 showing genomic region of INS gene (Figure from http://ghr.nlm.nih.gov/gene)

Cytogenetic Location: 11p15.5, short (p) arm of chromosome 11 at position 15.5

Molecular Location: base pairs 2,159,779 to 2,161,209 on chromosome 11
The first evidence for linkage and an association between VNTR and PCOS subjects revealed that the class III alleles were only associated with women who were anovulatory and hyperinsulinaemic (Waterworth et al., 1997). Another study on 74 UK women with PCOS reported an association between the class III allele and lower insulin sensitivity (K. Michelmore et al., 2001). In Slovene PCOS subjects, class III INS VNTR alleles were found to be more frequent and their interaction with body mass index was a significant predictor of serum insulin level (Ferk, Perme, & Gersak, 2008). On the other hand, no association between INS VNTR polymorphism and PCOS was reported in Czech (Vankova, Vrbikova, Hill, Cinek, & Bendlova, 2002) or Spanish women (Calvo, Telleria, Sancho, San Millan, & Escobar-Morreale, 2002). Subsequently, in a large-scale study using 255 nuclear families and 3,000 subjects from Irish and Finnish populations, INS-VNTR was not found to be a key factor in the pathogenesis and progress of PCOS (Powell, Haddad, Bennett, Gharani, Sovio, & Groves, 2005). A comparative study of INS VNTR between PCOS and tubal infertility groups found that INS VNTR genotypes are not associated with PCOS. However, they could have a certain influence on the phenotypic spectrum of the syndrome (Haller et al., 2007). No association between PCOS and INS-VNTR polymorphism was observed in either the Han Chinese (Y. Xu et al., 2009a) or Korean populations (Yun et al., 2012).

2. 2. Insulin receptor gene (INSR)

The insulin receptor is a heterotetrameric glycoprotein with two alpha and beta units. Its gene is located at chromosome 19p13.2, spanning 120Kb with 22 exons (Seino, Seino, Nishi, & Bell, 1989) [Figure 2.2]. The tyrosine kinase domain
of the receptor, which is necessary for insulin signal transduction, is encoded by exon 17–21. Alpha and beta subunits of the insulin receptor were derived by the proteolytic processing of a common 1,382 amino acid prepro-receptor (Ebina et al., 1985). Two compound heterozygote mutations which behave in a cis-dominant fashion to decrease mRNA transcription levels have been identified in the insulin-receptor gene of a patient with leprechaunism. Within this single allele there is a nonsense mutation at codon 897, while the other alleles map outside the coding sequence of the gene (Wardle, McLaughlin, Sykes, & Hull, 1987). More recently, the direct sequencing of all 22 exons of the INSR gene in three women with PCOS did not reveal any mutations (Sorbara et al., 1994). The screening of 22 hyperinsulinaemic patients for mutations of the insulin receptor gene revealed that these mutations are not involved in causing insulin resistance in UK PCOS subjects (Conway, Avey, & Rumsby, 1994). Furthermore, the screening of 24 severe insulin resistance patients revealed several mutations, but none of their missense or nonsense mutations contributed to the insulin resistance found in UK subjects with PCOS (Talbot et al., 1996). A His1058 C/T SNP at exon 17 of INSR is not associated with decreased insulin resistance in Chinese (Z. J. Chen et al., 2004; G. Villuendas et al., 2003); Korean (Farah-Eways, Reyna, Knochenhauer, Bartolucci, & Azziz, 2004); or Turkish women with PCOS (Unsal et al., 2009). However, this polymorphism did show a significant association with the lean rather than the obese US (S. Siegel et al., 2002) and Indian PCOS women (Glazener, Kelly, & Hull, 1987). A novel SNP in intron 21 (176477 C > T) of INSR showed strong association with the pathogenesis of PCOS in the Korean population (Sorbara et al., 1994).
A meta-analysis of eight studies comprising 795 cases and 576 controls found no significant evidence for an association between PCOS and \textit{INSR} His1058 C/T polymorphism (C. Moran, Reyna, Boots, & Azziz, 2004). By contrast, linkage analysis using STRs encompassing the INSR region of chromosome 19 did find evidence for an association between the D19S884 loci on \textit{INSR} (Kahsar-Miller, Boots, Bartolucci, & Azziz, 2004; Tan et al., 2006; Tucci et al., 2001). Furthermore, the DNA sequence surrounding D19S884 conferred in vitro promotes activity in lympho blastoid cell lines (Azziz et al., 2004). A recent study using pathway based tagging SNP identifies new \textit{INSR} SNPs associated with PCOS; moreover, a large replication cohort confirmed association of PCOS with rs2252673 (S. I. Taylor et al., 1990).
A family-based association study using 260 trios of Han Chinese origin did not reveal significant evidence of association or linkage of the \textit{INSR} gene to PCOS (Hull, 1987). According to a Chinese study, a novel T/C SNP at codon Cys1008 of the \textit{INSR} gene is associated with decreased insulin sensitivity in Chinese PCOS women. The study found that the association is not caused by the change of synthesis or secretion of the \textit{INSR} beta-subunit, but most probably by the effects of this novel SNP on the function of the \textit{INSR} beta subunit (Jin et al., 2006). A novel SNP in the \textit{INSR} gene, +176477 C > T, was associated with the pathogenesis of PCOS in a Korean population (E. J. Lee et al., 2008). The study found a significant association of C/T polymorphism at His1058 of \textit{INSR} with PCOS in lean rather than obese Indian women (Mukherjee, Shaikh, Khavale, Shinde, Meherji, & Shah, 2009).

\textbf{2. 3. Insulin receptor substrates (\textit{IRS})}

Tyrosine phosphorylation is the result of insulin binding to its receptor; this in turn leads to the phosphorylation of several protein and insulin receptor substrates (\textit{IRS}), including primarily \textit{IRS-1} and \textit{IRS-2} for initiating and coordinating multiple downstream pathways (Sun et al., 1991; Sun et al., 1995). A series of gene “knockout” experiments demonstrated the critical role of both \textit{IRS1} and \textit{IRS2} where both aid in activating multiple signaling pathways for the regulation of glucose homeostasis by insulin (Araki et al., 1993; Tamemoto et al., 1994). The human \textit{IRS1} gene contains the entire 5’ untranslated region and the protein coding region in a single exon and is localized on chromosome 2q36-37 by in situ hybridization (Araki et al., 1993) [\textbf{Figure 2.3}]. The \textit{IRS2} gene is mapped on chromosome 13q34 (Kalidas et al., 1998) [\textbf{Figure 2.4}].
The open reading frames of *IRS1* and *IRS2* predict a molecular weight of 131 and 136 kD. Arg972Gly, a common variant of *IRS1*, lies between two potential sites of tyrosine phosphorylation involved in binding the p85 subunit of the PI-3 kinase. Although the G972R variant is not associated with abnormal expression of the *IRS-1* protein (K. Takeuchi et al., 1997) it does impair signaling (Almind, Inoue, Pedersen, & Kahn, 1996). Asp1057Gly, a common *IRS2* variant, has not been associated with changes in insulin sensitivity in lean or obese adults (Almind et al., 1999). Although the initial study did not reveal any association of PCOS with the *IRS1* gene (Urbanek et al., 1999), many subsequent studies have been concentrated on Arg972Gly and Asp1057Gly polymorphisms in PCOS; this continuing interest is due to the complementary role of *IRS1* and *IRS2* in insulin signaling. The higher frequency of the *IRS1* variant was observed in adolescent girls with hyperandrogenism (Witchel, Smith, Tomboc, & Aston, 2001), but the G972R variant acted as a modifier locus among women who are heterozygous carriers of CYP21, which indicates its limited role in the development of PCOS (Witchel, Kahsar-Miller, Aston, White, & Azziz, 2005). Recently, attention has also been focused on insulin receptor substrates and the association with PCOS of SNPs at the *IRS1* and *IRS2* loci. The results, however, are contradictory. A slightly higher frequency of Arg972 was observed in PCOS women of Chilean (Sir-Petermann et al., 2004; Sir-Petermann et al., 2001) and Turkish populations (Dilek, Ertunc, Tok, Erdal, & Aktas, 2005). The *IRS1* Gly972Arg polymorphism is significantly associated with PCOS in the Japanese (T. Baba et al., 2007).
Cytogenetic Location: 2q36.3, long (q) arm of chromosome 2 at position 36.3
Molecular Location: base pairs 226,731,317 to 226,798,790 on chromosome 2

and Greek populations (Christopoulos et al., 2010). The IRS1 Gly972Arg has the highest frequency reported worldwide and is associated with insulin resistance and higher fasting insulin in Southern Italian women (Pappalardo et al., 2010). Furthermore, the IRS1 genotype also influenced the fasting insulin levels and HOMA indices in PCOS women on metformin therapy (Ertunc, Tok, Aktas, Erdal, & Dilek, 2005). No significant association between insulin receptor substrate genes and PCOS was reported in the French (El Mkadem et al., 2001), Spanish (G. Villuendas et al., 2005), German (Haap et al., 2005), Taiwanese (Leng, Karlsson, & Zierath, 2004), Chilean (Valdes et al., 2008), Slovak (Dravecka, Lazurova, & Habalova, 2010), Greek (Marioli et al., 2010), Indian (Dasgupta, Sirisha, Neelaveni, Anuradha, & Reddy, 2012) or Iranian populations (Rashidi, Azizy, Najmeddin, & Azizi, 2012). Very few studies reported an association between IRS2 Gly1057Asp and PCOS. The Gly1057Asp polymorphism influenced blood glucose levels in
non-diabetic Caucasian and African-American women with PCOS (Ehrmann et al., 2002). An analysis of US Caucasian women revealed three additional IRS2 SNPs that are associated with PCOS (rs7997595, rs7987237, rs1865434) (M. O. Goodarzi et al., 2011). A recent genome-wide association study (GWAS) of PCOS in Han Chinese women failed to detect associations between the polymorphism of the IRS gene and PCOS (Z. J. Chen et al., 2011). However, two independent meta-analyses suggest that IRS1 Gly972Arg polymorphism causes significant risk for PCOS, but that IRS2 Gly1057Asp polymorphism has not shown such risk (Ioannidis, Ikonomi, Dimou, Douma, & Bagos, 2010; Ruan, Ma, & Xie, 2012).

2.4. Insulin-like growth factors (IGFs)

The IGFs are peptide hormones secreted from many different cells and exhibiting a high sequence of similarity to insulin. There are two principal IGFs, known as IGF-
1 and IGF-2. Their functions include: mediation of growth hormone action; stimulation of growth of cultured cells; stimulation of the action of insulin; and involvement in development and growth. Each of these has a number of variant forms, a result of the use of alternative gene promoters and alternative splicing. The gene IGF2 is located on chromosome 11p15.5 (O'Dell & Day, 1998) [Figure 2.5]. A single nucleotide polymorphism (SNP) in the 3′ untranslated region of the IGF2 gene (ApaI; rs680) is known to increase IGF2 mRNA in leukocytes due to increased liverIGF2 expression and secretion. Together with IGF1 and IGF binding proteins, IGF2 stimulates adrenal and ovarian androgen secretion. The association between PCOS and G alleles of the ApaI polymorphism (IGF2 3′UTR GA; rs680) was first established in Spanish women (J. L. San Millan et al., 2004). A subsequent study found that the ApaI polymorphism in the IGF2 cluster in combination with the −108 polymorphism (rs705379) in PON1 increased the risk of PCOS in German women (Knebel et al., 2009). A recent study showed a predominance of ApaI GA + AA genotypes in younger Brazilian women with PCOS (Ramos Cirilo et al., 2012).

2.5. Peroxisome proliferator-activated receptor γ (PPARG)

Peroxisome proliferator-activated receptors are members of the nuclear receptor super family of ligand-activated transcription factors (Issemann & Green, 1990). The PPAR-γ2 is formed by an alternative mRNA splicing pathway and regulates the transcription and expression of numerous target genes. These genes have been shown to be involved in adipocyte differentiation, lipid and glucose metabolism, and
Figure 2.5. Location of IGF1 gene in Chromosome 12

Chromosome 12 showing genomic region of IGF1 gene (Figure from http://ghr.nlm.nih.gov/gene)

Cytogenetic Location: 12q23.2, long (q) arm of chromosome 12 at position 23.2
Molecular Location: base pairs 102,395,867 to 102,481,839 on chromosome 12

Atherosclerosis (A. C. Li & Glass, 2004). The gene coding for PPAR-γ has been mapped to chromosome 3q25 (Greene et al., 1995) [Figure 2.6]. The human PPAR-γ gene is composed of 9 exons; it spans more than 100 kb of genomic DNA (Fajas et al., 1997). A common C to G base exchange leads to the substitution of proline with alanine at codon 12, which has been associated with reductions in both DNA binding and transcriptional activity in vitro. Recent studies have indicated that the Ala12 allele is involved in increased insulin sensitivity by enhanced suppression of lipid oxidation, thereby permitting more efficient glucose disposal. Several studies have found similar genotype and allele frequencies of the PPAR-γ Pro12Ala polymorphism in PCOS. Women and healthy controls in Italy (F. Orio, Jr. et al., 2003; Orio Jr et al., 2004), Spain (J. L. San Millan et al., 2004), China (Wang et al., 2006), Turkey (Tok, Aktas, Ertunc, Erdal, & Dilek, 2005), Chile (Guzman, Erices,
Valdes, & Salzar, 2007), Korea (Chae et al., 2010), Greece (Christopoulos et al., 2010; Koika et al., 2009; Xita, Lazaros, Georgiou, & Tsatsoulis, 2009), Los Angeles (Antoine et al., 2007), Germany (Haap et al., 2005; Knebel et al., 2008), Poland (Bidzinska-Speichert et al., 2012) and Slovenia (Dragojevič, Marc, & Mlinar, 2008).

Although PPAR-γ Pro12Ala polymorphism is equally distributed in PCOS women and healthy controls, it showed a modifier effect on insulin resistance in both German (Hahn et al., 2005) and multi-ethnic populations (M. Hara et al., 2002). Contrary to these findings, however, some studies have shown that the Pro12Ala polymorphism is significantly more frequent in control subjects when compared with PCOS women, indicating a protective effect by the Ala allele against the development of PCOS in Finland (Korhonen et al., 2003), Turkey (M. Yilmaz et al., 2006), India (S. Dasgupta et al., 2012), and Korea (Gu & Baek, 2009). PCOS subjects carrying Pro12Ala showed higher leptin levels than the Pro12Pro and Ala12Ala genotypes, indicating that the single Ala12 allele may play a protective role in respect to hyperleptinemia (Bidzinska-Speichert et al., 2012). Although the protective trend of the G allele existed, a recent meta-analysis did not show a significant association between Pro12Ala and PCOS (Tang et al., 2012).

A meta-analysis using 17 case control studies from Europe and Asia supports the finding that the PPAR-γ Pro12Ala polymorphism is capable of reducing the PCOS in European but not in Asian women (H. Zhang, Bi, Hu, Lu, & Zhu, 2012). Yet another meta-analysis using 17 studies reported that the Ala12 variant would
Figure 2.6. Location of PPAR-G gene in Chromosome 3

Chromosome 3 showing genomic region of PPAR-G gene (Figure from http://ghr.nlm.nih.gov/gene)

Cytogenetic Location: 3p25.2, which is the short (p) arm of chromosome 3 at position 25.2
Molecular Location: base pairs 12,287,850 to 12,471,054 on chromosome 3.

decrease the risk of PCOS and result in lower BMI and fast insulin levels in Europeans, but would have no impact on HOMA-IR in PCOS patients (He, J., Wang, Liu, Liu, & Li, 2012).

2.6. Calpain-10 (CAPN10)

Calpains are calcium-dependent intracellular non-lysosomal proteases that are capable of hydrolyzing specific substrates involved in calcium-regulated signaling pathways (Emori, Kawasaki, Imajoh, Kawashima, & Suzuki, 1986). Calpain-10 is an atypical member of the calpain family and is expressed at the mRNA and protein levels by several tissue types including pancreatic β islet cells; liver; skeletal muscle; and adipocytes (Carlsson et al., 2005; Pihlajamaki et al., 2006). The gene encoding calpain-10 (CAPN10) consists of 15 exons and is located on chromosome 2q37.3 [Figure 2.7]. It was shown to be related to proinsulin
processing, insulin secretion and insulin resistance (Baier et al., 2000; Zusterzeel et al., 2001). CAPN10 variants are known to influence cholesterol levels, blood pressure values, and insulin resistance phenotypes in the Spanish population (Saez et al., 2008). Several SNPs in CAPN10 (UCSNP-63, −44, −43, −19) have been the focus of PCOS researches; however, the results are contradictory. CAPN10 UCSNPs associated with PCOS varies in different populations. The CAPN10 UCSNP-44 allele showed significant association in the populations of Spanish (Gonzalez et al., 2002; Gonzalez et al., 2003), Turkish (M. Yilmaz et al., 2009), and Indian women (S. Dasgupta et al., 2012). A significant association between the UCSNP-43 polymorphism and the PCOS metabolic phenotype was found in hirsute southern Brazilian patients (Wiltgen, Furtado, Kohek, & Spritzer, 2007) as well as Chilean PCOS women (Marquez, Pacheco, Valdes, & Salazar, 2008).

**Figure 2.7. Location of CAPN10 gene in Chromosome 2**

*Chromosome 2 showing genomic region of CAPN10 gene (Figure from http://ghr.nlm.nih.gov/gene)*

Cytogenetic Location: 2q37.3, which is the long (q) arm of chromosome 2 at position 37.3

Molecular Location: base pairs 240,586,716 to 240,599,109 on chromosome 2.

The UCSNP-45 C allele is associated with idiopathic hirsutism in Spanish PCOS women (Escobar-Morreale et al., 2002). The UCSNP-56 and ins/del-19 are
found to be in strong linkage disequilibrium and showed significant association with PCOS in German women. The TGG3AGCA and TGA2AGCA haplotypes showed both decreased and increased risk for PCOS (Vollmert et al., 2007). The more common allele of UCSNP-63 showed evidence for excess transmission in a single-locus transmission disequilibrium analysis of European trios. However, this association was not replicated in the case-control study from that region (Haddad et al., 2002). In contrast to these associations, none of the \textit{CAPN10} polymorphisms were associated with PCOS in German Caucasians (Haap et al., 2005) or Turkish adolescent girls (Unsal et al., 2009). Although there is no significant association between individual polymorphisms of \textit{CAPN10} and PCOS, neither the haplotype nor the diplotypes of this gene showed significant associations with PCOS in African-American (Ehrmann et al., 2002), Korean (J. Y. Lee et al., 2009) or Indian populations (S. Dasgupta et al., 2012). A recent meta-analysis using 11 case control studies demonstrated that the \textit{CAPN10} UCSNP-63 homozygous allele and the UCSNP-19 insert allele are protective factors for PCOS (M. Huang, Xiao, Zhao, Liu, & Chen, 2012).

\textbf{2. 7. Need for present study}

Under the pretext of aping the Western lifestyle, the Indian population has undergone a renaissance in such a way that is conducive to the increase in prevalence of PCOS among young girls (Dasgupta & Reddy, 2008). The incidence rates are steadily increasing with more environmental, physiological and genetic parameters being associated with the condition. Poor dietary choices coupled with a sedentary lifestyle leads to obesity that has been observed in women affected with
PCOS. In addition, it has been found that Asian Indians are more susceptible to insulin resistance and cardiovascular risks at a low body mass index (BMI) because of the presence of higher percentage of body fat and abnormal adiposity (Gill et al., 2012). Obesity has been linked to chronic anovulation and hyperinsulinemia, which in turn, has a negative effect on the ovulatory process by causing an increase in the levels of luteinizing Hormone (LH), thereby leading to premature cessation of ovulation. Insulin resistance (IR) is an intrinsic factor that has been observed in 50 – 70 % of the cases (Mukherjee & Maitra, 2010). IR induced hyperinsulinemia drives the production of excess androgen which causes hirsutism observed in many patients. Studies done on Indian population have suggested that abnormalities of the insulin receptor are more common in Indian women with PCOS compared to white women with PCOS (Norman et al. 1995). Hyperinsulinemia has also been found to correlate with a profile of increased cardiovascular risk factors in PCOS, independent of obesity (Mather et al. 2000). But in another study done in India they found no correlation between the fasting glucose / insulin ratio and the triglyceride levels. No correlation is reported between markers of obesity such as BMI and waist/hip ratio with various lipid parameters. But in PCOS women with insulin resistance, the lipid profile was significantly different compared to insulin-sensitive women (Anuradha et al. 2005). There is a paucity of published data on the prevalence of glucose intolerance and its genetic basis in women with PCOS in India. Hence this study was carried out to find genetic associations (with special interest in genes involved in insulin secretion and action and/or its signaling pathways) concerning polycystic ovary syndrome.