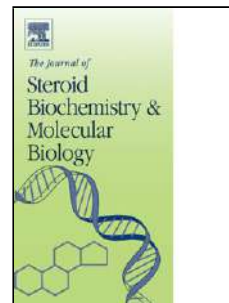


## Accepted Manuscript

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**Title Page**

Differential insulin and steroidogenic signaling in insulin resistant and non- insulin resistant human luteinized granulosa cells - a study in PCOS patients.

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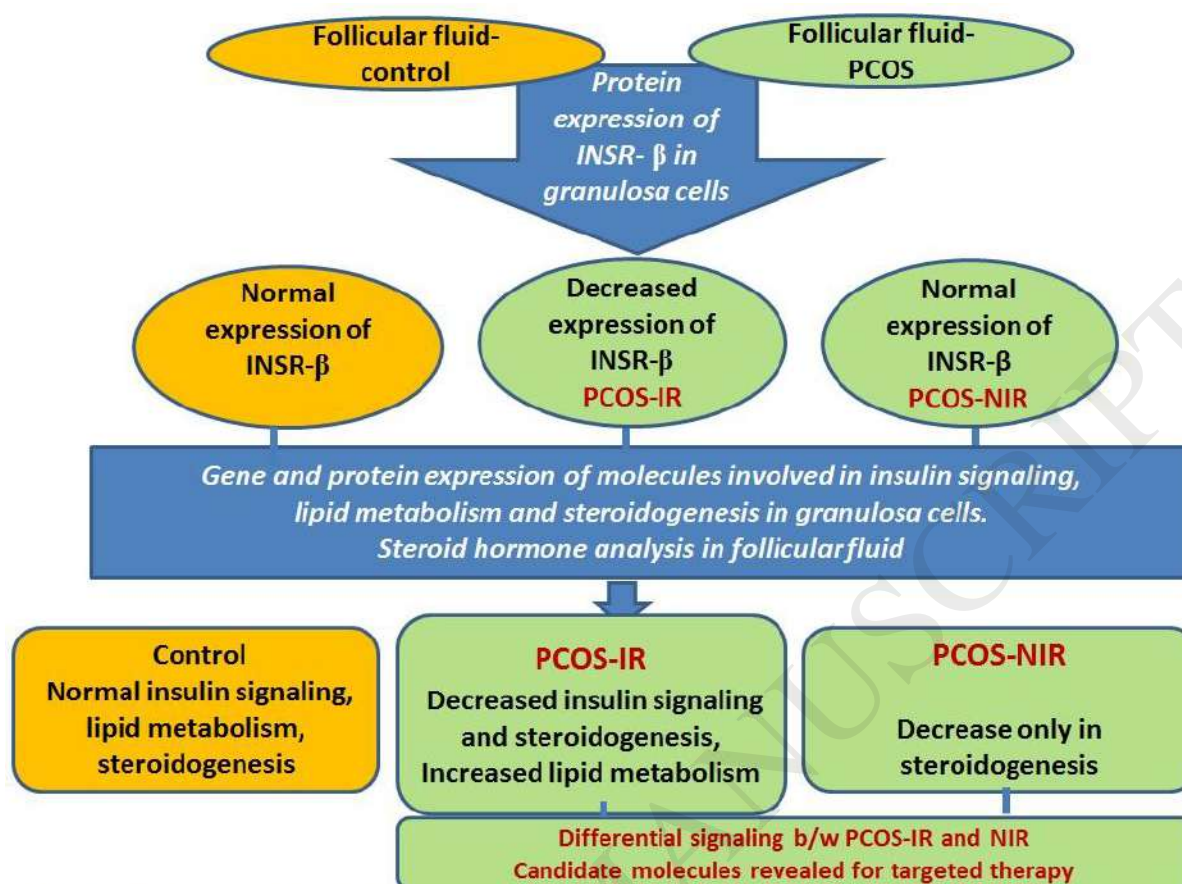
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## Graphical abstract



INSR-β : insulin receptor- β, PCOS- Polycystic ovarian syndrome, IR- Insulin Resistance , NIR- Non Insulin Resistance

## Research High lights

- Protein expression of INSR-β in granulosa cells to differentiate IR and NIR.
- Study reveals differential signaling between PCOS-IR and PCOS-NIR.
- Decrease in insulin signaling and steroidogenesis in PCOS-IR group.
- Decrease only in steroidogenesis in PCOS-NIR group.
- Unraveling candidate molecules for target therapy of PCOS phenotypes.

## Abstract

Insulin resistance (IR) is one of the significant aberrations in polycystic ovarian syndrome (PCOS), however is only observed in 70% – 80% of obese PCOS and 20% – 25% of lean PCOS. Hyperinsulinemia accompanies PCOS-IR along with hyperandrogenemia against normal insulin and androgen levels in PCOS-non insulin resistance (NIR). This could possibly be due to defects in the downstream signaling pathways. The study thus aims to unravel insulin and steroidogenic

signaling pathways in luteinized granulosa cells isolated from PCOS –IR and NIR vs matched controls. Luteinized granulosa cells from 30 controls and 39 PCOS were classified for IR based on a novel method of down regulation of protein expression of insulin receptor- $\beta$  (INSR-  $\beta$ ) as shown in our previous paper. We evaluated expression of molecules involved in insulin, steroidogenic signaling and lipid metabolism in luteinized granulosa cells followed by analysis of estradiol, progesterone and testosterone in follicular fluid. Protein expression of INSR-  $\beta$ , pIRS (ser 307), PI(3)K, PKC- $\zeta$ , pAkt, ERK1/2, pP38MAPK and gene expression of IGF showed differential expression in the two groups. Increased protein expression of PPAR- $\gamma$  was accompanied by up regulation in SREBP1c, FAS, CPT-1 and ACC-1 genes in PCOS-IR group. Expression of StAR, CYP19A1, 17  $\beta$ - HSD and 3  $\beta$ - HSD demonstrated significant decrease along with increase in CYP11A1, FSH-R and LH-R in both the groups. Follicular fluid testosterone increased and progesterone decreased in PCOS-IR group. This study shows how candidate molecules that were differentially expressed, aid in designing targeted therapy against the two phenotypes of PCOS.

**Keywords:** PCOS, insulin signaling, steroidogenic signaling, steroid hormones

## 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. As per statistics by National Women's Health Information Centre quotes about 5% - 10% of women of child bearing age (20-40) suffer from PCOS [1]. PCOS has variable features such as 1. Clinical or biochemical hyperandrogenism, 2. Chronic anovulation, 3. Polycystic ovary after exclusion of disorders of pituitary or the adrenal that could present in a manner similar to PCOS [2]. Besides being a gynecological disorder, PCOS is now accepted as metabolic syndrome with insulin resistance (IR), one of the most significant metabolic aberration.

The prevalence of IR in the general population is 10%–25%, whereas in PCOS patients, it is approximately 60%–70% [3]. 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 of obese PCOS women [4]. A higher number (70–80%) of obese PCOS (BMI >30) and a relatively lower number (20–25%) of 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) [3]. In consonant with these facts, the Rotterdam criteria has also considered anovulatory women with normal androgen levels and polycystic ovary as a distinct phenotype of PCOS. In this group of women, insulin sensitivity was observed to be normal [5]. According to the National Institute of Health 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 [3]. Many studies 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 [3]. Only one study 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 [6].

In the present study, we hypothesized that inspite of using clinical and biochemical parameters, PCOS patients can be classified as IR and NIR based on a novel molecular marker - insulin receptor-  $\beta$  (INSR-

$\beta$ ) expression on luteinized granulosa cells. Thus we investigated protein expression of INSR-  $\beta$  in luteinized granulosa cells, classified them as IR and NIR and further evaluated insulin signaling pathway. Genes involved in steroidogenesis, gonadotropin receptors, lipid metabolism, and insulin like growth factor (IGF) system were also evaluated. Steroid hormones were estimated in follicular fluid. Findings presented here demonstrate significant differences in key molecules involved in insulin signaling, IGF system, lipid metabolism and steroidogenic signaling in PCOS with and without insulin resistance which may act as candidate molecules for characterizing the defect and designing appropriate therapy.

## 2. Materials and Methods

### 2.1. Subjects

Human follicular fluid samples were collected, from August, 2012 to April 2015, after informed consent from patients undergoing *in vitro* fertilization (IVF) over the course of 32 months at Nova IVI Fertility Clinic, Ahmedabad, India. All the controls as well as patients underwent controlled ovarian hyperstimulation (COH) using flexible antagonist protocol. The follicular fluid devoid of oocyte was collected for the experiments.

**2.1.1. Inclusion criteria:** The diagnosis included donors, male factor infertility, tubal factor infertility and PCOS with an age ranging from 20 to 40 years.

**2.2.2. Exclusion criteria:** Patients with endometriosis and poor ovarian response were excluded from the study.

The study was approved by the Institutional Ethics committee for human research (IECHR), Faculty of Science, The M. S. University of Baroda, Vadodara (Ethical Approval Number FS/IECHR/BC/SG2).

### 2.2. Human granulosa-luteal cell isolation

Follicular aspirates from individual controls (n= 30) PCOS patients (n= 39) were centrifuged at 300 g at room temperature. Blood contaminants were removed from luteinized granulosa cells by Histopaque gradient centrifugation at 400 g at room temperature. The middle layer of cells was then collected and re-suspended in 10 ml volume of DMEM/F12 medium and washed twice by a further 5 min

centrifugation. The viability of cells was analysed at final stage by trypan blue exclusion dye method.

The cells were aliquoted for mRNA and protein expression analysis.

### 2.3. Total RNA Extraction and qRT-PCR

Total cellular RNA was isolated from follicular fluid luteinized granulosa cells and then reverse transcribed into first strand cDNA. qRT-PCR for lipogenic genes Sterol Regulatory Element Binding Protein (SREBP1c), Fatty acid synthase (FAS), Acetyl-CoA carboxylase 1 (ACC-1), Carnitine palmitoyl transferase I (CPT-1), Insulin like growth factor system (IGF-1, IGF-2, IGF-1R, IGF-2R) and gonadotropins receptors [follicle stimulating hormone receptor (FSH-R), luteinizing hormone receptor (LH-R)] study was performed on an Applied- Biosystem 7500-Real-Time PCR Sequence detection System using standard temperature cycling conditions. qRT-PCR for steroidogenic genes Steroidogenic Acute Regulatory protein (StAR), Cytochrome P450 side chain cleavage (CYP11A1), 3-beta hydroxy steroid dehydrogenase (3  $\beta$ -HSD), Aromatase (CYP19A1), 17-beta hydroxy steroid dehydrogenase (17  $\beta$ -HSD) was performed on Applied Biosystem 7500 FAST Real Time PCR Sequence detection System by predesigned primers from TaqMan gene expression assays. All qRT-PCR results were normalized to the level of  $\beta$ -actin and 18S rRNA determined in parallel reaction mixtures to correct any differences in RNA input. Fold changes in qRT-PCR gene expression were analyzed using 7500 Real time PCR software V.2.0.6 and Data assist software (Applied Biosystems Inc.) which led to a possible estimation of the actual fold change. Negative RT was performed with untranscribed RNA. (Primer sequence: Tables 1 and 2).

Table 1: Taqman Gene expression probes (Human)

S.No	Name	ID	Cat.No	amplicon length
1	StAR - Steroidogenic Acute Regulatory protein	Hs00986558_g1	4448892	68
2	CYP11A1-Cytochrome P450 side chain cleavage	Hs00897322_g1	4448892	90
3	CYP19A1-Aromatase	Hs00903410_m1	4448892	88
4	HSD3 B2 ( 3-beta hydroxy steroid dehydrogenase type-2)	Hs01080264_g1	4448892	77
5	HSD17B1 (17-beta hydroxy steroid dehydrogenase type-1)	Hs00907289_g1	4448892	93

Table 2: List of primers sequences (Human) with its amplicon size

Gene	Accession number	Sequence (5'→3')	Product size	Annealing Temperature
SREBP1c	NM_001005291	F: TGCATTTTCTGACACGCTTC R: CCAAGCTGTACAGGCTCTCC	171	60
ACC-1	NM_198834	F: TTTAAGGGGTGAAGAGGGTGC R: CCAGAAAGACCTAGCCCTCAAG	171	60
FAS	NM_004104	F: CACAGGGACAACCTGGAGTT R: ACTCCACAGGTGGGAACAAG	97	60
CPT-1	NM_001876	F: TCGTCACCTCTTCTGCCTTT R: ACACACCATAGCCGTCATCA	206	60
$\beta$ -Actin	NM_001101	F: ACTCTTCCAGCCTTCCTTCC R: CGTACAGGTCTTTGCGGATG	101	60
IGF-1	NM_000618.3	F: AGCAGTCTTCCAACCCAATTATTTAG R: AGATGCGAGGAGGACATGGT	83	56
IGF-1R	NM_000875.4	F: AAGGCTGTGACCCTCACCAT R: CGATGCTGAAAGAACGTCCAA	118	56
IGF-2	NM_000612.5	F: AGCAGTCTTCCAACCCAATTATTTAG R: GGACTGCTTCCAGGTGTCATATT	189	57
IGF-2R	NM_000876.2	F: AGCAGTCTTCCAACCCAATTATTTAG R: GAGACAAGTCAACAATAGAGCTTCCA	197	60
FSH-R	NM_000145.3	F: TTTCAAGAACAAGGATCCATTCC R: CCTGGCCCTCAGCTTCTTAA	336	60
LH-R	NM_000233.3	F: TTCAATGGGACGACTGACTT R: TGTGCATCTTCTCCAGATGTACGT	234	60

#### 2.4. Western blot analysis

Follicular fluid luteinized granulosa cells were suspended in cell lysis buffer composed of 62.5mM Tris-HCl, pH 6.8, 6M urea, 10% (v/v) glycerol, 2% (w/v) SDS, 0.00125% (w/v) bromophenol blue, freshly added 5% (v/v)  $\beta$ -mercaptoethanol and subjected to sonication on ice. Total protein content was quantified using Bradford assay (Bio-Rad Bradford Solution, USA). A total of 20  $\mu$ g protein was loaded on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, along with pre-stained molecular weight markers. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated overnight at 4°C with appropriate antibody dilution (Table: 3). The samples were then incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-goat or anti-mouse IgG and analyzed by Alliance 4.7 UVI Tec chemidoc.



Table 3: Antibody for Western Blotting

Name of Antibody	Company and Catalog No.	Mono/Poly clonal	Mol. Weight (kDa)	Isotype
PI(3)K p85(19H8)	Cell Signaling #4257	Mono	85	Rabbit
INSR - $\beta$	Cell Signaling #3025	Mono	95	Rabbit
pIRS1	Cell Signaling #2381	Poly	180	Rabbit
pAkt	Cell Signaling #4060	Mono	60	Rabbit
P38 MAPK	Cell Signaling #9212	Poly	43	Rabbit
P <sub>44/42</sub> MAPK (Erk1/2)	Cell Signaling #9102	Poly	42,44	Rabbit
Protein Kinase C- $\zeta$	Millipore #07-264	Poly	72	Rabbit
PPAR- $\gamma$	Cell Signaling #2435	Mono	53,57	Rabbit
StAR (Steroidogenic acute regulatory protein)	gifted by Prof. Stocco	Poly	30	Rabbit
CYP11A1	Santacruz		60	Goat
3 $\beta$ - HSD	Gifted by Prof. Van Luu THE	Poly	35	Rabbit
CYP19A1	Cell Signaling	Poly	58	Rabbit
17 $\beta$ - HSD	Gifted by Prof. Van Luu THE	Poly	35	Rabbit
$\beta$ -Actin	Thermo Scientific #MAI-91399	Mono	43	Mouse

## 2.5. Follicular fluid hormone analysis

The steroid hormones were measured in the follicular fluid devoid of cells by enzyme-linked immunosorbent assay (Diametra; Italy), according to the manufacturer's instructions. Each sample was assayed in duplicate. The assay sensitivity range was 8.68pg/ml for E<sub>2</sub>, 0.05ng/ml for P<sub>4</sub> and 0.07ng/ml for testosterone.

## 2.6. Statistical Analysis

The results are presented as mean  $\pm$  standard error of the mean. The data were statistically analyzed by employing one-way analysis of variance followed by Newman Keuls Multiple Comparison Test (GraphPad Prism; Graph Pad Software, Inc., La Jolla, CA). The minimum level of significance ( $P < 0.05$ ) was considered.

## 3. Results

### 3.1. Characterization of IR in granulosa cell and assessment of viability

Luteinized granulosa cells from individual control and PCOS follicular fluid samples were isolated and analyzed for expression of INSR- $\beta$  as done before [7, 8]. The samples having down regulation of INSR- $\beta$  were segregated as PCOS-IR, and rest having expression of INSR- $\beta$  similar to that of control as PCOS-NIR. Luteinized granulosa cells from control, PCOS-IR and PCOS-NIR were analyzed 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 compared to control, however the decrease was much more significant in PCOS-IR group ( $P < 0.01$ ) as compared to PCOS-NIR (Fig.1).

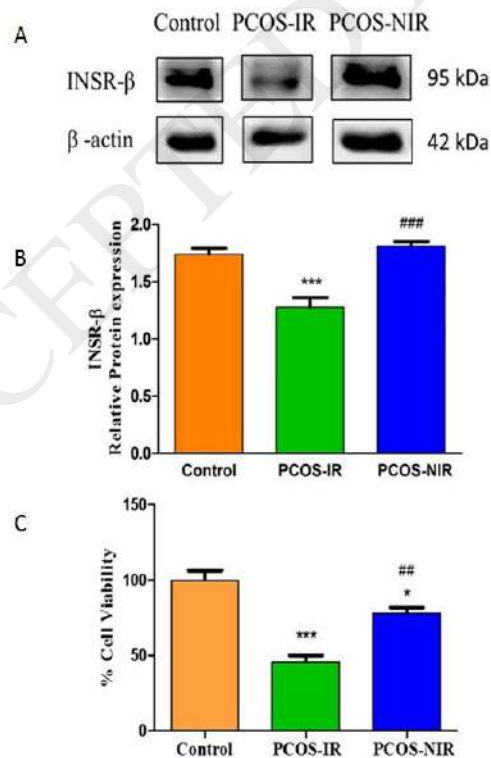
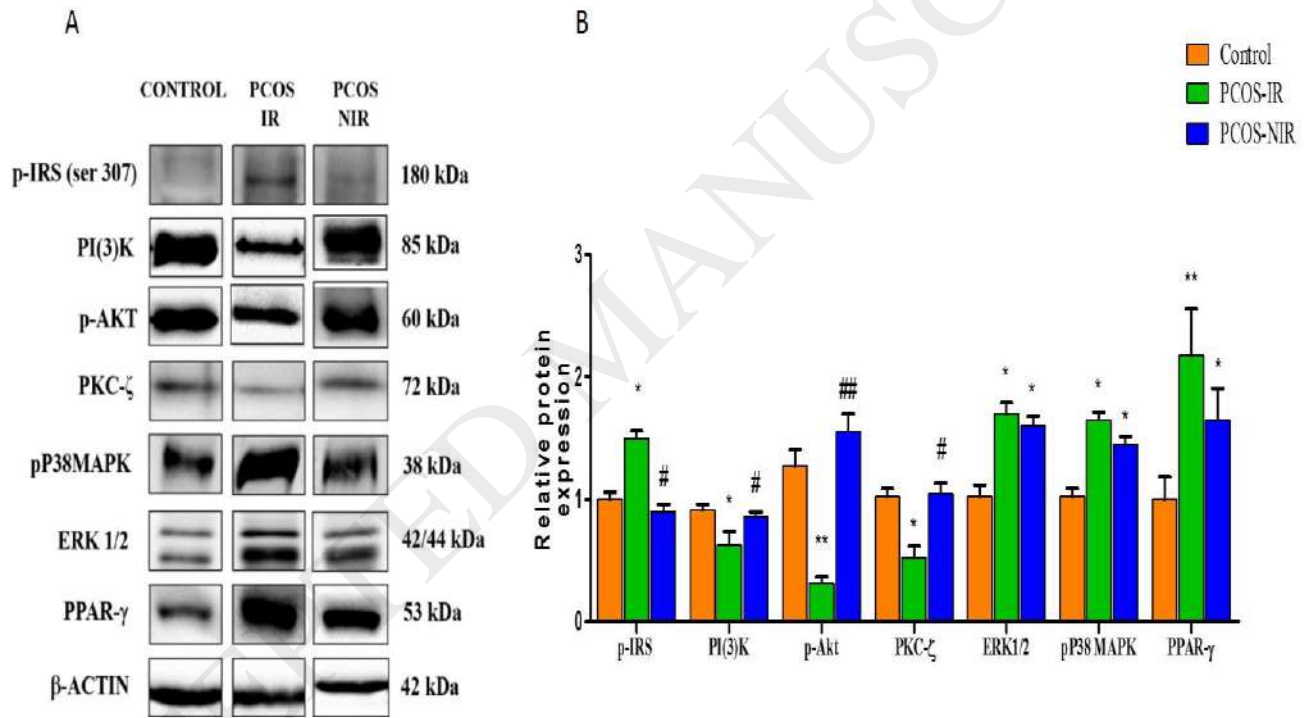


Figure 1: Characterizing human luteinized granulosa cells isolated from PCOS samples as IR and NIR. A. Protein expression of INSR-  $\beta$  in 39 PCOS and 30 controls was determined by western blot method. B). Densitometry of INSR-  $\beta$ . C). % granulosa cell viability was done by trypan blue exclusion dye method. \*\*\*  $P < 0.001$  PCOS-IR vs. control, ####  $P < 0.001$  PCOS-NIR vs PCOS-IR.\* $P < 0.05$  PCOS-NIR vs. Control, ##  $P < 0.01$  PCOS-NIR vs. PCOS-IR.

### 3.2. Alterations in Insulin signaling, lipid metabolism and IGF system in luteinized granulosa cells from PCOS-IR

To explore the in depth mechanism of insulin signaling in PCOS-IR and PCOS-NIR, protein expression of phospho insulin receptor substrate [pIRS(ser307)], phosphatidylinositol 3-kinase (PI(3)K), phospho protein kinase B (pAkt), protein kinase C (PKC- $\zeta$ ), extracellular regulatory kinase (ERK1/2), phospho P38 mitogen activated protein kinase (pP38MAPK) and Peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) from luteinized granulosa cells were analyzed by western blot method. Along with down regulated protein expression of INSR  $\beta$ ; significant decrease was observed in downstream candidate proteins such as PI(3)K ( $P < 0.05$ ), pAkt ( $P < 0.01$ ) and PKC- $\zeta$  ( $P < 0.05$ ) in PCOS-IR group as compared to control and PCOS-NIR group. The results demonstrated elevated protein expression of pIRS(ser 307) ( $P < 0.05$ ) in PCOS-IR group as compared to PCOS-NIR and control which signify down regulation of receptors. PCOS-IR and PCOS-NIR showed significant increase ( $P < 0.05$ ) in expression of ERK1/2 and pP38MAPK as compared to control group with no significant difference among the PCOS groups. Significant increase was observed in protein expression of PPAR- $\gamma$  in PCOS-IR ( $P < 0.01$ ) and PCOS-NIR ( $P < 0.05$ ) as compared to control group. As PCOS is associated with several metabolic disturbances, we further dissected whether any difference in lipid metabolism exists in PCOS IR group as compared to PCOS NIR. Thus expression of genes involved in lipid metabolism namely SREBP1c, FAS, ACC-1 and CPT-1 was analyzed by real time PCR. The results demonstrated that there was a significant increase in mRNA expression of SREBP1c ( $P < 0.001$ ), FAS ( $P < 0.001$ ), ACC-1 ( $P < 0.01$ ) and CPT-

1 ( $P < 0.01$ ) in PCOS-IR as compared to control and SREBP1c ( $P < 0.001$ ), FAS ( $P < 0.001$ ), ACC-1 ( $P < 0.01$ ) and CPT-1 ( $P < 0.01$ ) in PCOS-NIR as compared to PCOS-IR. The results demonstrated significant changes in lipid synthesis and oxidation in PCOS-IR condition with no change observed in PCOS-NIR group as compared to control. There was a remarkable down regulation of IGF-1 ( $P < 0.05$ ) with significant increase of IGF-2 ( $P < 0.05$ ) in PCOS-IR group. However when analyzed for the gene expression of their cognate receptors there was a marked up regulation in PCOS-IR group as compared to that of PCOS- NIR and control groups (Fig: 2).



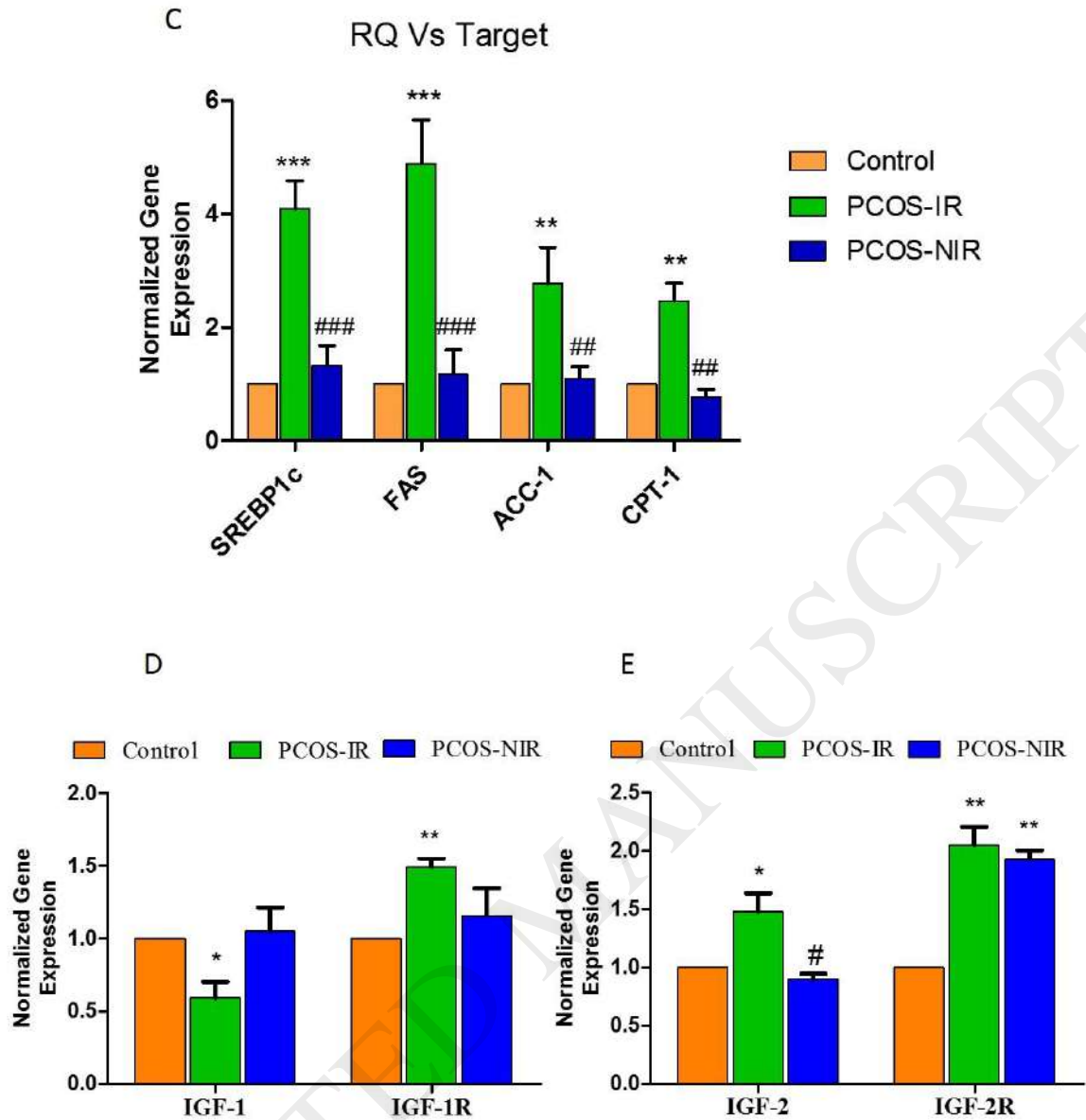
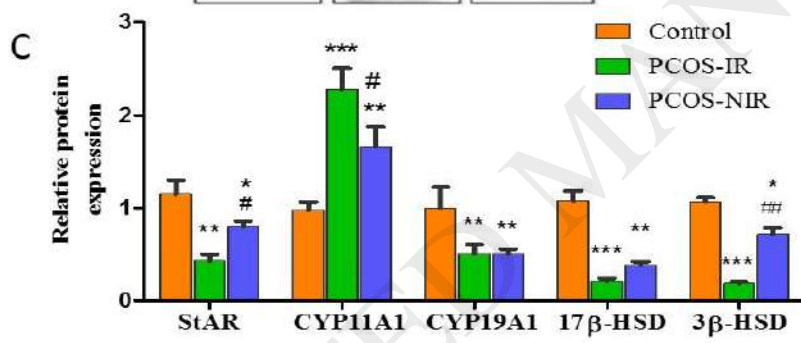
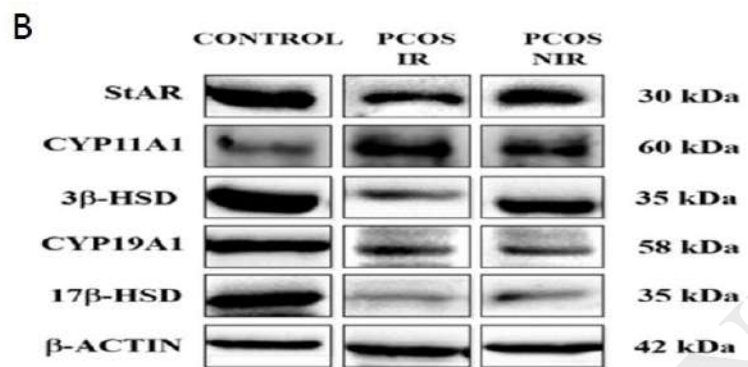
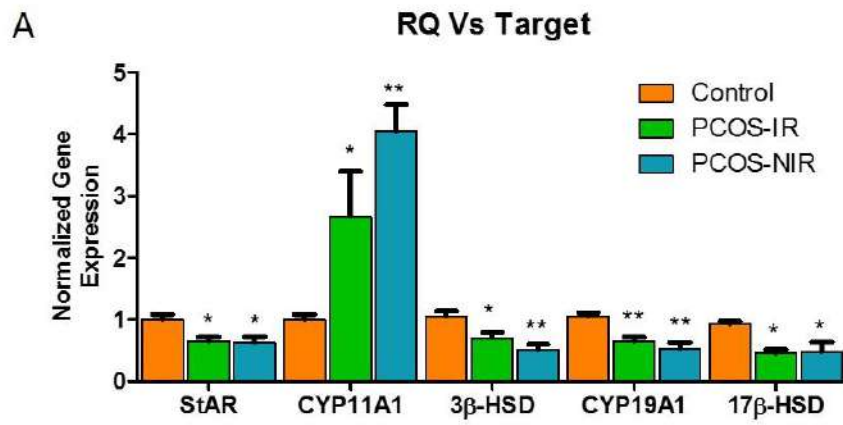


Figure 2: Expression of genes and proteins involved in insulin signalling cascade in human luteinized granulosa cells isolated from control, PCOS-IR and PCOS-NIR follicular fluid samples. A) Protein expression of pIRS(ser307), PI(3)K, pAkt, PKC- $\zeta$ , ERK1/2, p38MAPK and PPAR  $\gamma$  by Western blot method B) Densitometry of pIRS(ser307), PI(3)K, pAkt, PKC  $\zeta$ , ERK1/2, p38MAPK and PPAR- $\gamma$ . C) mRNA expression of SREBP1c, FAS, ACC-1, CPT-1 genes involved in fatty acid metabolism by qRT-PCR mRNA expression of D) IGF-1 and IGF1-R and E) IGF-2 and IGF-2R by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$  PCOS-IR, \*\*\* $P < 0.001$  vs. control, #  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$  PCOS-NIR vs. PCOS-IR.

### 3.3. Steroidogenic machinery is altered in PCOS-IR as well as PCOS-NIR

In order to elucidate the steroidogenic machinery StAR, CYP11A1, 3  $\beta$  HSD, CYP19A1 and 17  $\beta$  HSD were analyzed for gene and protein expression followed by gene expression of FSH-R and LH-R and steroid hormone levels. Significant changes included decrease in mRNA of StAR ( $P < 0.05$ ), 17  $\beta$  HSD ( $P < 0.05$ ), 3  $\beta$  HSD ( $P < 0.05$ ), CYP19A1 ( $P < 0.01$ ) and increase in CYP11A1 ( $P < 0.05$ ) in PCOS-IR group as compared to control. Similarly in PCOS-NIR group StAR ( $P < 0.05$ ), 17  $\beta$  HSD ( $P < 0.05$ ), 3  $\beta$  HSD ( $P < 0.01$ ), CYP19A1 ( $P < 0.01$ ) demonstrated significant decrease and increase in CYP11A1 ( $P < 0.01$ ) as compared to control. Expression of proteins in PCOS-IR group revealed decrease in StAR ( $P < 0.01$ ), 17  $\beta$  HSD ( $P < 0.001$ ), 3  $\beta$  HSD ( $P < 0.001$ ), CYP19A1 ( $P < 0.01$ ) and an increase in CYP11A1 ( $P < 0.001$ ) as compared to control. Similarly in PCOS-NIR group decrease was observed in protein expression of StAR ( $P < 0.05$ ), 17  $\beta$  HSD ( $P < 0.01$ ), 3  $\beta$  HSD ( $P < 0.05$ ), CYP19A1 ( $P < 0.01$ ) and increase in CYP11A1 ( $P < 0.01$ ) as compared to control. However significant decrease in protein expression of StAR ( $P < 0.05$ ), 3  $\beta$  HSD ( $P < 0.01$ ) and CYP11A1 ( $P < 0.05$ ) in PCOS-IR as compared to PCOS-NIR indicated decreased severity in PCOS-NIR group. Gene expression analysis of FSHR revealed significant increase in PCOS-IR ( $P < 0.01$ ) and PCOS-NIR ( $P < 0.05$ ) as compared to control. Significant increase was also revealed in gene expression of LHR in PCOS-IR ( $P < 0.001$ ) and PCOS-NIR ( $P < 0.05$ ) as compared to control. Along with these observations, when follicular fluid samples were analyzed for hormones, a considerable decrease was observed in progesterone concentration in PCOS-IR ( $P < 0.01$ ) as compared to control and PCOS-NIR groups ( $P < 0.05$ ) whereas significant increase was observed in the levels of testosterone in PCOS-IR ( $P < 0.01$ ) group as compared to control and PCOS-NIR ( $P < 0.05$ ). Estradiol levels however did not reveal any significant difference between control, PCOS-IR and PCOS-NIR groups. (Fig: 3).



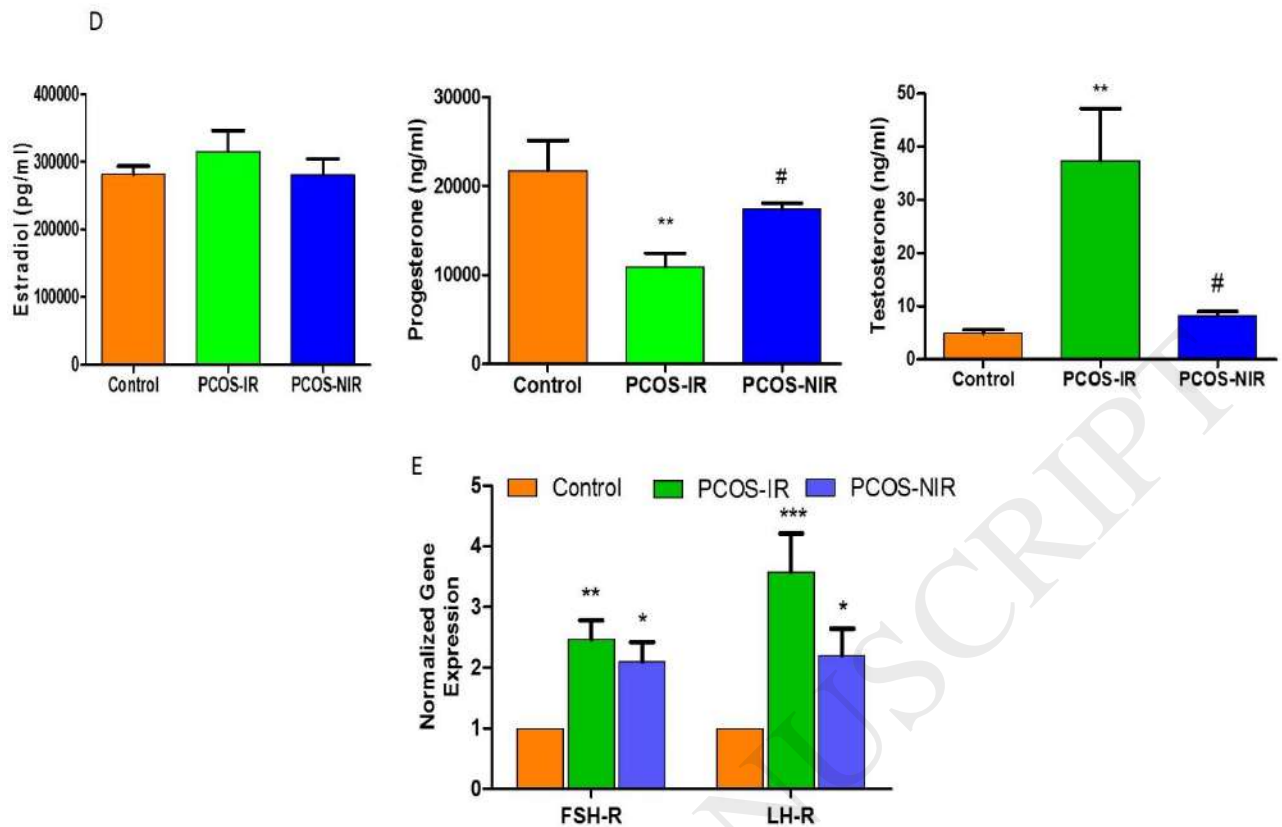


Figure 3: Steroidogenic profile of human luteinized granulosa cells isolated from control, PCOS-IR and PCOS-NIR samples. A) mRNA expression of StAR, CYP11A1, 3 $\beta$ -HSD, CYP19A1, 17 $\beta$ -HSD genes by qRT-FAST PCR B). Protein expression on StAR, CYP11A1, 3 $\beta$ -HSD, CYP19A1, 17 $\beta$ -HSD by Western blot method. C) Densitometry analysis using  $\beta$ -actin as endogenous control. D) Analysis of steroid hormones estradiol, progesterone and testosterone concentration in follicular fluid aspirates devoid of luteinized granulosa cells by ELISA. E) mRNA expression of FSH-R and LH-R genes in human luteinized granulosa cells by qRT-PCR \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 PCOS-IR and PCOS-NIR vs. control. #P < 0.05, ##P<0.01 PCOS-IR vs. PCOS-NIR.

#### 4. Discussion

Over production of androgens and insulin resistance have synergistic effect in many PCOS women, contributing to the alteration of functions in several tissues, including luteinized granulosa cells. This leads to changes in the expression of proteins related to tissue homeostasis and 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. To better understand granulosa cell death and protein expression in insulin and steroidogenic signaling, at first on the basis of down regulation of INSR- $\beta$  in luteinized granulosa cells, PCOS were segregated as



PCOS-IR and PCOS-NIR as done in our earlier experiments, which could help expand our knowledge about reproductive failure observed in these cases [7, 8]. Amongst several markers for IR, decrease in INSR- $\beta$  protein has been demonstrated in various insulin-target tissues such as liver, skeletal muscle, adipose tissue and kidney during IR condition [9]. The same has also been demonstrated in cultures of granulosa cells isolated from ovaries of PCOS-IR and has been correlated clinically with PCOS-IR patients [10]. In the present study, reduction in expression of INSR- $\beta$  was observed in 64% of LGC's isolated from follicular fluid aspirates of PCOS whereas rest 36% did not show INSR- $\beta$  reduction when compared to controls. The results claimed 60-80% of PCOS as IR and other PCOS as NIR which were in line with the literature [11]. Luteinized 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 [12]. Therefore drastic decrease in granulosa cell viability in PCOS-IR group as compared to PCOS-NIR group in the present study, can be correlated to down regulation of INSR-B leading to decreased survival and increased apoptosis in PCOS-IR group.

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-  $\beta$  [13, 14]. Thus, in the present study we opted to understand the protein expression of insulin signaling cascade in PCOS-IR and PCOS-NIR patients. Insulin mediates its actions via three major pathways: the PI(3)K pathway, implicated in the metabolic effects of insulin; the MAPK pathway, responsible for the mitogenic effects of insulin; and the PKC pathway [15]. Hence proteins that participate in the downstream insulin signaling pathway, specifically pIRS(ser 307), PI(3)K, pAkt, PKC  $\zeta$ , pP38 MAPK, ERK1/2 and PPAR  $\gamma$  were evaluated in the control, PCOS-IR and PCOS-NIR luteinized granulosa cells. 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 INSR- $\beta$ , PI(3)K along with increase in pIRS(ser 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 with a decrease in glycogen synthesis due to decreased pAKT and PKC- $\zeta$  as compared to control groups. Serine phosphorylation of IRS proteins reduces the ability of IRS proteins to attract PI(3)kinase, resulting in diminished glucose uptake and utilization in insulin target tissues. Thus, in contrast to a signal promoting tyrosine phosphorylation, excessive serine phosphorylation of IRS proteins could become detrimental for normal conductance of the metabolic insulin signaling downstream, causing insulin resistance [16].

Further in PCOS-NIR cells where, expression of INSR-  $\beta$ , PI(3)K, pAkt, PKC- $\zeta$  and pIRS(ser 307) were unchanged as compared to control group confirmed earlier findings that their activation was not

mandatory for ovarian steroidogenesis thus explaining a post insulin binding divergence of INSR  $\beta$  signaling and possibility of other pathways playing role in steroidogenesis [17].

In the present study, increase in the 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 thus contributing to an increase in reactive oxygen species in PCOS-IR and PCOS-NIR which can be responsible for decreased viability [11, 18-21]. 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 [18, 22, 23]. Other than the activation of p38MAPK functions as the mediator for gonadotropin releasing hormone-stimulated luteinizing hormone b promoter activity, thus explaining the mechanism by which luteinized granulosa cells would be undergoing premature luteinization in PCOS [24].

Luteinized granulosa cells play a pivotal role in the 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  $\gamma$ , SREBP1c, FAS, CPT-1 and ACC-1 whose expression in human luteinized granulosa cells support the existence of lipogenic and lipolytic activity in them [25, 26]. In the present study, we observed up-regulation of protein expression of PPAR  $\gamma$  accompanied by an up regulation in SREBP1c, FAS, CPT-1 and ACC-1 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 the insulin resistance, high circulating insulin continues to stimulate SREBP1c in liver [26]. The same paradigm might be responsible for IR luteinized granulosa cells leading to over production of fatty acids. Also due to the shift from normal situation to IR, the isoform SREBP1c precedes SREBP1a in human luteinized granulosa cells ultimately hindering the up regulation of StAR promoter activity and hence limiting the transfer of cholesterol for steroidogenesis [26]. ACC has a role in denovo lipogenesis (DNL) which produces malonyl-CoA, a major intermediate of fatty acid synthesis and an inhibitor of the fatty acid transporter CPT-1. However an increase in FAS as observed in PCOS-IR group leads to consumption of malonyl-CoA, thus limiting the accumulation of malonyl-CoA and consequent inhibition of CPT-1 leading to increase in  $\beta$ -oxidation. As DNL and  $\beta$ -oxidation of fatty acids are distinct pathways, fatty acid synthesis and  $\beta$ -oxidation can occur simultaneously, creating futile cycles in granulosa cells as observed in hepatocytes of non- alcoholic fatty liver disease [27]. Further, as hepatic DNL is positively correlated with insulin resistance, our observations for increased expression of FAS, CPT-1 and ACC-1 imply up regulation of denovo lipogenesis and  $\beta$ -oxidation in PCOS-IR group as compared to control and PCOS-NIR group. Taken together our results indicate

a major shift favoring fatty acid metabolism in luteinized granulosa cells rather than cholesterol production as a substrate for steroidogenesis.

After observing the effect of IR on insulin and lipid metabolism, we further expanded our studies to IGF system which plays an important role in the development of preantral to preovulatory follicles and apoptosis [28]. In human granulosa cells, IGF-2 being the predominant ligand as compared to IGF-1 is presumed to be the physiological ligand playing a significant role in folliculogenesis and embryonic development [29, 30]. IGF-1 has been reported to play a significant a role in promoting follicular growth, steroid secretion and anti-atretic function through regulation of cell cycle in human granulosa cells [31-34]. In the present study increase in IGF-2 expression in luteinized granulosa cells of PCOS-IR subjects correlates with the literature [30]. In IR condition, hyperinsulinemia suppresses hepatic insulin like growth binding protein (IGFBP1) production thus increasing the bioavailability of IGF-II as observed in PCOS-IR group in the present study [35]. The increase in IGF-2 peptide disrupts the folliculogenesis by pathologically increasing androgen production [36]. Further increase in IGF-2 peptide upregulates IGF1R further stimulating prematurely differentiation of granulosa cells to luteinized granulosa cells thus contributing to the pathogenesis of syndrome as observed under PCOS-IR condition as compared to PCOS-NIR in the present study [31, 37]. Up regulation of IGF-2R has been associated with type 2 diabetes and insulin resistance. Studies on IGF2R as a susceptibility gene for type 2 diabetes have implicated an insertion/deletion variant in the 3'UTR region of IGF2R resulting in a polymorphism that would likely change expression of IGF-2R and decrease the affinity of IGF-2 towards it [38]. A study by mass action kinetic model has revealed significantly higher IGF-2R: IGF-1R ratio for IGF-2R to be an effective suppressor of IGF-2 mediated activation [39, 40]. These findings from the literature suggest that the role of IGF-2R might be minor in counteracting IGF-2 in altered states as observed in the present study.

StAR protein plays the first significant step in steroidogenesis by transferring cholesterol from outer to the inner mitochondrial membrane [41]. Decrease in gene and protein expression of StAR was in line with the reports indicating reduced gonadal steroidogenesis [42, 43]. CYP11A1 is the key enzyme that initiates the rate limiting step in steroidogenesis by the conversion of cholesterol to pregnenolone and 3 $\beta$ -HSD in the biosynthesis of progesterone. In PCOS human luteinized granulosa cells the expression of CYP11A1 is upregulated whereas that of 3 $\beta$ -HSD is downregulated which is in accordance with earlier study [44]. Moreover, reports have also unraveled that overexpression of CYP11A1 cause defects in luteal phase development further hampering mitochondrial production and decreasing progesterone synthesis as observed in the present study [45]. The synthesis of estradiol is dependent on CYP19A1 and 17 $\beta$ -HSD [46]. The luteinized granulosa cells from follicular fluid samples of classic PCOS i.e irrespective of insulin resistance, are hyper responsiveness to LH increases 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 17 $\beta$ -HSD [47]. 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, epidermal growth factor, tumour necrosis factor- $\alpha$  and 5 $\alpha$ -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 [48].

Steroid hormones in the follicular fluid play an important role in the physiology of follicular growth, oocyte maturation and ovulation [49]. 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 and Bone morphogenetic protein are oocyte derived factors and inhibit progesterone production induced by FSH and 8-bromo-cAMP in luteinized granulosa cells during controlled hyper stimulation in IVF. These factors are reported to be highly expressed in oocytes of PCOS patients undergoing controlled ovarian hyperstimulation during IVF thus contributing to decreased progesterone as observed in the present study [50, 51]. 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 [6].

The gonadotropin receptors, FSHR and LHR 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 [52]. In the present study increase in FSHR and LHR along with down regulation 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 [53]. 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 [53]. 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.

### 5.5 .Conclusion

The present study discloses decrease in insulin signaling proteins related to metabolism, lipogenic genes and steroidogenesis in PCOS-IR group as against decrease only in steroidogenesis in PCOS-NIR group.

These differential signaling molecules ultimately might be involved in the prevalence of hyperinsulinemia and hyperandrogenemia in PCOS-IR group and intrinsic ovarian defects in PCOS-NIR group leading to follicular arrest, poor oocyte growth as well as quality observed in different PCOS phenotypes (Fig. 4 and Table 4). The study would aid in designing candidate molecules for targeted therapy towards the two different types of PCOS thus decreasing probability of reproductive failures.

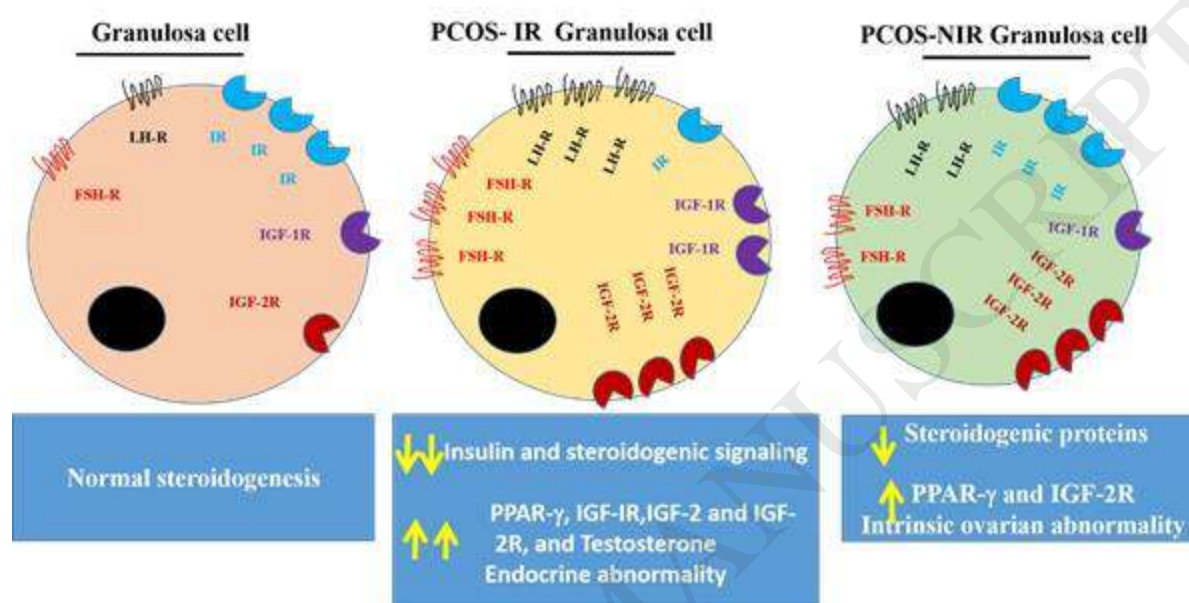


Figure 4: Schematic figure showing difference in signaling between control, PCOS-IR and PCOS-NIR luteinized granulosa cells.

Table 4: Metabolic changes in PCOS-IR and PCOS-NIR

(+: present, ∇: Decrease, Δ: Increase)

Name	Luteinized granulosa cells		
	Control	PCOS-IR	PCOS-NIR
Proteins			
INSR β	+	∇	+
PI(3)K	+	∇	+
pIRS-1	+	Δ	+
pAkt	+	∇∇	+
P38 MAPK	+	Δ	Δ
P <sub>44/42</sub> MAPK (Erk1/2)	+	Δ	Δ

Protein Kinase C $\zeta$	+	$\Delta$	+
PPARG	+	$\Delta\Delta$	$\Delta$
<b>Genes</b>			
SREBP1c	+	$\Delta\Delta\Delta$	+
FAS	+	$\Delta\Delta\Delta$	+
ACC-1	+	$\Delta\Delta$	+
CPT-1	+	$\Delta\Delta$	+
IGF1	+	$\nabla$	+
IGF1R	+	$\Delta\Delta$	+
IGF2	+	$\Delta$	+
IGF2R	+	$\Delta\Delta$	$\Delta\Delta$

**5.6.Declaration of Interest:** MB, AD, PS, MB, SG have nothing to declare. PS: Dr. Pawan Singal is the holder of the Dr. Naranjan S Dhalla Chair in Cardiovascular Research supported by the St. Boniface Hospital & Research Foundation.

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### 5.8.Authors Contribution

MB-contributed towards conception and design, performance of the experiments, analysis and interpretation of the data and drafting of the manuscript. AD- contributed towards performance of the experiments. PS and MB - Contributed in providing follicular fluid samples and

participated in discussion of the study. PS- Contributed towards critical reviewing of the manuscript. SG- Contributed towards conception and design, interpretation of the data and critical reviewing of the manuscript.

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