Chapter 6

To elucidate mechanism underlying insulin and steroidogenic signalling in insulin resistant and non-insulin resistant human luteinized granulosa cells.

6.1 Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder among women of reproductive age and is frequently associated with infertility in women. Poly cystic ovaries may be observed in anovulatory women, ovulatory women and at times in women with regular menstrual cycles attending *in vitro* fertilization clinics (Almahbobi et al. 1996). The statistics by National Women's Health Information Centre (NWHIC) quotes about 5-10% of women of child bearing age (20-40) to have PCOS. The disorder was first reported by Stein and Leventhal in 1936 in a small population of overweight women. PCOS is a heterogeneous disorder with variable features such as 1. Clinical or biochemical hyperandrogenism, 2. Chronic anovulation 3. PCO after exclusion of disorders of pituitary, ovary or the adrenal that could present in a manner similar to PCOS (Fig. 6.1) (Diamanti-Kandarakis and Dunaif 2012).

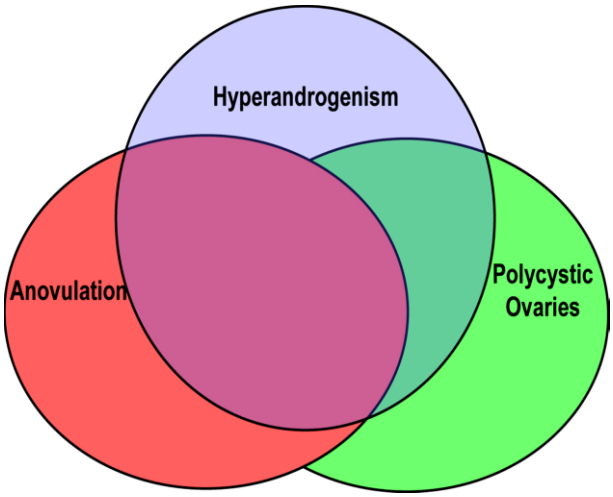


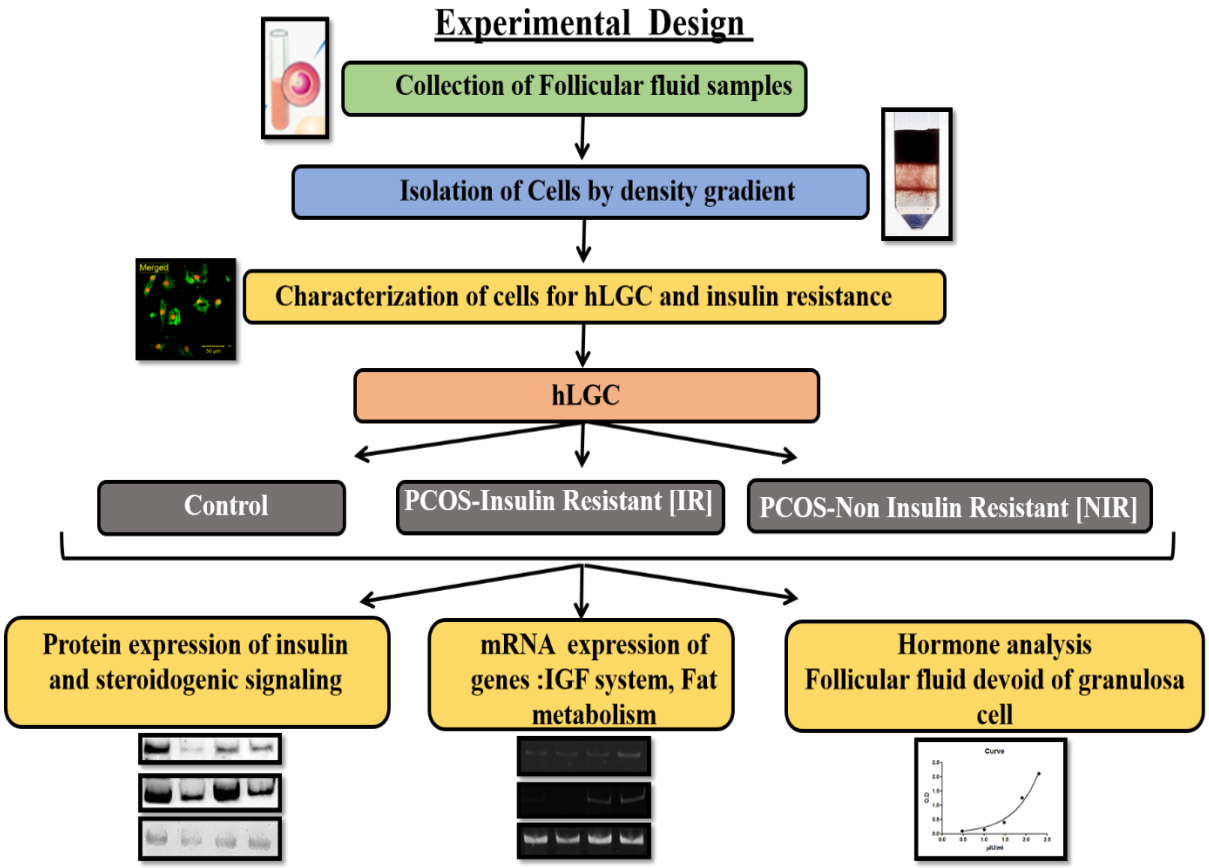
Figure 6. 1: The diagnostic criteria for PCOS includes two or more of these features. (Diamanti-Kandarakis and Dunaif 2012).

Considering these as the diagnostic features, PCOS was given several phenotypes by different groups such as National Institute of Child Health and Development (NICHD), Rotterdam and Androgen Excess Society (AES) which accounted for its increased prevalence from 10 to 20% globally, including India where the numbers have doubled in last 10 years (Azziz et al. 2009; Diamanti-Kandarakis and Dunaif 2012). Apart from being a gynaecological disorder, PCOS is

now accepted to have several metabolic problems amongst which insulin resistance (IR) is one of the most significant metabolic aberration (Ergen et al. 2012).

The prevalence of IR in the general population is 10–25%, whereas in PCOS it is approximately 60–70% (Marshall and Dunaif 2012). Approximately, 20–50% of the women with PCOS are normal weight or are lean, and the pathophysiology of the disorder in these women differs from that in obese women (Verit and Erel 2008). Similarly 70–80% of obese PCOS (BMI >30) and 20–25% lean PCOS (BMI<25) are IR, and the pathophysiology of the disorder in these women may differ from that in non - insulin resistant PCOS (PCOS-NIR) (Marshall and Dunaif 2012). Continued with these facts, the Rotterdam criteria has also considered anovulatory women with normal androgen levels and PCO as a distinct phenotype of PCOS. In this group of women insulin sensitivity was observed to be normal (Azziz et al. 2009). According to the NIH criteria, IR is a common and not universal feature of PCOS and seems to be in the range of lesser common findings in the additional phenotypes of PCOS diagnosed using the Rotterdam criteria (Marshall and Dunaif 2012). Many studies in literature have demonstrated IR in both obese as well as lean PCOS women. On the other hand, several studies have revealed normal insulin sensitivity in PCOS and lean PCOS women using the gold standard "hyperinsulinemic euglycemic clamp" when compared to reproductively normal control women (Marshall and Dunaif 2012). Only one study in the literature has reported anthropometric and endocrine differences between IR and NIR women with PCOS and has further demonstrated hyperinsulinemia to accompany PCOS-IR patients with hyperandrogenemia (Meirow et al. 1996). Taking this into account it is an intriguing problem to unravel signalling mechanisms underlying PCOS –IR and NIR conditions and furthermore to evaluate candidate molecules for characterizing the defect and designing appropriate therapy. Thus human luteinized granulosa cells (hLGC's), harvested from follicular aspirates obtained at egg collection for *in vitro* fertilization (IVF), provide a practical model for studying insulin and steroidogenic action (Richardson et al. 2005). Thus in the present chapter we investigated insulin receptor-β (INSR-β) at first to classify them as IR and NIR and then evaluated expression of proteins involved in insulin and steroidogenic signalling, expression of genes involved in IGF system, along with steroid hormone levels in follicular fluid.

6.2 Experimental Design



6.3 Results

6.3.1 Characterization of IR in granulosa cell and assessment of viability

Granulosa cells from individual PCOS follicular fluid samples were isolated and analysed for expression of INSR-β. The samples having down regulation of INSR-β were segregated as PCOS-IR, and rest having expression of INSR-β similar to that of control as PCOS-NIR. The control, PCOS-IR and PCOS-NIR granulosa cells were analysed for their viability by trypan blue exclusion dye. The viability of luteinized granulosa cells was significantly decreased in PCOS-IR ($P < 0.001$) as well as PCOS-NIR ($P < 0.05$) group, however the decrease was much more significant in PCOS-IR group ($P < 0.01$) as compared to PCOS-NIR (Fig.6.2).

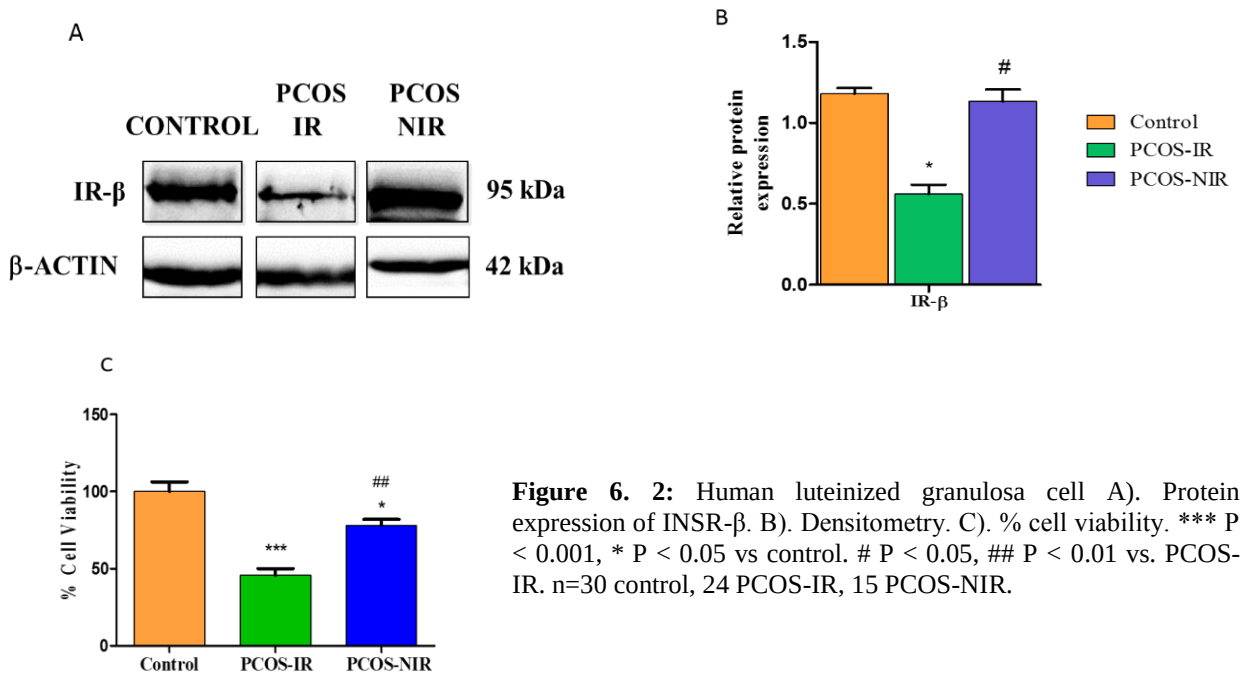


Figure 6. 2: Human luteinized granulosa cell A). Protein expression of INSR-β. B). Densitometry. C). % cell viability. *** P < 0.001, * P < 0.05 vs control. # P < 0.05, ## P < 0.01 vs. PCOS-IR. n=30 control, 24 PCOS-IR, 15 PCOS-NIR.

6.3.2 Insulin signalling is altered in PCOS-IR and PCOS-NIR

To explore the in depth mechanism of insulin signalling in control, PCOS-IR and PCOS-NIR hLGC's, the protein expression of p-IRS (307), PI(3)K, p-Akt, PKC-ζ, ERK1/2,pP38MAPK and PPAR-γ was analyzed by western blot. Along with down regulated protein expression of INSR-β; downstream candidate proteins such as PI(3)K, p-Akt and PKC-ζ also showed significant decrease ($P < 0.05$ and $P < 0.01$) in PCOS-IR group as compared to control and PCOS-NIR group. PCOS-IR and PCOS-NIR showed significant increase ($P < 0.05$) in expression of ERK1/2 and pP38MAPK as compared to control group. The results demonstrated elevated protein expression of p-IRS (307) ($P < 0.05$) in PCOS-IR group as compared to PCOS-NIR and control.

Significant increase was observed in protein expression of PPAR- γ in PCOS-IR ($P < 0.01$) and PCOS-NIR ($P < 0.05$) as compared to control group (Fig: 6.3).

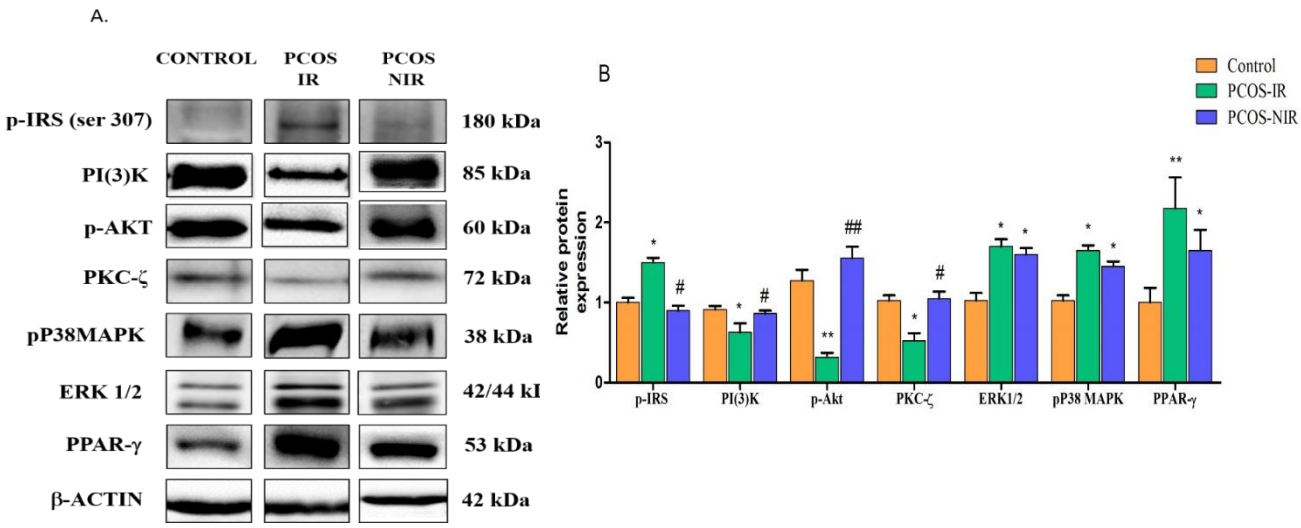


Figure 6. 3: Expression of insulin signalling proteins in control, PCOS-IR and PCOS-NIR. A) Western blot B) Densitometry * P < 0.05, ** P < 0.01 vs. control. $^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01 vs. PCOS-IR. n=30 control, 24 PCOS-IR, 15 PCOS-NIR (20 µg protein).

6.3.3 Fat metabolism genes are altered in PCOS-IR

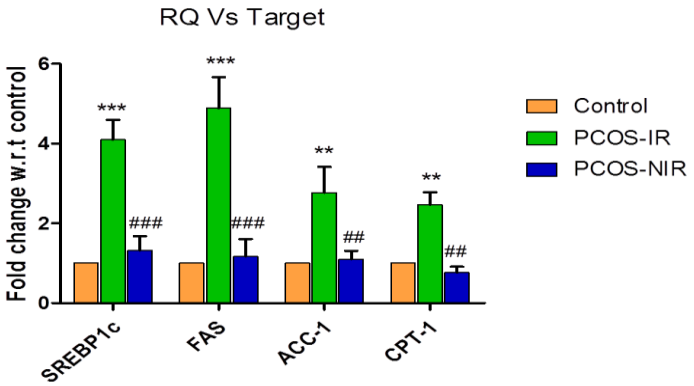


Figure 6. 4: Expression of fatty acid metabolism genes in control, PCOS-IR and PCOS-NIR. *** P < 0.001 and ** P < 0.01 vs. control, $^{\#\#\#}$ P < 0.001 and $^{\#\#}$ <0.01 vs. PCOS-IR. n=30 control, 24 PCOS-IR, 15 PCOS-NIR.

Fat acid synthesis, sterol synthesis, fatty acid oxidation and IR are tightly interconnected. As mentioned earlier PCOS is associated with several metabolic disturbances, we dissected whether any difference in fat metabolism exists in PCOS IR group as compared to PCOS NIR. Thus the expression of fatty acid metabolism genes namely SREBP1c, FAS, ACC-1 and CPT-1 were analysed by real time PCR. The results demonstrated that there was a significant increase in mRNA expression in SREBP1c, FAS, ACC-1 and CPT-1 in PCOS-IR as compared to control (*** $P < 0.001$, ** $P < 0.01$) and PCOS-NIR ($^{\#\#}P < 0.01$) group. The results clarified that fatty

acid synthesis and oxidation was in PCOS-IR condition as no change in these candidate genes were observed in NIR group as compared to control (Fig: 6.4).

6.3.4. Expression of steroidogenic genes and proteins is altered in PCOS-IR and NIR

Altered steroid hormone levels are the major culprit for the development of PCOS. Thus to elucidate further, expression of steroidogenic genes and proteins expression mainly of StAR, CYP11A1, 3β-HSD, CYP19A1 and 17β-HSD were analysed by real time PCR and western blot respectively. Significant decrease was observed in mRNA of StAR ($P < 0.05$) and protein of StAR ($P < 0.01$ and $P < 0.05$) in PCOS-IR as well as PCOS-NIR as compared to control. However CYP11A1 mRNA and protein demonstrated significant increase ($P < 0.05$, $P < 0.01$) and ($P < 0.001$, $P < 0.01$) respectively in PCOS-IR compared to PCOS-NIR and control. There was a down regulation in 17β-HSD and 3β-HSD mRNA ($P < 0.05$) as compared to control in both the groups along with decrease in protein expression ($P < 0.001$). Significant decrease ($P < 0.01$) was also observed in mRNA and protein expression of CYP19A1 in PCOS-IR and PCOS-NIR groups as compared to control (Fig: 6.5).

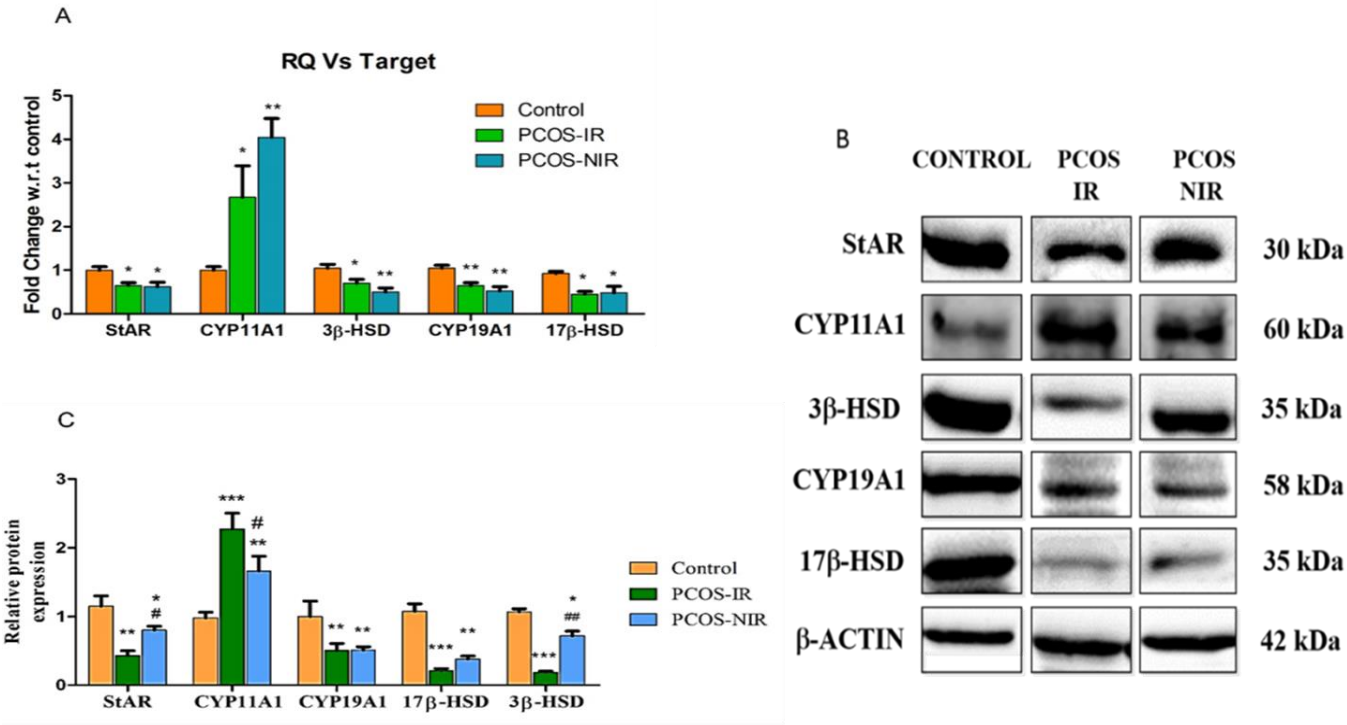


Figure 6. 5: Expression of genes and proteins involved in steroidogenesis in control, PCOS-IR, PCOS-NIR. A. mRNA expression B. Western blot study using β- actin as endogenous control C. Densitometry analysis. *** P < 0.001, ** P < 0.01, * P < 0.05 vs. control, ### P < 0.001, ## P <0.01, # P < 0.05 vs. PCOS-IR. n=30 control, 24 PCOS-IR, 15 PCOS-NIR.

6.3.5 Steroid hormones are altered in follicular fluid from PCOS-IR and PCOS-NIR

Alterations in the level of steroid hormones were determined and it was observed that estradiol did not reveal any significant difference between control, PCOS-IR and PCOS-NIR groups. However a considerable decrease ($P < 0.01$) was observed in progesterone concentration in PCOS-IR group as compared to control and PCOS-NIR group, with a significant increase ($P < 0.01$ and $P < 0.05$) in the levels of testosterone in PCOS-IR group as compared to control and PCOS-NIR respectively (Fig:6.6).

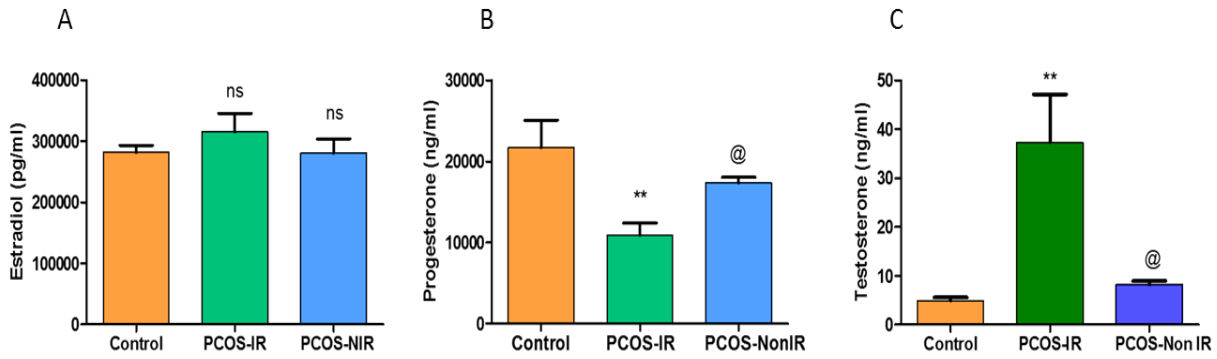


Figure 6. 6: Steroid hormone concentration in follicular fluid aspirates. A. estradiol, B. progesterone and C. testosterone. ** P < 0.01 vs. control, @ P < 0.05 vs. PCOS-IR. n=30 control, 24 PCOS-IR, 15 PCOS-NIR

6.3.6 mRNA expression of FSH-R and LH-R are altered in PCOS-IR and NIR

Gonadotropin receptors also play an important role in steroidogenesis. After observing significant decrease in steroidogenic proteins, gene expression of FSH-R and LH-R was analysed by quantitative real time PCR. The analysis revealed significant increase ($P < 0.01$ and $P < 0.05$) in FSH-R and ($P < 0.001$ and $P < 0.05$) LH-R in PCOS-IR and PCOS-NIR respectively as compared to control. (Fig: 6.7).

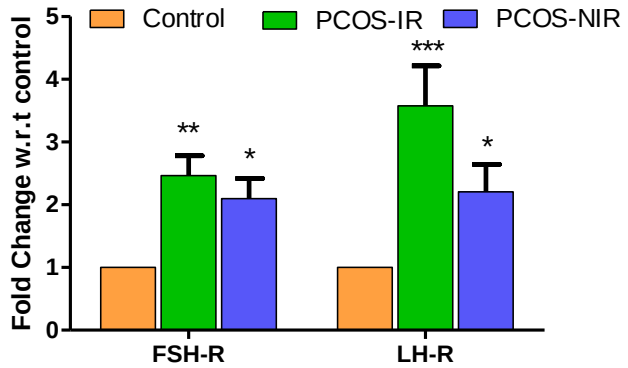


Figure 6. 7: Expression of FSH-R and LH-R genes in PCOS-IR and PCOS-NIR. *P < 0.05, ***P < 0.001 vs. control. n=30 control, 24 PCOS-IR, 15 PCOS-NIR.

6.3.7 Insulin like growth system (IGF) is altered in PCOS-IR and NIR

IGF signalling is known to be altered during PCOS condition however its status in PCOS with and without IR is not clear. Hence IGF-1, IGF-2 and their receptors IGF-1R and IGF-2R were analysed in hGLC's of PCOS-IR and PCOS-NIR by qRT-PCR. There was a remarkable down regulation of IGF-1 ($P < 0.05$) in PCOS-IR whereas IGF-2 demonstrated significant increase ($P < 0.05$) in PCOS-IR group. However when analysed for the gene expression of its cognate receptors there was a marked up regulation in PCOS IR group as compared to that of PCOS NIR and control group (Fig: 6.8).

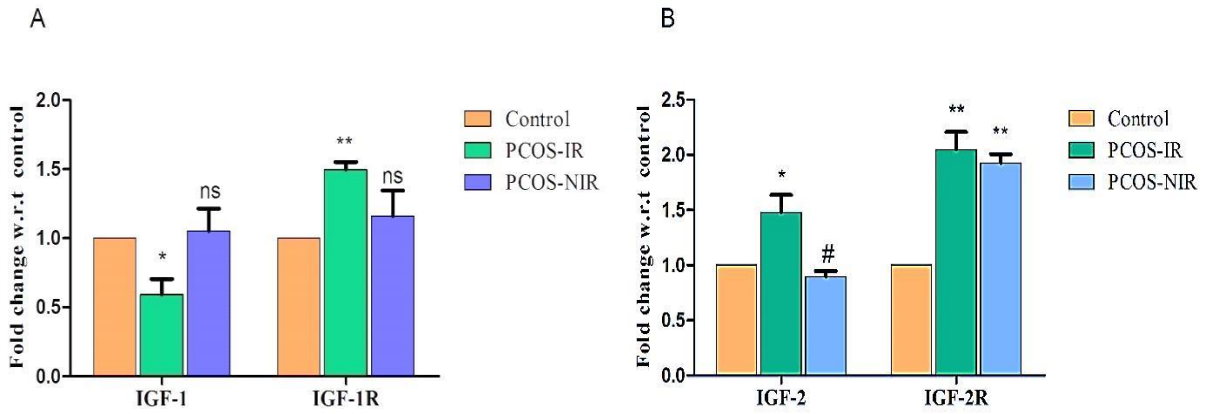


Figure 6. 8: Expression of IGF's and their receptors in PCOS-IR and PCOS-NIR. A. IGF-1 and IGF-1R and B. IGF-2 and IGF-2R *P < 0.05, **P < 0.01 vs. control. ns= non significant. n=30 control, 24 PCOS-IR, 15 PCOS-NIR.

6.4 Discussion

Over production of androgens and insulin resistance have synergetic effect in many PCOS women contributing to the alteration of functions in several tissues, including granulosa cells. This leads to change in the expression of proteins related to tissue homeostasis, intracellular steroid bioavailability. It has also been described that hyperinsulinemia in PCOS could possibly be due to defects in the expression and/or activity of proteins downstream from the insulin receptor. Thus, understanding granulosa cell death and protein expression in insulin and steroidogenic signalling in PCOS-IR and PCOS-NIR patients could help expand knowledge about reproductive failure observed in the majority of these cases. Granulosa cells are the major somatic cells that are involved in steroidogenesis, apoptosis and provide nutrition for the development of oocyte. Several studies have reported decrease in granulosa cell viability in PCOS (Niu et al. 2014). Drastic decrease in granulosa cell viability in PCOS-IR group as

compared to PCOS-NIR group in the present study could be co related to down regulated INSR-β leading to decreased survival and increased apoptosis in PCOS-IR.

Presence of hyperinsulinemia in PCOS-IR but not in PCOS-NIR has been reported earlier which could possibly be due to some defect in proteins downstream from the INSR-β (Dunaif 1997; Schinner et al. 2005). Thus, in the present study we opted to understand the protein expression of insulin signalling cascade in PCOS-IR and PCOS-NIR patients. Insulin mediates its actions via three major pathways: the PI-3K pathway, implicated in the metabolic effects of insulin; the MAPK pathway, responsible for the mitogenic effects of insulin; and the PKC pathway (Baillargeon and Nestler 2006). To better understand insulin activity in these cells, in the present investigation at first on the basis of down regulation of INSR- β in hLGC's, PCOS were segregated as PCOS-IR and PCOS-NIR following which proteins that participate in the downstream insulin signalling pathway, specifically p-IRS (307), PI(3)K, p-Akt, PKCζ, pP38 MAPK, ERK1/2 and PPARγ were evaluated between control, PCOS-IR and PCOS-NIR hLGC's. Activation of these signaling proteins leads to translocation of GLUT-4 from cytoplasm to membrane thus helping in glucose uptake by the cell during folliculogenesis. Decreased expression of IR-β, PI(3)K, p-Akt and PKCζ along with increase in p-IRS (307) in PCOS-IR group suggested a lowered GLUT4 vesicle translocation to the cell periphery, eventually leading to a deficient glucose entering into the cell as compared to control groups. Further in PCOS-NIR cells where, expression of IR-β, PI(3)K, p-Akt, PKCζ and p-IRS (307) were unchanged as compared to control group confirmed the findings of the literature that their activation was not mandatory for ovarian steroidogenesis thus explaining a post insulin binding divergence of IR-β signalling and possibility of other pathways playing role in steroidogenesis (Poretsky et al. 2001).

In the present study increase in expression of pP38 MAPK and P44/42 MAPK was observed in both PCOS-IR and PCOS-NIR groups as compared to control suggesting roles of other stimuli. Osmotic shock, inflammatory cytokines, heat shock, oxidative stress etc overexpress pP38 MAPK and ERK pathways upregulating thioredoxin-interacting protein (TXNIP) thus contributing to an increase in reactive oxygen species in PCOS-IR and PCOS-NIR which can be responsible for decreased viability (Evans 2007; Lin et al. 2009; Ito et al. 2010; Seto-Young et al. 2011; Kaur et al. 2012). Studies have revealed constitutive activation or involvement of various other hormones in the MAPK-ERK pathway for contributing to resistance to insulin's

metabolic actions in PCOS-IR by increasing ser/thr phosphorylation (Corbould et al. 2006; Lin et al. 2009; Tee and Miller 2013). Other than these activation of p38MAPK functions as the mediator for GnRH-stimulated *Lhb* promoter activity, thus explaining the mechanism by which granulosa cells would be undergoing premature luteinisation in PCOS (Sharma et al. 2011).

Granulosa cells play a pivotal role in uptake of cholesterol, fatty acids and other lipids, many of which act as substrates for developing oocyte and steroid synthesis after luteinization. Insulin performs these important aspects through the transcription factors such as PPAR-γ, sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), carnitine palmitoyltransferase-1 (CPT-1) and acetyl coA carboxylase (ACC-1) whose expression in human granulosa cells support the existence of lipogenic and lipolytic activity in them. (Shimomura et al. 1997; Christenson et al. 2001; Richardson et al. 2005). In the present study we observed up regulation of protein expression of PPAR-γ accompanied by up regulation in SREBP1c, FAS, CPT-1 and ACC genes in PCOS-IR group as compared to control and PCOS-NIR. The increase in SREBP1c is supported by the fact that in liver despite of insulin resistance, high circulating insulin continues to stimulate SREBP-1c in liver, the same paradigm might be responsible for IR granulosa cells as well leading to over production of fatty acids. Also due to the shift from normal situation to IR, the isoform SREBP1c precedes SREBP1a in human granulosa cells ultimately hindering the up regulation of StAR promoter activity and hence limiting the transfer of cholesterol for steroidogenesis (Christenson et al. 2001). Further, our observations for increased expression of FAS, CPT-1and ACC imply up regulation of fatty acid metabolism. Taken together our results indicated a major shift in hormonal balance that favours fatty acid synthesis in granulosa cells where rather greater need appears for cholesterol production as a substrate for steroidogenesis.

StAR protein plays first and significant step in steroidogenesis by transferring cholesterol from outer to the inner mitochondrial membrane (Park et al. 2015). Decrease in gene and protein expression of StAR was in line with reported literature indicating reduced gonadal steroidogenesis (Jakimiuk et al. 2001a; Petrescu et al. 2001). CYP11A1 is the key enzyme that intitiates the rate limiting step in steroidogenesis by conversion of cholesterol to pregnenolone and 3 β-HSD in the biosynthesis of progesterone. In PCOS human luteinized granulosa cells the expression of CYP11A1 is up regulated whereas that of 3 β-HSD is down regulated which is in accordance with earlier study (Doldi et al. 2000; Jakimiuk et al. 2001b). Moreover reports have

also unravelled that overexpression of CYP11A1 leads to defects in luteal phase development further hampering mitochondrial production and decreasing progesterone synthesis as observed in the present study (Chien et al. 2013). The synthesis of estradiol is dependent on CYP19A1 and 17β-HSD (Miro et al. 1995). The granulosa cells from follicular fluid samples of classic PCOS i.e irrespective of insulin resistance, are hyper responsiveness to LH rose their proliferating capacity by undergoing luteinisation and restricting the growth of follicle to a diameter of ~ 4-7mm leading to absence or very low expression of CYP19A1 and 17β HSD (Jonard and Dewailly 2004). Other than this presence of several proteins in follicular fluid such as high molecular weight FSH receptor binding inhibitor, inhibin-a subunit precursor, insulin-like growth factor binding proteins (IGFBPs), epidermal growth factor (EGF), tumour necrosis factor-a (TNF-a) and 5a-androstane-3,17-dione reflect that the physiological microenvironment in follicles from polycystic ovaries inhibit the expression of CYP19A1 mRNA as observed in both the groups of PCOS in the present study (Jakimiuk et al. 1998).

Steroid hormones in the follicular fluid play an important role in the physiology of follicular growth, oocyte maturation and ovulation (de Resende et al. 2010). Estradiol is important for follicular growth whereas progesterone plays an important role in maintenance of pregnancy. In the present study estradiol concentrations were not different as compared to any of the PCOS types whereas decrease in concentration of progesterone was demonstrated in PCOS-IR as well as PCOS-NIR group as compared to control with less significant decrease in PCOS-NIR. In the follicles, the periovulatory period shifts the steroidogenic mission of the Graffian follicle from estrogenic synthetic tissue to predominantly progesterone synthetic tissue leading to recruitment of paracrine/endocrine factors. Growth Differentiation Factor -9 (GDF-9) and Bone morphogenetic protein (BMP-15) are oocyte derived factors and inhibit progesterone production induced by FSH and 8-bromo-cAMP in granulosa cells during controlled hyper stimulation in IVF reported to be highly expressed in oocytes of PCOS patients undergoing COH during IVF (Otsuka et al. 2000; Yamamoto et al. 2002; Zhao et al. 2010; de Resende et al. 2012).Testosterone levels proved to be very high in PCOS-IR group which is attributed to reduced CYP19A1 activity that leads to piling up of the androgens thus confirming previous theories of association of hyperandrogenism with hyperinsulinemia (Meirow et al. 1996).

The gonadotropin receptors, FSH-R and LH-R play a significant role in folliculogenesis and ovulation respectively. Their polymorphic variants observed in PCOS are strongly associated

with its clinical features such as increased levels of gonadotrophic hormones and the presence of hyperandrogenism thus leading to severity of the disorder (Valkenburg et al. 2009). In the present study increase in FSH-R and LH-R along with downregulation of most of the steroidogenic proteins, ultimately decreasing progesterone synthesis in PCOS-NIR group indicate intrinsic defect in steroidogenesis. Moreover studies in literature have demonstrated increased LH/FSH ratio in normo insulinemic PCOS patients (Li et al. 2010). This finding along with our findings for PCOS-NIR group indicate that the dysfunction in PCOS-NIR might be at the level of hypothalamus-pituitary. Further studies in literature have demonstrated normal LH/FSH ratio in hyper insulinemic PCOS patients (Li et al. 2010). This finding along with our findings of decreased insulin signalling and steroidogenic signalling in PCOS-IR group indicate that the dysfunction might be caused by metabolic disorder. (Legro et al. 1998; Li et al. 2010).

After observing for the effect of IR on insulin, fat metabolism and steroidogenic signalling, we further expanded our studies to IGF system which plays an important role in the development of preantral to preovulatory follicles and apoptosis (Silva et al. 2009). In human granulosa cells IGF-II plays a significant role as against IGF-1 in folliculogenesis and embryonic development. Hyperinsulinemia accompanying IR suppresses hepatic insulin like growth binding protein (IGFBP-1) production thus increasing the bioavailability of IGF-II and IGF-1R as observed in PCOS-IR group in the present study. The increase in IGF-II peptide disrupts the folliculogenesis and up regulation of IGF-1R further stimulates differentiation in granulosa cells thus contributing to the pathogenesis of syndrome (Mantzoros 2006; Mehta et al. 2013; Livingstone and Borai 2014). Increase in IGF-II peptide up regulates IGF-2R which in turn sequesters IGF-II for internalization limiting the activation of IGF1R and INSR-β by IGF-2 peptide thus inhibiting the growth of granulosa cells as observed in the present study in PCOS-IR and PCOS-NIR patients (Spicer and Aad 2007; Brown et al. 2008; Kaur et al. 2012).

Summarizing the results, PCOS-IR group demonstrated decrease in insulin signalling proteins related to metabolism, genes involved in fatty acid metabolism and steroidogenesis as against PCOS-NIR group where only steroidogenesis showed a decrease. Increased testosterone levels increased presence of hyper androgenism in both the groups. In conclusion, prevalence of hyperinsulinemia and hyperandrogenemia in PCOS-IR and intrinsic ovarian defects accompanied by defects at defects at hypothalamus and pituitary ultimately would affect the oocyte growth and quality (Fig. 6.9).

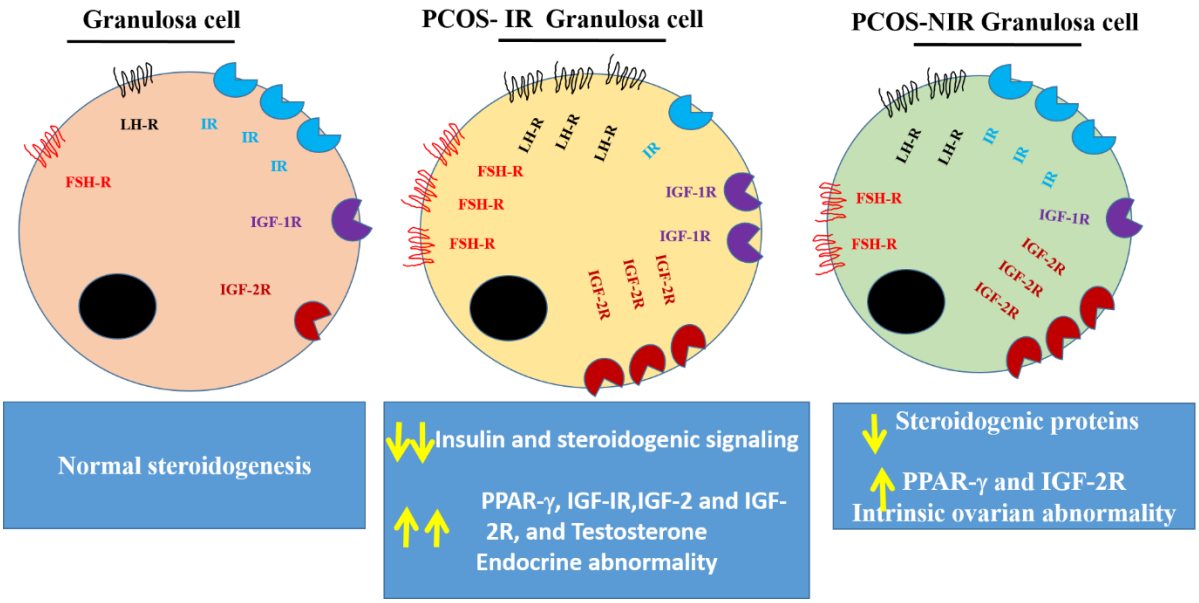


Figure 6. 9: Schematic figure showing difference in signalling between control, PCOS-IR and PCOS-NIR luteinized granulosa cells.

6.5 References

Almahbobi G, Anderiesz C, Hutchinson P, McFarlane JR, Wood C, Trounson AO. 1996. Functional integrity of granulosa cells from polycystic ovaries. *Clinical endocrinology* **44**: 571-580.

Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE. 2009. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. *Fertility and sterility* **91**: 456-488.

Baillargeon J-P, Nestler JE. 2006. Commentary: polycystic ovary syndrome: a syndrome of ovarian hypersensitivity to insulin? *The Journal of clinical endocrinology and metabolism* **91**.

Brown J, Delaine C, Zaccheo OJ, Siebold C, Gilbert RJ, van Boxel G, Denley A, Wallace JC, Hassan AB, Forbes BE. 2008. Structure and functional analysis of the IGF-II/IGF2R interaction. *The EMBO journal* **27**: 265-276.

Chien Y, Cheng W-C, Wu M-R, Jiang S-T, Shen C-KJ, Chung B-c. 2013. Misregulated progesterone secretion and impaired pregnancy in Cyp11a1 transgenic mice. *Biology of reproduction* **89**: 91.

Christenson LK, Osborne TF, McAllister JM, Strauss III JF. 2001. Conditional response of the human steroidogenic acute regulatory protein gene promoter to sterol regulatory element binding protein-1a 1. *Endocrinology* **142**: 28-36.

Corbould A, Zhao H, Mirzoeva S, Aird F, Dunaif A. 2006. Enhanced mitogenic signaling in skeletal muscle of women with polycystic ovary syndrome. *Diabetes* **55**: 751-759.

de Resende L, dos Reis R, Ferriani R, Vireque A, Santana L, Martins WP. 2010. [Concentration of steroid hormones in the follicular fluid of mature and immature ovarian follicles of patients with polycystic ovary syndrome submitted to in vitro fertilization]. *Revista brasileira de ginecologia e obstetricia: revista da Federacao Brasileira das Sociedades de Ginecologia e Obstetricia* **32**: 447-453.

de Resende LOT, Vireque AA, Santana LF, Moreno DA, de Sá Rosa ACJ, Ferriani RA, Scrideli CA, Reis RM. 2012. Single-cell expression analysis of BMP15 and GDF9 in mature oocytes and BMPR2

in cumulus cells of women with polycystic ovary syndrome undergoing controlled ovarian hyperstimulation. *Journal of assisted reproduction and genetics* **29**: 1057-1065.

Diamanti-Kandarakis E, Dunaif A. 2012. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. *Endocrine reviews* **33**: 981-1030.

Doldi N, Grossi D, Destefani A, Gessi A, Ferrari A. 2000. Polycystic ovary syndrome: evidence for reduced 3β-hydroxysteroid dehydrogenase gene expression in human luteinizing granulosa cells. *Gynecological endocrinology* **14**: 32-37.

Dunaif A. 1997. Insulin Resistance and the Polycystic Ovary Syndrome: Mechanism and Implications for Pathogenesis 1. *Endocrine reviews* **18**: 774-800.

Ergen K, Yildiz F, Ozcan M, Cekmen M, Ta Utkan T, Karakoc Y. 2012. Oxidative Stress Status, Metabolic Profile and Cardiovascular Risk Factors in Patients with Polycystic Ovary Syndrome. *Medicine Science* **1**.

Evans JL. 2007. Antioxidants: do they have a role in the treatment of insulin resistance? *Indian Journal of Medical Research* **125**: 355.

Ito M, Miyado K, Nakagawa K, Muraki M, Imai M, Yamakawa N, Qin J, Hosoi Y, Saito H, Takahashi Y. 2010. Age-associated changes in the subcellular localization of phosphorylated p38 MAPK in human granulosa cells. *Molecular human reproduction* **16**: 928-937.

Jakimiuk AJ, Weitsman SR, Brzechffa PR, Magoffin DA. 1998. Aromatase mRNA expression in individual follicles from polycystic ovaries. *Molecular human reproduction* **4**: 1-8.

Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. 2001a. Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *J Clin Endocrinol Metab* **86**: 1318-1323.

Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. 2001b. Luteinizing Hormone Receptor, Steroidogenesis Acute Regulatory Protein, and Steroidogenic Enzyme Messenger Ribonucleic Acids Are Overexpressed in Thecal and Granulosa Cells from Polycystic Ovaries 1. *The Journal of Clinical Endocrinology & Metabolism* **86**: 1318-1323.

Jonard S, Dewailly D. 2004. The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. *Human Reproduction Update* **10**: 107-117.

Kaur S, Archer KJ, Devi MG, Kriplani A, Strauss III JF, Singh R. 2012. Differential gene expression in granulosa cells from polycystic ovary syndrome patients with and without insulin resistance: identification of susceptibility gene sets through network analysis. *The Journal of Clinical Endocrinology & Metabolism*.

Legro RS, Driscoll D, Strauss JF, Fox J, Dunaif A. 1998. Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. *Proceedings of the National Academy of Sciences* **95**: 14956-14960.

Li Y, Wei L, Xiong Y, Liang X. 2010. [Effect of luteinizing hormone vs follicular stimulating hormone ratio on anti-Mullerian hormone secretion and folliculogenesis in patients with polycystic ovarian syndrome]. *Zhonghua fu chan ke za zhi* **45**: 567-570.

Lin Q, Poon SL, Chen J, Cheng L, HoYuen B, Leung P. 2009. Leptin interferes with 3′, 5′-cyclic adenosine monophosphate (cAMP) signaling to inhibit steroidogenesis in human granulosa cells. *Reprod Biol Endocrinol* **7**.

Livingstone C, Borai A. 2014. Insulin-like growth factor-II: its role in metabolic and endocrine disease. *Clinical endocrinology* **80**: 773-781.

Mantzoros CS. 2006. *Obesity and diabetes*. Springer Science & Business Media.

Marshall JC, Dunaif A. 2012. All Women With PCOS Should Be Treated For Insulin Resistance. *Fertility and Sterility* **97**: 18.

Mehta BN, Chimote NM, Chimote MN, Chimote NN, Nath NM. 2013. Follicular fluid insulin like growth factor-1 (FF IGF-1) is a biochemical marker of embryo quality and implantation rates in in vitro fertilization cycles. *Journal of human reproductive sciences* **6**: 140.

Meirow D, Yossepowitch O, Rosler A, Brzezinski A, Schenker J, Laufer N, Raz I. 1996. Insulin Resistant and Nonresistant Polycystic Ovary Syndrome Represent Two Clinical and Endocrinological Subgroups. *Obstetrical & gynecological survey* **51**: 233-235.

Miro F, Smyth CD, Whitelaw PF, Milne M, Hillier SG. 1995. Regulation of 3 beta-hydroxysteroid dehydrogenase delta 5/delta 4-isomerase and cholesterol side-chain cleavage cytochrome P450 by activin in rat granulosa cells. *Endocrinology* **136**: 3247-3252.

Niu Z, Lin N, Gu R, Sun Y, Feng Y. 2014. Associations between insulin resistance, free fatty acids, and oocyte quality in polycystic ovary syndrome during in vitro fertilization. *The Journal of Clinical Endocrinology & Metabolism* **99**: E2269-E2276.

Otsuka F, Yao Z, Lee T-h, Yamamoto S, Erickson GF, Shimasaki S. 2000. Bone morphogenetic protein-15 Identification of target cells and biological functions. *Journal of Biological Chemistry* **275**: 39523-39528.

Park S-Y, Gomes C, Oh S-D, Soh J. 2015. Cadmium up-regulates transcription of the steroidogenic acute regulatory protein (StAR) gene through phosphorylated CREB rather than SF-1 in K28 cells. *The Journal of toxicological sciences* **40**: 151-161.

Petrescu AD, Gallegos AM, Okamura Y, Strauss JF, 3rd, Schroeder F. 2001. Steroidogenic acute regulatory protein binds cholesterol and modulates mitochondrial membrane sterol domain dynamics. *J Biol Chem* **276**: 36970-36982.

Poretsky L, Seto-Young D, Shrestha A, Dhillon S, Mirjany M, Liu H-C, Yih MC, Rosenwaks Z. 2001. Phosphatidyl-Inositol-3 Kinase-Independent Insulin Action Pathway (s) in the Human Ovary 1. *The Journal of Clinical Endocrinology & Metabolism* **86**: 3115-3119.

Richardson MC, Cameron IT, Simonis CD, Das MC, Hodge TE, Zhang J, Byrne CD. 2005. Insulin and human chorionic gonadotropin cause a shift in the balance of sterol regulatory element-binding protein (SREBP) isoforms toward the SREBP-1c isoform in cultures of human granulosa cells. *The Journal of Clinical Endocrinology & Metabolism* **90**: 3738-3746.

Schinner S, Scherbaum W, Bornstein S, Barthel A. 2005. Molecular mechanisms of insulin resistance. *Diabetic Medicine* **22**: 674-682.

Seto-Young D, Avtanski D, Varadinova M, Park A, Suwandhi P, Leiser A, Parikh G, Poretsky L. 2011. Differential roles of MAPK-Erk1/2 and MAPK-p38 in insulin or insulin-like growth factor-I (IGF-I) signaling pathways for progesterone production in human ovarian cells. *Hormone and metabolic research* **43**: 386.

Sharma S, Sharma PM, Mistry DS, Chang RJ, Olefsky JM, Mellon PL, Webster NJ. 2011. PPARG regulates gonadotropin-releasing hormone signaling in LbetaT2 cells in vitro and pituitary gonadotroph function in vivo in mice. *Biology of reproduction* **84**: 466-475.

Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *Journal of Clinical Investigation* **99**: 838.

Silva J, Figueiredo J, Van den Hurk R. 2009. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* **71**: 1193-1208.

Spicer L, Aad P. 2007. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biology of reproduction* **77**: 18-27.

Tee MK, Miller WL. 2013. Phosphorylation of human cytochrome P450c17 by p38α selectively increases 17, 20 lyase activity and androgen biosynthesis. *Journal of Biological Chemistry* **288**: 23903-23913.

Valkenburg O, Uitterlinden A, Piersma D, Hofman A, Themmen A, de Jong F, Fauser B, Laven J. 2009. Genetic polymorphisms of GnRH and gonadotrophic hormone receptors affect the phenotype of polycystic ovary syndrome. *Human reproduction*: dep113.

Verit FF, Erel O. 2008. Oxidative stress in nonobese women with polycystic ovary syndrome: correlations with endocrine and screening parameters. *Gynecologic and obstetric investigation* **65**: 233-239.

Yamamoto N, Christenson LK, McAllister JM, Strauss III JF. 2002. Growth differentiation factor-9 inhibits 3′ 5′-adenosine monophosphate-stimulated steroidogenesis in human granulosa and theca cells. *The Journal of Clinical Endocrinology & Metabolism* **87**: 2849-2856.

Zhao S-Y, Qiao J, Chen Y-J, Liu P, Li J, Yan J. 2010. Expression of growth differentiation factor-9 and bone morphogenetic protein-15 in oocytes and cumulus granulosa cells of patients with polycystic ovary syndrome. *Fertility and sterility* **94**: 261-267.