Universidad Rey Juan Carlos

DOCTORAL THESIS

The mitochondrial dysfunction in the granulosa cells of women with PCOS is caused by alterations in oxidative phosphorylation (OXPHOS) and the unfolded protein response, and leads to follicular dysfunction, particularly in insulin-resistant women.

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Rare sono le persone che usano la mente, poche coloro che usano il cuore,

uniche coloro che le usano entrambi.

Rita Levi-Montalcini

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Resumen

El síndrome de ovario poliquístico (SOP) es el trastorno ginecológico benigno más prevalente entre mujeres, afectando alrededor de un 4% al 21% a nivel mundial y causando infertilidad. Con mayor frecuencia (hasta 8-10 veces más) las mujeres con SOP requieren de técnicas de reproducción asistida para poder embarazarse y concebir en comparación con mujeres sin la patología. Las mujeres nacen con aproximadamente 400.000 folículos primordiales, detenidos en la profase I de la meiosis, en su corteza ovárica. En la pubertad, y con cada oleada posterior de desarrollo folicular, se activan cohortes de folículos primordiales. En los folículos primarios resultantes, comienza la comunicación bidireccional entre el ovocito y su capa circundante de células cuboidales de la granulosa (CG) para coordinar el desarrollo posterior. En el estadio de folículo secundario, las CG aumentan la expresión de receptores de FSH, lo que permite a estos folículos responder más eficazmente a las gonadotropinas, mientras que la capa de teca en desarrollo empieza a expresar receptores de LH y a producir andrógenos. De hecho, la interacción entre estos dos compartimentos foliculares durante las fases preantral y antral del desarrollo del folículo es fundamental para una esteroidogénesis adecuada. Los folículos en crecimiento secretan cantidades crecientes de estradiol, que estimula positivamente a la hipófisis anterior para que produzca LH. En circunstancias normales, se seleccionan los folículos dominantes de mayor tamaño (que producen más estradiol), mientras que los folículos más pequeños sufren oleadas de atresia. Sin embargo, estos procesos se interrumpen en las mujeres con síndrome de ovario poliquístico (SOP), lo que conduce a la disfunción ovárica, la detención folicular, y la morfología de ovario poliquístico (PCOM).

El SOP se caracteriza por tener varias etiologías posibles al igual que diferentes manifestaciones clínicas. Las mujeres SOP pueden presentar amenorrea, oligomenorrea o hipomenorrea; ovarios agrandados y/o quísticos (la denominada morfología de ovario poliquístico), hirsutismo, acné y alopecia androgénica, asociada con hiperandrogenismo; infertilidad; y varias condiciones dermatológicas. En base a diferentes combinaciones de estos síntomas junto a criterios clínicos, en

1990, los NIH establecieron los criterios diagnósticos del SOP para incluir el hiperandrogenismo (producción/secreción excesiva de andrógenos) y/o la hiperandrogenemia (aumento de andrógenos circulantes), la oligoanovulación, y excluir los trastornos relacionados. En 2003, el grupo de consenso ESHRE/ASRM (Rotterdam) añadió el recuento de folículos antrales como criterio diagnóstico clínico, y acordó que el diagnóstico de SOP debería basarse en la exclusión de trastornos relacionados y en dos de los tres criterios siguientes: oligoanovulación, signos clínicos y/o bioquímicos de hiperandrogenismo y ovarios poliquísticos. Una revisión sistemática realizada por la Androgen Excess PCOS (AE-PCOS) Society concluyó que el SOP se caracterizaba principalmente por el hiperandrogenismo. En este sentido, argumentaron que el SOP puede diagnosticarse de forma fiable clínicamente, con hirsutismo (a pesar de que las escalas visuales son subjetivas) y/o bioquímicamente, midiendo los niveles de testosterona libre (aunque reconocieron que los métodos podían ser imprecisos e insensibles). En 2012 el panel de consenso del NIH estableció cuatro fenotipos de SOP, si bien las guías internacionales más recientes abocaron por incluir la resistencia a insulina (RI) y la hiperinsulinemia como características claves del SOP.

La morfología de ovario poliquístico se caracteriza por presentar un mayor número de folículos detenidos en todas las etapas del desarrollo y ovocitos de calidad comprometida. Es resultado de una relativa deficiencia de FSH y un exceso de LH y LHR, lo que provoca que las células de la teca produzcan más andrógenos (dehidroepiandrosterona y 17-hidroxiprogesterona) y en última instancia una diferenciación terminal de las células de la granulosa de los folículos en desarrollo. En conjunto, estos procesos contribuyen a la anovulación y el aumento del número de andrógenos circulantes que caracterizan a las pacientes SOP. Respecto a esto último, si bien se observa un aumento de los niveles de andrógenos circulantes en aproximadamente el 60-80% de las pacientes con SOP, el hiperandrogenismo bioquímico no siempre se correlaciona con las manifestaciones de hiperandrogenismo clínico. Por otro lado, la resistencia insulina independiente de la obesidad es considerada otro factor determinante de la patogénesis, ya que conlleva un incremento de testosterona

libre y una estimulación de la biosíntesis de andrógenos en las células de la teca y un aumento de LHR. Debido a todos estos factores, es necesario adherirse a criterios de evaluación específicos y mantener historiales detallados de las pacientes para poder diferenciar entre las posibles etiologías del SOP.

La resistencia a la insulina y la consiguiente hiperinsulinemia en las mujeres con SOP pueden contribuir a un equilibrio redox alterado que promueve el estrés oxidativo en las células de la granulosa (CG). La hiperandrogenicidad de las mujeres con SOP aumenta aún más los niveles de estímulos profibróticos, agravando el estrés celular en las CG de los foliculos antrales y promoviendo la apoptosis. Así pues, el SOP puede aumentar la producción de especies reactivas de oxígeno y la disfunción mitocondrial, lo que, a su vez, puede activar respuestas intracelulares al estrés, como la respuesta mitocondrial a proteínas no plegadas (UPRmt) y la respuesta endoplasmática a proteínas no plegadas (UPRer). Las funciones del retículo endoplásmico (RE) rugoso (caracterizado por los ribosomas que cubren su membrana) incluyen la síntesis, el plegamiento, la modificación y el transporte de proteínas al citoplasma. Por su parte, el RE liso se encarga de la producción y el metabolismo de lípidos y esteroides. Cuando la carga de trabajo de plegamiento de proteínas excede la capacidad del RE, la acumulación resultante de proteínas desplegadas o mal plegadas en el lumen conduce al estrés del RE, que provoca un importante estrés en la células. Esta respuesta activa varias vías de transducción de señales, que promueven el plegamiento correcto o la degradación de las proteínas desplegadas y modulan una variedad de funciones celulares. En particular, la UPRer busca principalmente restaurar la homeostasis, sin embargo, si el estrés del RE no se puede resolver, puede inducir la muerte celular programada. Las cuatro acciones principales de laUPRer incluyen: (i) atenuar la traducción del ARNm para reducir la síntesis de proteínas, (ii) amplificar la síntesis de chaperonas del RE para aumentar la capacidad de plegamiento de proteínas, (iii) producir factores de degradación asociados al RE para eliminar las proteínas irreparablemente mal plegadas, e (iv) inducir la apoptosis.

En recientes años se ha promovido el estudio del efecto del estrés oxidativo provocado por la acumulación de proteínas mal plegadas en relación a la patogénesis del SOP. Como parte del **estudio I** se llevó a cabo una revisión bibliográfica para revelar que la disfunción mitocondrial en el SOP se ha relacionado con la resistencia a insulina y una peor competencia ovocitaria, además de anormalidades en otros tipos celulares como los leucocitos. La disfunción mitocondrial puede ser resultado de uno o varios factores simultáneamente: la presencia de diversas mutaciones en ADN mitocondrial de células de la granulosa en mujeres con SOP; la incapacidad por parte de la célula de responder al estrés oxidativo, en concreto una acumulación perjudicial de especies reactivas de oxígeno (ROS); y de una mala respuesta a la acumulación de proteínas desplegadas (UPRmt). Asimismo, otra respuesta a proteínas desplegadas ocurre en el retículo endoplasmático (UPRer) y puede influir en la competencia celular. Las mujeres con SOP suelen tener sobrepeso u obesidad y presentan una serie de aberraciones metabólicas asociadas a la disfunción mitocondrial. En las células somáticas y los ovocitos de las mujeres con SOP se observa un aumento de la ROS y del estrés oxidativo, asociados a mutaciones en las regiones del ADNmt que codifican los genes de la oxfosis y los ARNt, así como a una disminución del número de copias del ADNmt. Estas anomalías metabólicas y el fracaso de UPRer y UPRmt para restablecer la homeostasis pueden dar lugar a la activación de vías apoptóticas en varios tipos de células, incluidas las células de la granulosa, y acelerar el crecimiento de los folículos y la generación de ovocitos sanos. El fracaso en restaurar la homeostasis celular por parte de ambas respuestas desencadena procesos de apoptosis en las células de la granulosa. No obstante, es necesario llevar a cabo más estudios para desvelar los mecanismos específicos de la patogénesis del SOP en relación con la disfunción mitocondrial, y evaluar la respuesta a las proteínas desplegadas en pacientes con RI y sin RI.

Como parte del **estudio II**, y con el objetivo de caracterizar los perfiles transcriptómicos de células de la granulosa y células de la sangre periférica entre mujeres con SOP, jóvenes pacientes bajo-respondedoras (BR) y normo-respondedoras (NR), reclutamos un total de 60 pacientes (n=20/grupo)

sometidas a estimulación ovárica controlada. Tras una extracción de ARN seguida de una secuenciación y análisis bioinformático para revelar genes diferencialmente expresados y funciones enriquecidas entre grupos, desvelamos varios genes con potencial efecto en la patogénesis del SOP. Entre las células de la granulosa de pacientes SOP y NR, revelamos la disregulación de *MBE2M* y *MTMR9* ambos asociados a procesos apoptóticos, mientras que entre pacientes SOP y BR, determinamos una sobre-expresión de genes de asociados a la fosforilación oxidativa en la mitocondria, que puede relacionarse con una disminución del ADNmt y el estrés oxidativo. Entre las células de la sangre periférica en pacientes SOP y NR, encontramos la sobre-expresión del componente ARN de la telomerasa en pacientes SOP, que si bien puede deberse al estrés oxidativo; y la regulación a la baja del gen *RAB4B*, cuya deficiencia se ha asociado al desarrollo de una resistencia a insulina; en la comparación entre SOP y BR, destacamos *ZPF57* y *PXDN*, ambos regulados a la baja y relacionados con el desarrollo de la matriz extracelular y la metilación de ADN, respectivamente. En muchos aspectos, los numero de folículos y los patrones de respuesta a la estimulación controlada de las mujeres con SOP y BR parecen estar en los extremos opuestos de un mismo espectro. Por lo tanto, merece la pena investigar si estos dos importantes trastornos relacionados con la fertilidad se desarrollan como consecuencia de una perturbación en una única vía (es decir, regulación al incremento o a la baja de grupos de genes similares). Nuestro estudio es un paso en esta dirección y nuestros hallazgos preliminares sugieren diferencias específicas entre las mujeres con SOP y BR. Se necesitan futuros estudios con un tamaño de muestra adecuado dirigidos a subgrupos bien definidos de estos trastornos extremadamente heterogéneos para delinear vías génicas compartidas que puedan explotarse para identificar líneas terapéuticas.

En el **estudio III**, y para evaluar la expresión de genes implicados en las respuestas a las proteínas desplegadas en la mitocondria y retículo endoplasmático de células de la granulosa, reclutamos 60 pacientes; 40 de ellas con SOP (20 con RI y 20 sin RI) y 20 NR. Tras un protocolo de transcripción reversa a partir del ARN extraído de células de la granulosa y el posterior análisis estadístico entre

grupos, desvelamos la sobreexpresión, especialmente significativa en el grupo de pacientes SOP con RI, de las chaperonas *HSP10* y *HSP40* de la respuesta UPRmt. Estas chaperonas promueven la supervivencia celular en caso de estrés debido a proteínas mal plegadas, lo que explicaría un intento de las CGs de responder a altas condiciones de estrés que pueden desencadenar la apoptosis celular. Las proteínas de choque térmico (HSP) promueven la supervivencia celular actuando como chaperonas moleculares que ayudan a plegar correctamente las proteínas mal plegadas nacientes y acumuladas por el estrés, para evitar su acumulación. De hecho, las HSP mitocondriales desempeñan una función protectora que permite a las células sobrevivir en condiciones letales. Sorprendentemente, las HSP pueden contrarrestar tanto las vías apoptóticas intrínsecas como extrínsecas, (i) modulando varias vías de señalización fuera de las mitocondrias, (ii) controlando la liberación de moléculas pro-apoptóticas de las mitocondrias, y (iii) previniendo la activación de las caspasas y la consiguiente apoptosis. *HSP10*, además, suprime la poliubiquitinación del *IGF1R*, impidiendo su degradación. Como resultado, contribuye potencialmente a la patogénesis del SOP en las pacientes con RI incrementando la biogénesis de andrógenos en las células de la teca, debido a un aumento en la adquisición de receptores de LH. Similarmente, en las mujeres SOP, especialmente con RI, presentaron una mayor expresión de *IRE1*, *ATF4* y de *XBP1* que los controles, siendo reguladores de chaperonas (como *Bip*) que promueven mecanismos para aliviar el estrés debido a proteínas desplegadas en el RE. Tanto para las pacientes con RI y sin RI, la sobreexpresión de *CHOP*, factor proapoptótico, indica que no pudo recuperarse la homeostasis en las CGs, probablemente conllevando un estallido apoptótico. Nuestros hallazgos sugieren que los GC de las mujeres con SOP (con o sin IR) están metabólicamente alterados y regulan los genes UPRer y UPRmt. Nuestro estudio contribuye a la comprensión de los mecanismos moleculares que subyacen a los cambios patológicos que se producen en el microambiente folicular de las mujeres con SOP.

En vista de los resultados obtenidos en los estudios II y III y en línea con la revisión bibliográfica del estudio I, demostramos que la disfunción folicular relacionada con el SOP está mediada por

alteraciones en diferentes mecanismos moleculares en las mitocondrias y en retículo endoplasmático de células de la granulosa. Estas complejidades en el desarrollo folicular reafirman la necesidad de llevar a cabo un mayor número de estudios con los que desarrollar futuras estrategias terapéuticas dirigidas a aliviar el estrés celular, especialmente en pacientes con resistencia a insulina, y, en última instancia, mejorar la competencia de desarrollo de los ovocitos de pacientes con SOP.

Abstract

Polycystic ovarian syndrome (PCOS) is a prevalent benign gyneacological disorder, affecting approximately 4-20% women worldwide and causing infertility. To achieve pregnancy and a live birth, women with PCOS require assisted reproduction techniques 8-10x more than healthy women. PCOS is a heterogeneous disorder, with elusive etiologies and different clinical presentations. Women with PCOS may present with amenorrhea, oligomenorrhea, or hypomenorrhea; enlarged and/or cystic ovaries (the polycystic ovarian morphology [PCOM]); hirsutism, acne or oily skin, and androgenic alopecia associated with clinical hyperandrogenism; infertility; and several dermatological conditions. In 2012, the NIH consensus panel established four phenotypes of PCOS, based on different combinations of these clinical and metabolic criteria, and these provided the foundation for the most recent international guidelines (2018), which additionally included insulin resistance (IR) and hyperinsulinemia as key PCOS characteristics. The PCOM is characterized by a higher number of follicles arrested at all developmental stages, often containing immature oocytes with compromised quality. This phenotype results from the relative deficiency in FSH, which impedes follicle growth and synthesis of estrogen from the granulosa cells (GCs), and excess in LH and LH receptor, that lead to the terminal differentiation of the GCs. Collectively, these processes contribute to the anovulation and elevated circulating androgen levels experienced by patients with PCOS. Specifically, the aberrant neuroendocrine signaling causes the theca cells to produce more androgens and secrete higher levels of dehydroepiandrosterone and 17-hydroxyprogesterone in response to LH, compared to normoresponder patients. While augmented circulating androgen levels are observed in approximately 60–80% of patients with PCOS, biochemical hyperandrogenism does not always correlate with manifestations of clinical hyperandrogenism, thus it is immediately to establish focused evaluation criteria and maintain detailed patient histories to be able to differentiate possible etiologies. On the other hand, the bioavailability of testosterone is enhanced by IR, via reduction of sex hormone binding globulin levels in serum, and hyperinsulinaemia has been

associated with the antral follicle dysfunction and anovulation in PCOS, though the exact mechanisms remain unclear. Clinically, almost 75% of PCOS patients present varying levels of IR, which is a major determinant of PCOS pathogenesis, independent of obesity. Insulin signaling through the insulin-like growth factor receptors (IGFRs), which are overexpressed in the ovaries of obese women with PCOS, is postulated to stimulate the theca production of androgens, lead to hyperplasia of the thecal/stromal compartment, promote the premature acquisition of the LH receptors, and ultimately, leads to early follicular luteinization. Despite the clinical implications of IR in women with PCOS, IR testing is not routinely performed for these patients, and there is currently no agreement regarding the best methodology for diagnosing IR and characterizing its severity in PCOS.

There is increasing interest for elucidating the connection between oxidative stress and PCOS pathogenesis. For **Study I**, we conducted a literature review to compile evidence on how mitochondrial dysfunction in women with PCOS is related to IR, diminished oocyte competence and metabolic abnormalities in leukocytes. Mitochondrial dysfunction can be driven by the presence of one or several factors simultaneously: mutations in the mitochondrial DNA of GCs; cellular inability to respond to oxidative stress, specifically a detrimental accumulation of reactive oxygen species (ROS); and a poor response to the accumulation of unfolded proteins (UPRmt). Likewise, another response to unfolded proteins occurs in the endoplasmic reticulum (UPRre) and may influence GCs competency. Failure to restore cellular homeostasis by both responses triggers apoptosis processes in GCs. However, further studies are needed to unravel the specific mechanisms of PCOS pathogenesis in relation to mitochondrial dysfunction, and the response to unfolded proteins in IR and non-IR patients.

As part of **Study II**, and with the aim of characterizing the transcriptomic profiles of granulosa cells and peripheral blood cells among PCOS patients, young poor responders (YPRs) and normoresponders (YNRs) patients, we recruited a total of 60 patients (nºPCOS = 20; nºYPRs = 20; nºYNRs = 20) undergoing controlled ovarian stimulation. After RNA extraction followed by

sequencing and bioinformatics analysis to reveal differentially expressed genes and enriched functions between groups, we uncovered several genes with potential effect on PCOS pathogenesis. Among granulosa cells from PCOS and YNRs patients, we revealed dysregulation of *MBE2M* and *MTMR9* both associated with apoptotic processes, while among PCOS and YPRs patients, we determined an overexpression of oxidative phosphorylation-associated genes in mitochondria, which may be related to decreased mtDNA and oxidative stress. Among peripheral blood cells in PCOS and YNRs patients, we found over-expression of the RNA component of telomerase in PCOS patients, which may be due to oxidative stress; and down-regulation of the *RAB4B* gene, whose deficiency has been associated with the development of insulin resistance. In the comparison between PCOS and YPRs, we highlighted *ZPF57* and *PXDN*, both downregulated in the PCOS group and related to extracellular matrix development and DNA methylation, respectively.

In **Study III**, we recruited 60 patients: 40 of them with PCOS (20 with IR and 20 without IR) and 20 YNRs to compare the expression of genes involved in the responses to proteins unfolded in the mitochondria and endoplasmic reticulum of granulosa cells. After a reverse transcription protocol from RNA extracted from granulosa cells and subsequent statistical analysis between groups, we revealed the overexpression, especially significant in the group of PCOS patients with IR, of the chaperones *HSP10* and *HSP40* of the UPRmt response. These chaperones promote cell survival under stress due to misfolded proteins that we found overexpressed in GCs of patients, which would explain an attempt of GCs to respond to high stress conditions leading to apoptosis. *HSP10*, furthermore, suppresses IGF1R polyubiquitination, preventing its degradation, and potentially contributing to the pathogenesis of PCOS in IR patients by increasing androgen biogenesis in theca cells, due to an increase in LH receptor acquisition. Similarly, PCOS women, especially with IR, had higher expression of *IRE1*, *ATF4* and *XBP1* than controls, being chaperone regulators (such as *Bip*) that promote stress-relieving mechanisms due to accumulation of unfolded proteins in the ER. For both

IR and non-IR patients, overexpression of *CHOP*, a pro-apoptotic factor, indicates that homeostasis could not be recovered in GCs, probably leading to an apoptotic burst.

In view of the results obtained in **Studies II and III**, and in line with the literature review of **Study I**, we demonstrate that PCOS-related follicular dysfunction is mediated by alterations in different molecular mechanisms in the mitochondria and endoplasmic reticulum of the granulosa cells. These complex factors linked to the proper development of follicles reaffirm the need for further studies to develop therapeutic strategies aimed at alleviating cellular stress, especially in patients with insulin resistance, and ultimately improving the developmental competence of oocytes in PCOS patients.

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List of abbreviations

AE-PCOS	Androgen Excess PCOS
AMH	Anti-müllerian hormone
ART	Assisted reproductive technology
ATF4/6	Activating transcription factor 4/6
ATP	Adenosine triphosphate
BMI	Body mass index
Bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CHOP	CCAAT/enhancer-binding protein homologous protein
COS	Controlled ovarian stimulation
COX2	Cytochrome C oxidase II
CYT B	Cytochrome B
DEG	Differentially expressed gene
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1
DOR	Diminished ovarian response
DR5	Death receptor 5
dTMP	2'-deoxythymidine 5'-monophosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic reticulum
ESHRE	European Society of Human Reproduction and Embryology
ETC	Electron transport chain
FDA	United States Food and Drug Administration
FDR	False discovery rate
FPG	Fasting plasma glucose
FPKM	Fragments per kilobase of exon per million fragments mapped
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Granulosa cell
GnRH	Gonadotrophin releasing hormone
GnRHa	Gonadotrophin releasing hormone agonist
GO	Gene Ontology
GRP78	78 kDa glucose-regulated protein
h	Hour
hCG	Human chorionic gonadotropin
HOMA	Homeostasis model assessment
HP-HMG	Highly purified human menopausal gonadotropin
HSP	Heat shock protein
IGFR	Insulin-like growth factor receptor
IR	Insulin resistance
IRE1	Inositol-requiring enzyme-1
IR-PCOS	Insulin resistance and polycystic ovarian syndrome
ITH	Transcription initiation sites for the heavy strand [of mtDNA]
ITL	Transcription initiation sites for the light strand [of mtDNA]
IU	International unit
LH	Luteinizing hormone

lncRNA	Long non-coding RNA
M	Million
MFRP	Membrane Frizzled-Related Protein
MII	Metaphase II
Min	Minute
µL	Microliter
mL	Milliliter
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTMR9	Myotubularin Related Protein 9
MT-RNR1/2	Mitochondrially-encoded 12S ribosomal RNAs
ND1-6	Mitochondrially-encoded NADH dehydrogenase subunits 1-6
Ng	Nanogram
NIH	National Institute of Health
nIR-PCOS	Non-insulin resistance and polycystic ovarian syndrome
nM	Nanomolar
OHSS	Ovarian hyperstimulation syndrome
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovarian syndrome
PERK	ER-membrane protein kinase RNA-like ER kinase
POR	Poor ovarian response
qRT-PCR	Quantitative real-time polymerase chain reaction
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
SHBG	Sex hormone binding globulin
SIRT1	Sirtuin 1
TERC	Telomerase RNA component
tRNA	Transfer ribonucleic acid
TUDCA	Tauroursodeoxycholic acid
UBE2M	Ubiquitin Conjugating Enzyme E2 M
UPR	Unfolded protein response
UPR^{er}	Endoplasmic reticulum unfolded protein response
UPR^{mt}	Mitochondrial unfolded protein response
XBP1	X-box-binding protein 1
YNR	Young normoresponder
YPR	Young poor responder

CHAPTER 1: INTRODUCTION

1.1. Ovarian function

The female ovaries are small bilateral pelvic organs that provide two key functions for sexual reproduction. Each month, they produce mature oocytes for fertilization and synthesize the ovarian hormones (estrogen and progesterone), which are not only required to establish and maintain a healthy pregnancy, but also contribute to growth, metabolism, sexual function, and visible secondary sexual characteristics. Notably, the ovarian hormones negatively regulate the hypothalamic-pituitary-ovarian axis (1). The pulsatile secretion of the gonadotrophin releasing hormone (GnRH) stimulates synthesis and secretion of gonadotrophins from the anterior pituitary gland. Low frequency pulses of GnRH stimulates the production of follicle stimulating hormone (FSH) during the follicular phase of the menstrual cycle, while the surge of high frequency pulses of GnRH stimulates the production of luteinizing hormone (LH) to induce ovulation and initiate the subsequent luteal phase (2).

1.1.1. Folliculogenesis and follicular steroidogenesis

Women are born with approximately 400,000 primordial follicles (3), arrested at prophase I of meiosis, in their ovarian cortex. At puberty, and with every subsequent wave of follicular development, cohorts of primordial follicles are activated. In the resulting primary follicles, bidirectional communication begins between the oocyte and their surrounding layer of cuboidal granulosa cells (GCs) to coordinate subsequent development (4, 5). At the secondary follicle stage, the GCs increase expression of FSH receptors, allowing these follicles to respond more effectively to gonadotrophins (5, 6), while the developing theca layer begins to express LH receptors and produce androgens (7). Indeed, the cross-talk between these two follicular compartments during the preantral and antral stages of follicle development is fundamental for proper steroidogenesis (8) **(Figure 1)**. Growing follicles secrete increasing amounts of estradiol, which positively stimulates the anterior pituitary gland to produce LH. Under normal circumstances, the larger dominant follicles (which produce more estradiol) are selected for, while the smaller follicles undergo waves of atresia. However, as will be described in detail in the following sections of this Ph.D. dissertation, these

processes are disrupted in women with polycystic ovarian syndrome (PCOS), leading to ovarian dysfunction, follicular arrest, and the polycystic ovarian morphology (PCOM).

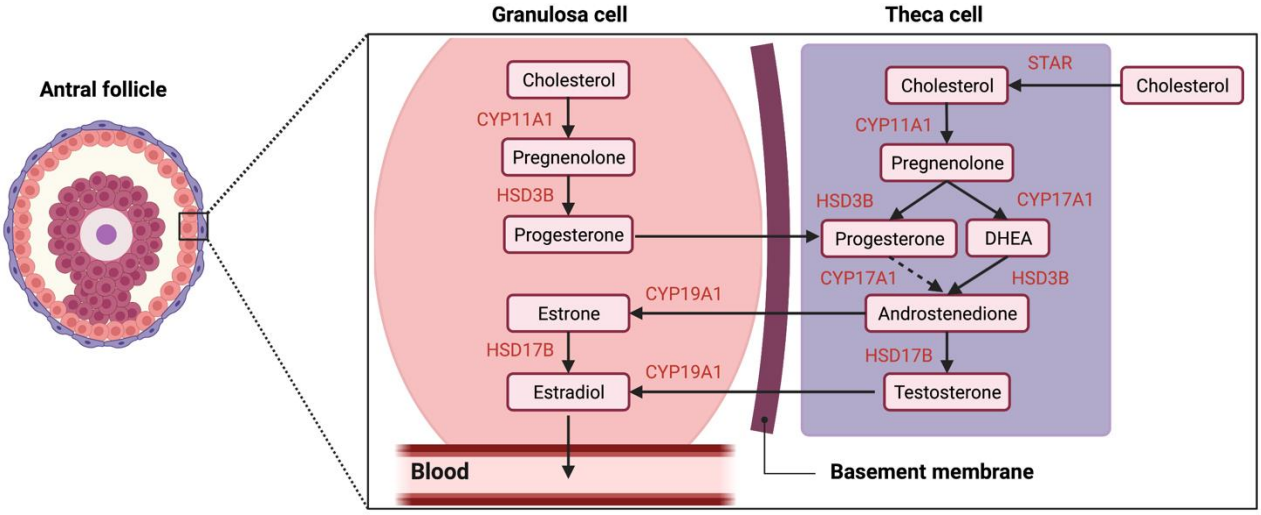

Figure 1: Normal follicular steroidogenesis. During each menstrual cycle, the granulosa and theca cells surrounding the oocyte produce progestagens, androgens and estrogens. In response to intracellular signaling cascades induced by the luteinizing hormone, STAR and CYP11A1 respectively transport and convert cholesterol into pregnolonone, a precursor of progesterone. During the follicular phase, progesterone from the granulosa cells is transported into the theca cells to be converted into androgens by CYP17A1 (an enzyme which is exclusively expressed in theca cells). Subsequently, the androgens are then returned to the granulosa cells to be converted to estrogens by CYP19A1 (an enzyme which is exclusively expressed in the granulosa cells). Increasing local estrogen biosynthesis in the ovary contributes to the cyclical recruitment of the dominant follicle for ovulation. Meanwhile, smaller follicles (which produce less estradiol) undergo waves of atresia. CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; CYP17A1, Cytochrome P450 Family 17 Subfamily A Member 1; CYP19A1, Cytochrome P450 Family 19 Subfamily A Member 1 (also known as aromatase); DHEA, dehydroepiandrosterone; HSD3B, 3β-Hydroxysteroid dehydrogenase; HSD17B, 17β-Hydroxysteroid dehydrogenase; STAR, steroidogenic acute regulatory protein.

1.1.2. Oocyte developmental competence

Oocyte quality or developmental competence is defined by an oocyte's ability to undergo cytoplasmic and nuclear maturation, such that it is then able to be fertilized normally, develop into an embryo, and ultimately produce viable offspring (9-11). Growing oocytes undergo a myriad of cytoplasmic changes during maturation, including an increase in the number of mitochondria and ribosomes for protein and RNA synthesis, relocation of the Golgi apparatus and endoplasmic reticulum (ER) to the periphery of the oocyte for their respective involvement in cortical granule formation and exocytosis (12). In terms of the nuclear maturation, the resumption of meiosis until

metaphase II (MII) is mediated by the condensation of maternal chromosomes, formation of meiotic spindles, and asymmetric cell division to extrude the first polar body (13).

Remarkably, the GCs adjacent to the oocyte are important determinants of oocyte development potential (14), as they produce a functional syncytium that provides important bidirectional communication with the oocyte during maturation, and maintains it quiescent until ovulation (15, 16). Indeed, the transzonal projections from the GCs to the oocyte plasma membrane direct unique paracrine signaling molecules that assist oocyte metabolism and development, while the oocytes produce specific factors that facilitate GC differentiation and function (11, 12, 17-20).

1.2. Polycystic ovarian syndrome

Polycystic ovary syndrome (PCOS) is the most prevalent benign gynecological disorder; it affects between 4-21% of women globally (21) and causes infertility (22, 23). Women with PCOS require assisted reproductive technology (ART) eight to ten times more frequently than those without PCOS (24, 25).

The key features of PCOS, which will be described in detail in the following sections, are depicted in **Figure 2**.

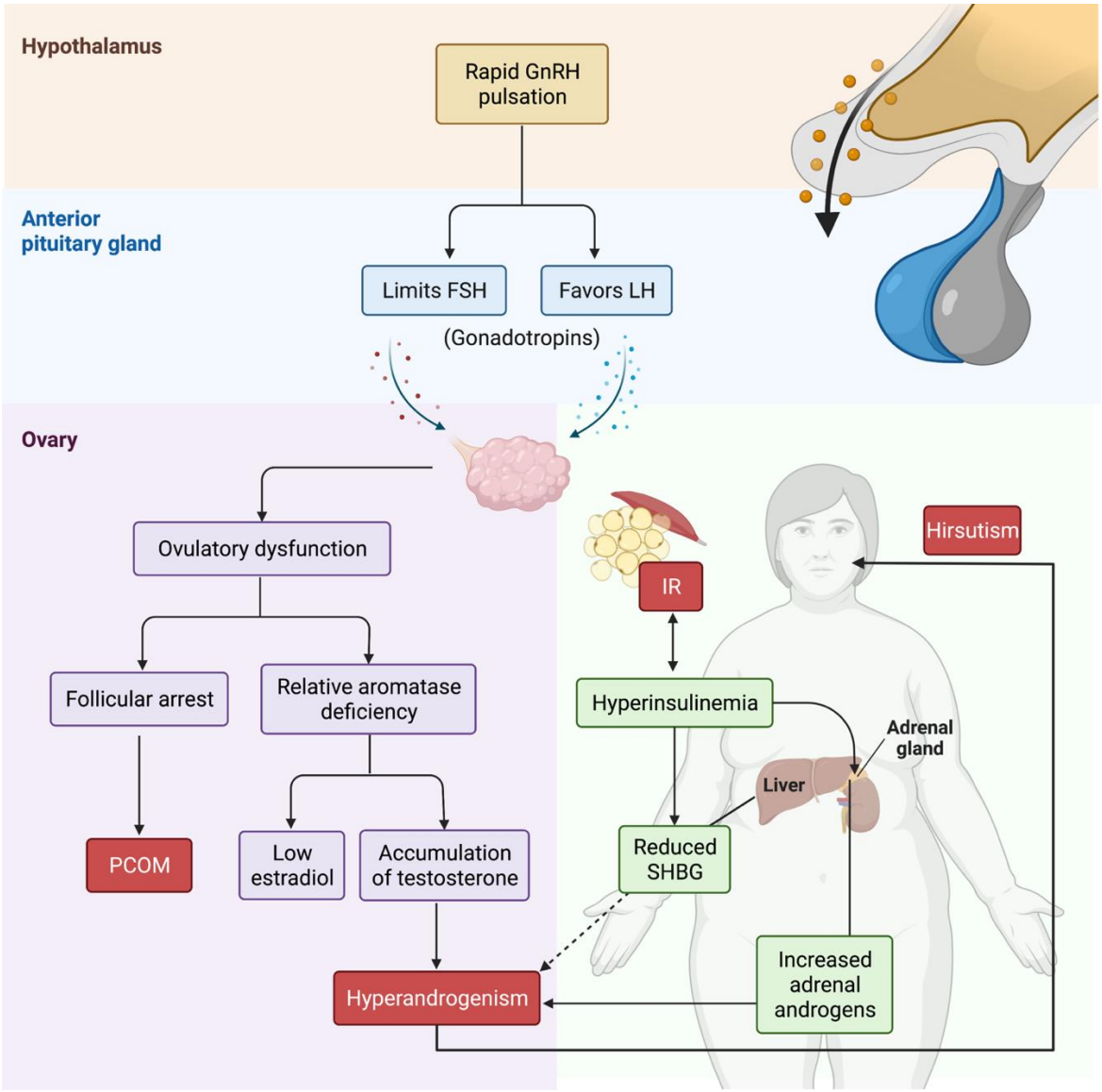

Figure 2: Polycystic ovarian syndrome. FSH, follicle stimulating hormone; GnRH, Gonadotropin releasing hormone; IR, insulin resistance; LH, luteinizing hormone; PCOM, polycystic ovarian morphology; SHBG, sex hormone binding globulin.

1.2.1. The evolution of the clinical classification of PCOS

PCOS is a heterogeneous disorder, with elusive etiologies and different clinical presentations. Women with PCOS may present with amenorrhea, oligomenorrhea, or hypomenorrhea; enlarged and/or cystic ovaries (the PCOM; whose pathophysiology will be described in detail in section 1.2.2); hirsutism (defined as hair growth in different androgen-sensitive sites), acne or oily skin, and androgenic alopecia, associated with hyperandrogenism (described in detail in section 1.2.3); infertility; and dermatological conditions such as skin tags and acanthosis nigricans (characterized by

the thickening and darkening of the upper skin layers, which results in a velvety appearance) on the back of the neck, in the armpits and under the breasts (28). As such, it has been difficult to reach a global consensus on which key features of PCOS to include in its diagnosis (21).

In 1990, the NIH established PCOS diagnostic criteria to include hyperandrogenism (excessive production/secretion of androgens) and/or hyperandrogenemia (increased circulating androgens), oligo-anovulation, and exclude related disorders (29). In 2003, the ESHRE/ASRM (Rotterdam) consensus group (30) added the antral follicle count as a clinical diagnostic criterion, and agreed that a PCOS diagnosis should be based on the exclusion of related disorders and two of the following three criteria: oligo-anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries. A systematic review by the Androgen Excess PCOS (AE-PCOS) Society concluded that PCOS was mainly characterized by hyperandrogenism. In this regard, they argued PCOS can reliably be diagnosed clinically, with hirsutism (despite the visual scales being subjective) and/or biochemically, by measuring free testosterone levels (even though they recognized the methods could be inaccurate and insensitive) (31).

Based on these considerations, the 2012 NIH consensus panel (21) established four phenotypes of PCOS, based on different combinations of clinical and metabolic criteria (**Figure 3**). As reviewed by Lizneva *et al.* (21), the evidence gathered from clinical populations suggests that "classic PCOS" (phenotypes A and B), "ovulatory PCOS" (phenotype C) and "nonhyperandrogenic PCOS" (phenotype D) were respectively associated with severe, intermediate and mild symptomology. Interestingly, international epidemiological studies have shown that half of the clinical PCOS populations are classified as phenotype A, while phenotypes B, C and D have almost equal prevalence (21). Conversely, in medically unbiased (i.e., "natural" or unselected) populations, ~66% of PCOS patients were classified as phenotypes B and C, while phenotypes A and D were the least prevalent (21). Further, the higher metabolic risks observed in PCOS patients that clinically present with higher obesity and hyperandrogenemia (with respect to the unselected populations),

reinforce the need for a rapid diagnosis and subphenotype classification to provide personalized treatment, prevent long-term health consequences related with PCOS, and ultimately improve quality of life (32).

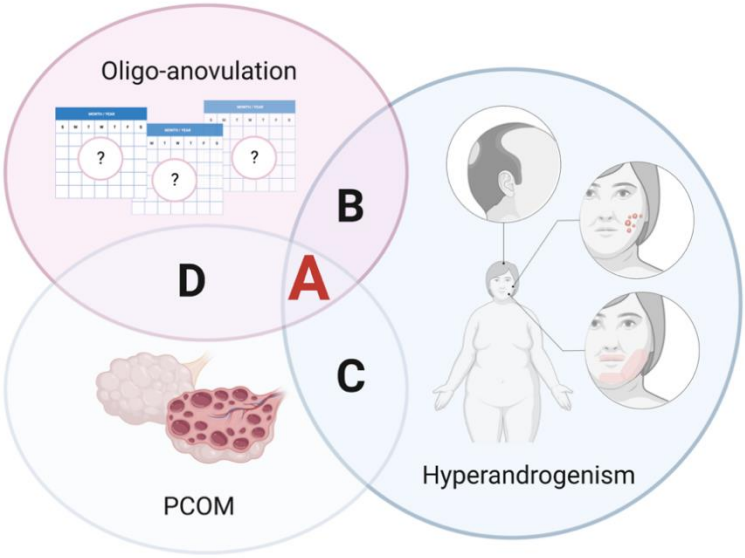

Figure 3: Phenotypes of polycystic ovarian syndrome. The four phenotypes (A-D) established by the NIH consensus panel (21) were as follows: phenotype A, characterized by hyperandrogenism (clinical and biochemical), chronic ovulatory dysfunction and the polycystic ovary morphology (PCOM); phenotype B, characterized by hyperandrogenism (clinical and biochemical) and chronic ovulatory dysfunction; phenotype C, characterized by hyperandrogenism (clinical and biochemical) and PCOM; and phenotype D, characterized by ovulatory dysfunction and PCOM. Within the context of PCOS, hyperandrogenism presents as androgenic alopecia, excessive acne, and hirsutism.

Table 1 summarizes the differential diagnoses for PCOS, as established by the Endocrine Society in 2013 (33), which range from endocrinopathies to malignant etiologies (34). Once these conditions are ruled out, patients should subsequently be screened for hypertension, dyslipidemia (using a lipid panel), type 2 diabetes mellitus (using a two-hour oral glucose tolerance test), obstructive sleep apnea (in overweight patients), and depression, due to their association with PCOS (34).

Table 1: Recommended diagnostic workup for patients presenting with hyperandrogenism (including hirsutism, androgenic alopecia, and excessive acne), and ovulatory dysfunction.

SYMPTOMS	TEST	CONDITION TO RULE OUT
missed period, nausea and vomiting	Pregnancy	Pregnancy
weight gain, tiredness, slow movements and thoughts, muscle cramps	Serum TSH	Thyroid dysfunction
oligomenorrhea	Prolactin	Hyperprolactinemia
oligomenorrhea, infertility, acne, and premature pubarche	17-hydroxyprogesterone*	Nonclassical congenital adrenal hyperplasia
Low body weight, eating disorder, or excessive exercise	Serum LH, FSH, and estradiol	Hypothalamic amenorrhea
Hot flashes and urogenital symptoms	Serum FSH and estradiol	Primary ovarian insufficiency
Severe virilization (change in voice, clitomegaly, rapid onset)	Total testosterone* DHEA-S* Ovarian ultrasound CT or MRI of adrenals	Androgen-secreting tumor
Buffalo hump, purple striae, hypertension	Salivary/urinary cortisol Dexamethasome suppression	Cushing syndrome
Change in hat/glove size, protruding jaw, impaired vision	ILGF-1	Acromegaly

CT, computed tomography; DHEA-S, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; ILGF-1, insulin-like growth factor 1; LH, luteinizing hormone; MRI, magnetic resonance imaging; TSH, thyroid stimulating hormone. *Ideally measured in the morning, during the follicular phase.

Notably, the most recent international guidelines (from 2018) (35) acknowledged insulin resistance (IR) as another key feature of PCOS (but did not yet recommend clinical testing for IR), and emphasized that serum anti-müllerian hormone (AMH) levels should not yet be used as an alternative to ultrasound-based diagnoses, or as a single test for the diagnosis of PCOS, due to the limitations of AMH measurement (36). Finally, the AE-PCOS (31) established that the PCOM can be diagnosed using a threshold of ≥25 follicles [when an ultrasound with maximal resolution (≥8 MHz) is employed] or ovarian volume ≥ 10 mL. The international guidelines (35) agreed with the latter, but revised the follicle count to ≥20 follicles per ovary (in at least one ovary).

Despite these progressive improvements in the clinical classifications of PCOS over the last decades, a more in-depth understanding of the molecular mechanisms underlying PCOS

pathophysiology can help identify the dysregulated pathways that potentially contribute to the spectrum of PCOS pathogenesis.

1.2.2. Dysfunctional folliculogenesis and compromised oocyte competence

The PCOM is characterized by a higher number of follicles arrested at all developmental stages (37, 38), often containing immature oocytes with compromised quality (39). Notably, the relative deficiency of FSH (40, 41) impedes follicle growth and synthesis of estrogen from the GCs, whereas the excess of LH, and inappropriate acquisition of LH receptors in small follicles, induces the terminal differentiation of the GCs (42-44). Together, these processes lead to the premature arrest of follicle development (resulting in a pool of small follicles, range 2–8 mm in size), the inability to select a dominant follicle, and ultimately, the anovulation experienced by patients with PCOS (42, 45, 46).

Following controlled ovarian stimulation with exogenous FSH (using clomiphene or human recombinant FSH), women with PCOS develop a larger number of follicles as a result of an exacerbate response, but end up with a similar number of embryos, clinical pregnancy and live birth rate (per initiated cycle) as normoresponders, due to the lower fertilization potential and higher risk of miscarriage, respectively (39, 47-52). Further, chromosomal abnormalities were significantly higher among the miscarriages of PCOS patients compared to controls (61.3% vs. 47.8%) (53). Collectively, these findings suggest that the quality of oocytes and embryos of women with PCOS may be compromised, and that factors other than anovulation may contribute to their PCOS-related infertility.

1.2.3. Hyperandrogenism

Hyperandrogenism, or androgen excess, is a common reproductive endocrinopathy affecting 5-10% of reproductive-aged women (54). Clinical hyperandrogenemia is characterized by hirsutism (hair growth in different androgen-sensitive sites), acne, androgenic alopecia and different degrees of virilization, which leads to psychological morbidity and negatively affects quality of life (54, 55).

Interestingly, these manifestations can be present without biochemical hyperandrogenism, which is defined as an elevated serum level of one or more androgens (i.e., total testosterone [≥2.1 nmol/L], free testosterone [≥0.03 pmol/L], dehydroepiandrosterone sulfate [DHEAS; ≥6.7 μmol/L], and androstenedione [≥8.6 nmol/L]) (56). However, since some patient populations (e.g., Asian women) can have naturally elevated androgen levels without showing clinical manifestations, and clinical findings can range in severity between patients or change within the same patient over time, it is important to establish focused evaluation criteria and maintain detailed patient histories to be able to differentiate possible etiologies (54).

Augmented circulating androgen levels are observed in approximately 60–80% of patients with PCOS (55), but have also been associated with idiopathic hirsutism, non-classical congenital adrenal hyperplasia, IR, acanthosis nigricans, ovarian or adrenal androgen-secreting neoplasms, androgenic drug intake, Cushing's syndrome and hyperprolactinemia (54). In PCOS, aberrant neuroendocrine signaling causes the theca cells to produce more androgens and secrete higher levels of dehydroepiandrosterone and 17-hydroxyprogesterone in response to LH, compared to normoresponder patients (36, 57-59). In this regard, anti-androgens alone or in combination with hormonal contraceptives can be used to alleviate symptoms (60).

1.2.4. Insulin resistance

Clinically, almost 75% of PCOS patients present varying levels of IR (61, 62), which results from abnormal insulin signaling and metabolic dysfunction in insulin-responsive tissues, and is associated with a substantial risk for developing type 2 diabetes mellitus, coronary artery disease (63, 64), and possibly even PCOS-related infertility (61, 65). IR is a major determinant of PCOS pathogenesis (66), independent of obesity (67), because it enhances the bioavailability of testosterone (68) by reducing the serum level of sex hormone binding globulin (SHBG; produced by the liver). In this regard, hyperinsulinaemia has been associated with the antral follicle dysfunction and anovulation in PCOS (42, 69-71). Although the mechanisms of insulin signaling in PCOS remain

unclear, insulin signaling through the insulin-like growth factor receptors (IGFRs) is postulated to stimulate the theca production of androgens (42, 68), lead to hyperplasia of the thecal/stromal compartment in women with PCOS (69), and promote the premature acquisition of the LH receptors, which leads to early follicular luteinization (72). Notably, a recent study suggested that the insulin receptor is overexpressed in the ovaries of obese women with PCOS, and repressed in metabolic tissues, respectively contributing to androgen excess and the peripheral IR (73).

Although IR is also associated with increased abdominal body fat over a wide range of body mass index (BMI) values (29), the relationship between IR and BMI in women with PCOS remains controversial. Some studies suggest that non-obese women with PCOS may have increased IR independent of BMI (46), while others report a correlation between weight and IR (74, 75). On one hand, the greater proportion of adipocytes in normal-weight women with PCOS likely contributes to peripheral IR and associated hyperinsulinemia (74). While on the other hand, obesity is associated with ovulatory dysfunction, in addition to altered metabolism and mitochondrial dysfunction in oocytes, culminating in impaired oocyte quality (76). Oocytes of obese mice fed a high-fat diet have abnormal morphologies, lower adenosine triphosphate (ATP) production, and higher levels of detrimental reactive oxygen species (ROS) contributing to the oxidative stress in oocytes and embryos. Interestingly, weight loss (from dietary restriction) significantly reduced ROS production and oxidative damage to lipids, proteins, and amino acids in leukocytes (77). Smits *et al.* (2021) (78)similarly observed that high-fat diets had negative effects on the metabolic health, oocyte quality and pregnancy rates of mice.

Despite the clinical implications of IR in women with PCOS, IR testing is not routinely performed for these patients, and there is currently no agreement regarding the best methodology for diagnosing IR and characterizing its severity in PCOS (79).

1.2.4.1. The homeostasis model assessment (HOMA) and its diagnostic potential

The homeostasis model assessment (HOMA) was first developed in 1985, by Matthews *et al.* (80), to quantify IR using the concentration of fasting plasma glucose (FPG) and insulin (or C-peptide) concentrations. Insulin levels depend on the pancreatic β-cell response to glucose concentrations, while glucose concentrations are regulated by insulin-mediated glucose production via the liver. Thus, deficient β-cell function will echo a diminished response of β-cells to glucose-stimulated insulin secretion. Similarly, IR is reflected by the diminished suppressive effect of insulin on hepatic glucose production. The HOMA model has proved to be a robust clinical and epidemiological tool that assesses the glucose-insulin homeostasis through a set of simple, mathematically derived nonlinear equations. The equation is derived from the use of the insulin-glucose product, divided by a constant, as follows:

$$\mathrm{HOMA} = \frac{\text{insulin (mIU/L)} \times \text{FPG (mmol/L)}}{22.5}$$

In 2018, Lewandowski *et al.* (81) argued that women with PCOS have higher HOMA values than the general population. This was supported by Wiweko *et al.* (2018), who found a positive correlation between serum AMH and HOMA IR values – which were significantly higher for the most severe PCOS phenotype (A; manifesting with hyperandrogenism, the PCOM and ovulatory dysfunction; **Figure 3**).

1.3. The pathophysiology of PCOS

1.3.1. Mitochondrial dysfunction: clinical implications and a glimpse into the molecular aspects

Metabolic abnormalities and mitochondrial dysfunction in women with PCOS likely contribute to reproductive challenges. Increased peripheral IR and type 2 diabetes mellitus are associated with alterations in mitochondrial function, dynamics, and biogenesis (82), and GC

mitochondrial dysfunction in women with diabetes was associated with reduced oocyte competence and worse pregnancy outcomes (83). In women with PCOS, impairment of mitochondrial function was observed in leukocytes and endothelial cells (84, 85). Similarly, Skov *et al.* (86) reported reduced expression of mitochondrial oxidative phosphorylation (OXPHOS) genes in the skeletal muscle of women with PCOS. Collectively, these data support the hypothesis that abnormalities in mitochondrial function and ensuing abnormal metabolic milieu play a role in the pathogenesis of PCOS and underlie the associated infertility.

Mitochondrial DNA (mtDNA) is highly conserved among mammals (87). Human mitochondria contain circular double-stranded mtDNA of approximately 16.5 kb. Among the 37 encoded genes, 2 are for the small and large subunits of ribosomal RNA (rRNAs; 12S and 16S, respectively), and 22 are for transfer RNAs (tRNAs) that are sufficient to translate the 13 polypeptides required for OXPHOS (88) (**Figure 4**).

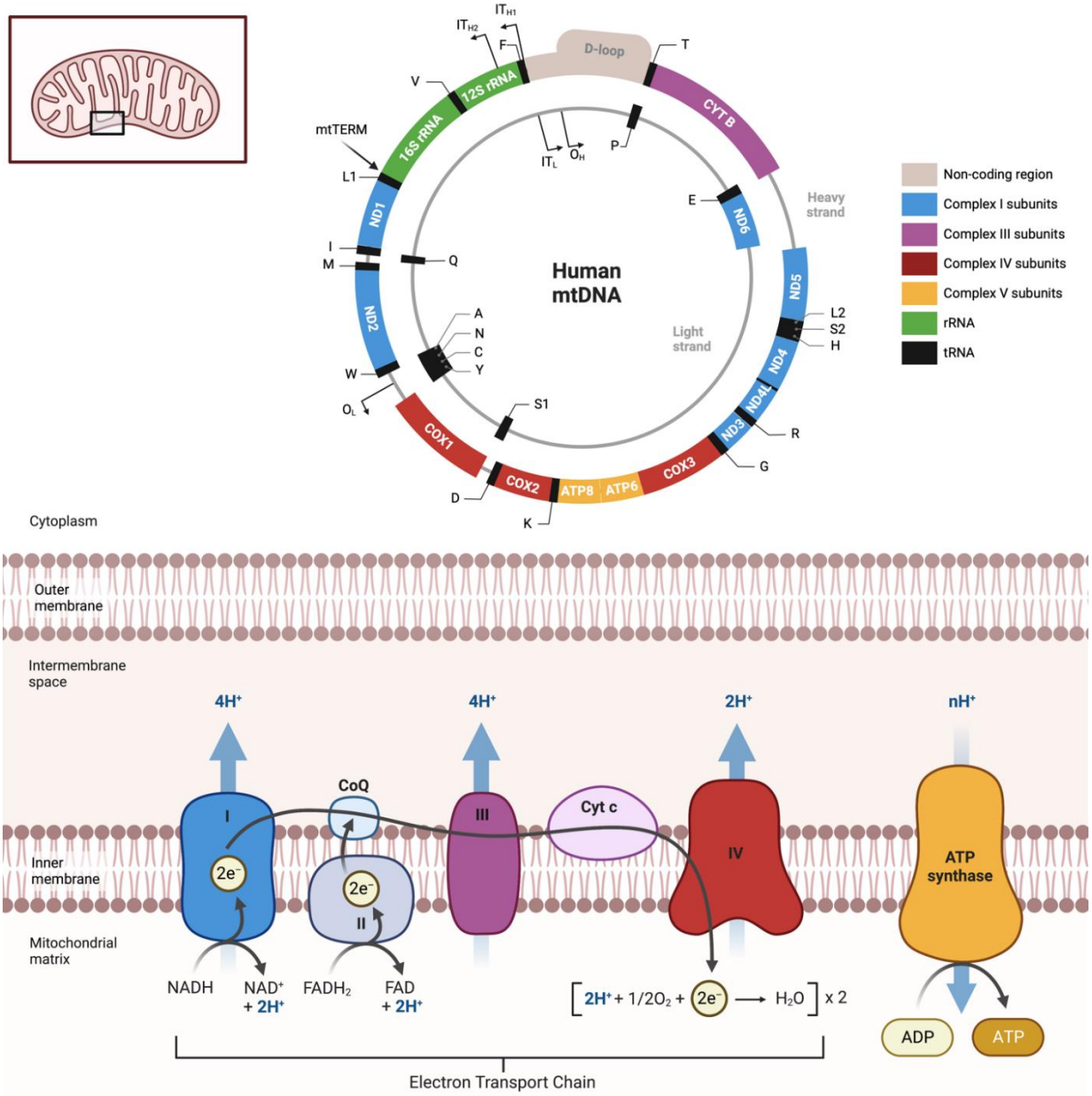

Figure 4: Mitochondrial DNA and cellular respiration. **(Top)** Human mitochondrial DNA (mtDNA) sequence map. The outer "heavy" strand contains most of the mitochondrial genes that encode the components required for oxidative phosphorylation, while the inner "light" strand contains a single polypeptide (ND6) and eight transfer RNAs (tRNAs). The displacement loop (D-loop), a short nucleic acid strand (complementary to the light strand) displaces the heavy strand (88) in the non-coding region and contains the major transcription initiation sites for the heavy and light strands (IT_{H1} and IT_L, respectively) (88). The clockwise direction of DNA synthesis is indicated with the arrows showing the origins of H-strand (O_H) and L-strand (O_L) replication, while the counterclockwise direction of RNA synthesis is indicated by the arrows depicting the initiation of transcription sites (IT_L, IT_{H1}, IT_{H2}). The 22 tRNA genes are labelled with the single letter code of the amino acid. **(Bottom)** The electron transport chain across the inner mitochondrial membrane. NADH and $FADH_2$ pass their electrons to the electron transport chain, turning back into NAD^+ and FAD, to be used in glycolysis and the citric acid cycle. The path of the protons (H+) pumped back into the cell, at each stage of the process, is indicated by thick blue arrows. The curved arrow drawn across protein complexes I-IV indicates the path of the electrons (e-), which ultimately are transferred to molecular oxygen, that, in turn, splits in half and accepts protons from the matrix to form water. ADP, adenosine diphosphate; ATP, Adenosine 5′-triphosphate; ATP 6/8, mitochondrially-encoded ATP synthase membrane subunit 6/8; CoQ, coenzyme Q; COX 1-3, mitochondrially-encoded cytochrome C oxidase subunits I-III, respectively; CYT B, cytochrome B; Cyt C, cytochrome C; ND1-6, mitochondrially-encoded NADH dehydrogenase subunits 1-6, respectively.

Given its proximity to the electron transport chain (ETC), the mtDNA is directly affected by the oxidative damage produced by the ROS, and is susceptible to more mutations than nuclear DNA, due to the absence of protective histones and inefficient DNA repair (89-91). Although ROS are a natural by-product of OXPHOS, their aberrant accumulation is problematic. As will be discussed in detail in the following sections, overproduction and/or excessive presence of ROS produces mitochondrial dysfunction, reduces the mtDNA copy number (which ultimately limits cellular respiration), and generally promotes apoptosis through cell damage (**Figure 5**).

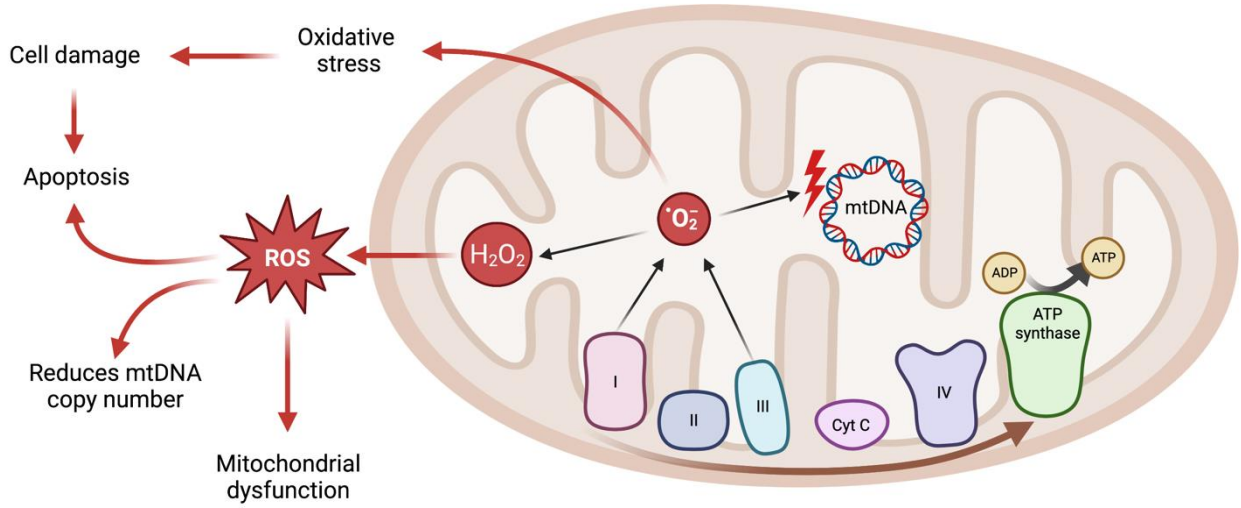

Figure 5: Cellular responses to the accumulation of reactive oxygen species. A simplified version of the electron transport chain is depicted (with the arrow along the mitochondrial membrane showing the flow of electrons through complex I-IV; see **Figure 4** for more details) to show its contribution to the production of reactive oxygen species (ROS). ADP, adenosine diphosphate; ATP, Adenosine 5′-triphosphate; Cyt C, cytochrome C; H_2O_2, hydrogen peroxide; mtDNA, mitochondrial DNA;$^{\bullet}O_2^-$, superoxide radical.

Mitochondrial DNA expression is largely controlled by the displacement loop (D-loop), in which a short nucleic acid strand (complementary to the light strand) displaces the heavy strand (88). Given that the D-loop (the 1118-bp sequence between nucleotides 577 and 16,028) contains the major transcription initiation sites for the heavy and light strands (IT_{H1} and IT_L, respectively) (88), mutations in this region could affect the replication, recombination, or membrane association of mtDNA (92). In addition to mutations in the D-loop region, mutations in genes that encode the OXPHOS proteins, tRNAs and rRNAs were also identified in women with PCOS (**Figure 6**). Zhuo *et al.* (93) assessed

peripheral blood cells in 57 women with PCOS and 38 controls, and identified sixteen variants in the D-loop, seven in the 12S rRNA, three in 16S rRNA, six tRNA polymorphisms among women with PCOS (i.e., $tRNA^{Gln}$ T4395C, $tRNA^{Cys}$ G5821A, $tRNA^{Asp}$ A7543G, $tRNA^{Lys}$ A8343G, $tRNA^{Arg}$ T10454C, and $tRNA^{Glu}$ A14693G), in addition to other variants that occurred in mitochondrial polypeptide-coding genes, suggesting that mitochondrial dysfunction might be involved in the pathogenesis of PCOS.

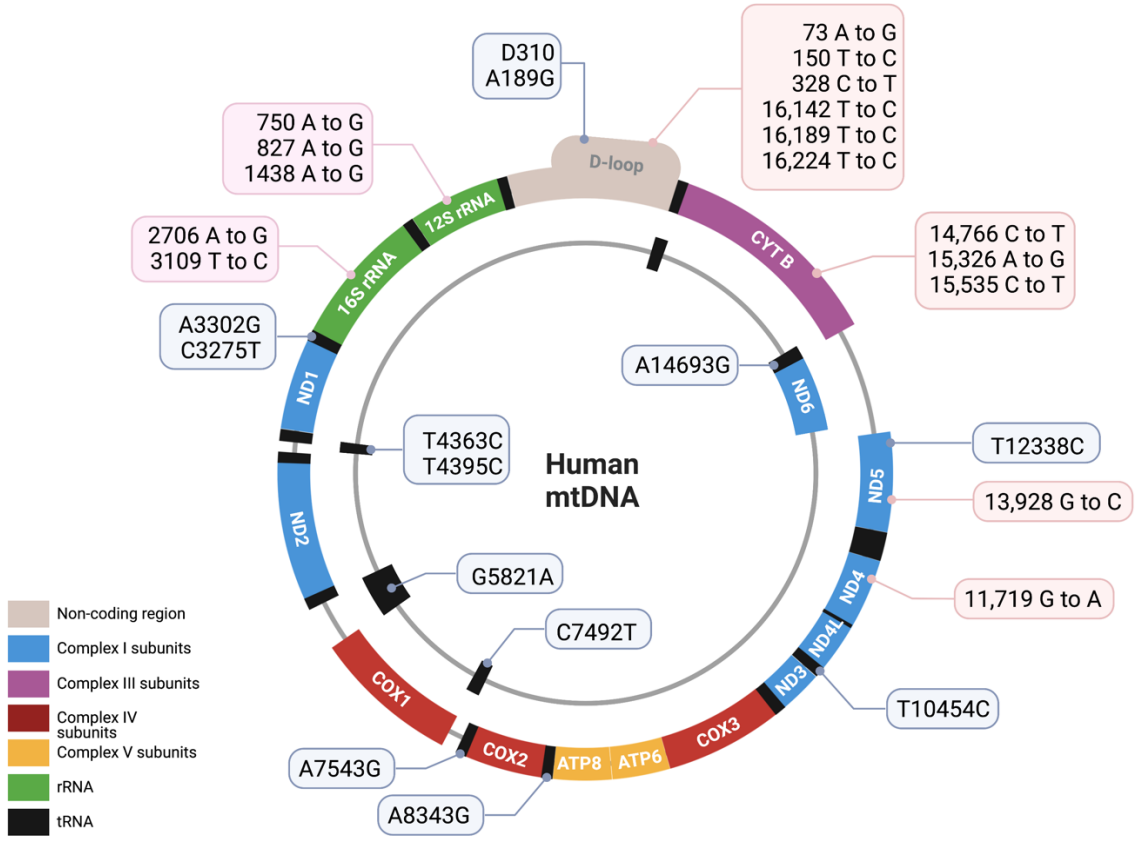

Figure 6: PCOS-related mutations in the human mtDNA. Note, the PGC-1α rs8192678 "Ser" allele was also associated with PCOS. Variants and single nucleotide polymorphisms compiled from previous publications (93-97).

Accordingly, additional tRNA variants were detected in PCOS patients with IR (94-96). Biochemical analysis of patients with variants C3275T and T4363C ($tRNA^{Leu}$, $tRNA^{Glu}$) revealed deficiency of mtDNA and ATP production and increasing number of ROS (94), while in another study the A3302G mutation ($tRNA^{Leu}$) was associated with the failure of mt-tRNA metabolism and

lower mtDNA copy number in comparison to healthy controls (95). In women with PCOS, a decrease in the expression of genes involved in OXPHOS was demonstrated in skeletal muscle, leading to the hypothesis that alterations in mitochondrial function could underlie some of the pathological characteristics of IR (53). To support this hypothesis, the function of mitochondrial complex I in the ETC was found to be reduced in PCOS patients compared to controls matched for age and body mass index, indicating that in some women with PCOS oxygen consumption is impaired (99). These observations suggest a potential pathway through which mtDNA mutations could contribute to the pathogenesis of PCOS.

1.3.2. Reduced mitochondrial DNA copy number

Damage to mtDNA is associated with mitochondrial dysfunction, leading to a reduction of cellular metabolic activity and activation of apoptotic cell death (37). In this regard, maintaining an adequate quantity of mtDNA is essential to preserve mitochondrial function and support cell growth (100). The mtDNA copy number, the relative measure of the number or mass of mitochondria present in the cell, negatively correlated with the intracellular ROS levels and was found to be significantly lower in women with PCOS (101, 102), particularly those with tRNA variants (95). Further, in women with PCOS, mtDNA quantity negatively correlated with IR (even after correcting for IR) (85), and positively correlated with SHBG (85), which regulates androgen bioactivity (103) in addition to inversely correlating with fasting glucose, insulin, and IR (104). In murine models of IR, MII oocytes exhibited lower mtDNA copy numbers, impaired ATP biosynthesis, and reduced fertilization rates (105). Taken together, these findings suggest that the lower mtDNA content in women with IR-PCOS may reflect the oxidative stress, DNA damage and overall disrupted mitochondrial function resulting from the excessive presence of ROS and limited ATP biosynthesis (106).

1.3.1. Effect of oxidative stress on the granulosa cells of women with PCOS

Elevated oxidative and metabolic stress have been reported for women with PCOS (107, 108) and are postulated to play a role in the pathogenesis of this disorder (109, 110). The follicular fluid

contains proteins, sugar, ROS, antioxidants, and hormones whose concentration reflects the status of the GCs and directly affects the maturation and quality of the oocytes (111-114). The imbalance between antioxidant factors and ROS in the follicular fluid affects oocyte quality, fertilization, and embryonic development (115). Further, women with PCOS presented with increased total malondialdehyde levels; lower total antioxidant capacity and thiol concentrations compared to healthy women (116, 117); reduced glutathione (118); a decrease in the level of haptoglobin, a protein with antioxidant properties (119). Consistent with these findings, in women with PCOS who have augmented free testosterone levels, oxidative stress may increase susceptibility to DNA damage and adversely affect GCs, which play an important role during oocyte maturation and developmental competence, ovulation and fertilization (120).

The GCs' metabolic activity has important implications for oocyte quality (121), as metabolic dysfunction in GCs may lead to abnormal folliculogenesis in women with PCOS (122). For example, the almost quadrupled ROS levels in the GCs of women with PCOS were related to significantly increased apoptosis (123), and multiple clinical studies associated the GCs (123) and leukocytes (84, 124) of women with PCOS with increased ROS production and decreased antioxidant concentrations. In this context, the accumulation of ROS in the GCs could compromise the function of mitochondrial oxidative metabolism and lead to abnormal morphology, programmed cell death, and follicular atresia (125). Moreover, impairment of OXPHOS has been associated with IR, growing insulin secretion from β-cells and dysregulation of fatty acid metabolism in mice and humans (126). These mechanisms likely contribute to overproduction of ROS, GC injury, and impaired follicle development.

1.3.2. The role of the unfolded protein responses in PCOS

In response to mitochondrial dysfunction, the overproduction of ROS, oxidative stress, or the accumulation of mis- or unfolded proteins cells activate the mitochondrial unfolded protein response (UPR^{mt}) and ER unfolded protein response (UPR^{er}), in an attempt to restore cellular homeostasis (127).

1.3.2.1. The mitochondrial unfolded protein response (UPR^{mt})

Besides the 13 ETC proteins encoded by mtDNA (**Figure 4**), the vast majority of mitochondrial proteins (e.g., chaperones, proteases, antioxidants, mitochondrial protein import components) are encoded in the nucleus and are shuttled from the cytoplasm, across the outer and inner mitochondrial membranes (128-132). Under normal circumstances, the mitochondrial chaperone system [which includes heat shock proteins (HSPs)] promotes mitochondrial homeostasis (mitostasis) by shuttling and re-folding misfolded proteins to prevent their accumulation within the mitochondrial matrix. However, under certain conditions, the chaperones become overloaded, leading to an accumulation of misfolded or unassembled proteins, and induction of the UPR^{mt}. The UPR^{mt} is an adaptive transcriptional response, activated by multiple forms of mitochondrial perturbations, including impediments in mitochondrial translation, protein synthesis, or OXPHOS, alterations in mitochondrial proteostasis or metabolism, mtDNA mutations, ROS, and impairment in mitochondrial protein import machinery (128, 133-136). The UPR^{mt} is mediated through several intertwining intracellular signaling pathways that regulate translation of genes which result in increased folding capacity, antioxidant capacity, and protein quality control, or decreased protein folding load (137). For the scope of this thesis dissertation, we will focus on the canonical UPR^{mt} signaling, which promotes transcription of nuclear-encoded chaperones and proteases, and the sirtuin axis, that relies on several nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases (SIRTs) to orchestrate mitochondrial biogenesis and mitostasis (**Figure 7**). These pathways have emerged as key processes in oogenesis, such that failure of the UPR^{mt} resulted in the accumulation of damaged proteins, compromised OXPHOS, led to the overproduction of ROS, and ultimately, oocyte dysfunction in mice (132).

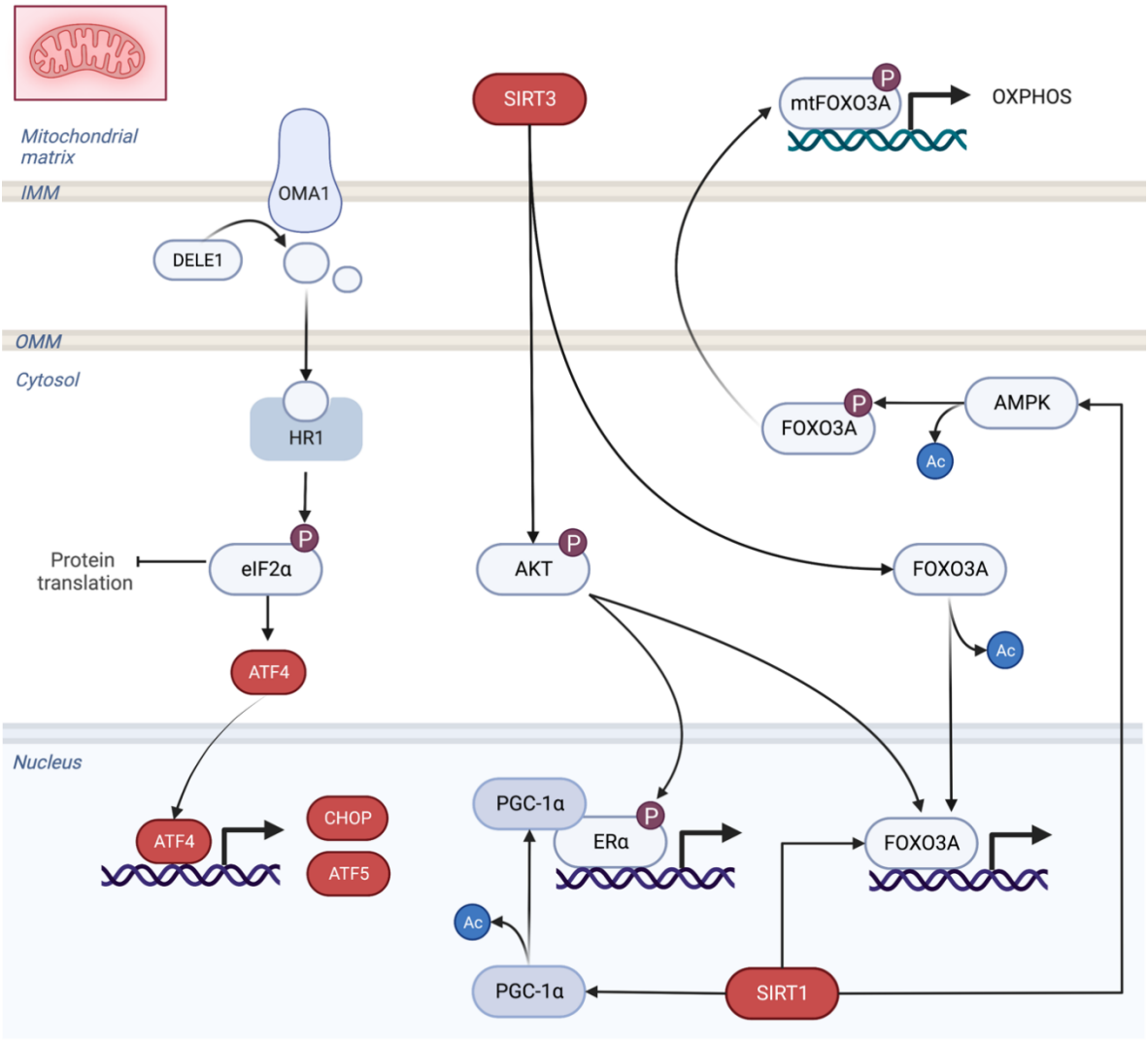

Figure 7: The mitochondrial unfolded protein response. Inefficient protein folding, or the accumulation of mis- or unfolded proteins in the cytoplasm activates the mitochondrial unfolded protein response (UPR^{mt}) to restore mitochondrial function and promote cell survival (134). In the canonical axis, OMA1 zinc metallopeptidase (OMA1) cleaves DAP3 binding cell death enhancer 1 (DELE1) into a fragment that can cross through the outer mitochondrial membrane to bind and activate the eukaryotic translation initiation factor 2 alpha kinase 1 (HRI) in the cytosol. In turn, activated HRI then phosphorylates eukaryotic translation initiation factor-2α (eIF2α), that briefly attenuates global protein translation while upregulating activation transcription factor 4 (ATF4), which regulates activation transcription factor 5 (ATF5) and CCAAT/enhancer-binding protein homologous protein (CHOP) transcription (138, 139). On the other hand, nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases (SIRTs) orchestrate transcription of both nuclear and mitochondrial DNA. For example, oxidative damage stimulates the SIRT3/AKT/FOXO3A signaling (140) or sirtuin 3 (SIRT3)-mediated deacetylation of forkhead boxo 3a (FOXO3A) to permit its nuclear translocation and transcriptional activity. Similarly, stress conditions also cause SIRT1-mediated deacetylation of AMP-activated protein kinase (AMPK) (141), activating it to promote phosphorylation of FOXO3a (on Ser30). Consequently, FOXO3a translocates into the mitochondria to initiate transcription of oxidative phosphorylation genes (142). Nuclear SIRT1 additionally activates FOXO3 transcriptional activity (142) to upregulate genes involved in mitostasis (143). Further, SIRT1 also deacetylates the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) to sequester it in the nucleus (144). Notably, PGC-1α is a master regulator, which binds to and co-activates the nuclear estrogen receptor alpha (ERα) to drive the expression of genes involved in mitochondrial biogenesis (145, 146), among others. Finally, ERα-mediated transcription is

also promoted through phosphorylation by AKT (147). IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane.

1.3.2.2. The endoplasmic reticulum unfolded protein response (UPR^{er})

The ER is the principal site for folding and maturation of transmembrane, secretory and ER-resident proteins. Most intracellular proteins are post-translationally folded in the cytosol with the assistance of chaperones, while membrane and secreted proteins are folded and matured in the ER. However, conditions such as hyperglycemia, oxidative stress, cytotoxicity, inflammation, pharmacological agents, pathogens, altered calcium homeostasis, nutrient deprivation, and genetic mutations can increase the demand for protein folding or attenuate the protein folding capacity of the ER (148), leading to the accumulation of incorrectly folded proteins in the ER (149), and in turn, ER stress. In response to acute stress, the cell activates the UPR^{er} (150, 151), mediated by ER-membrane protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor 6 (ATF6) (152, 153) (**Figure 8**). Notably, in cases of prolonged stress, when the UPR^{er} is unable to restore homeostasis, pro-apoptotic pathways are activated to ensure the survival of the organism (154, 155).

ER stress has been detected in the GCs of late-stage follicles of healthy patients (156), and to a greater extent in patients with PCOS compared to non-PCOS controls (150). Further, hyperandrogenism has not only been linked to ER stress in the GCs of antral follicles, but also an upregulation of pro-apoptotic mechanisms (157). These findings suggest that cellular hemostasis could not be restored by the UPR^{er}, and that hyperandrogenism-related ER stress contributes to the growth arrest of antral follicles in PCOS by promoting GC apoptosis.

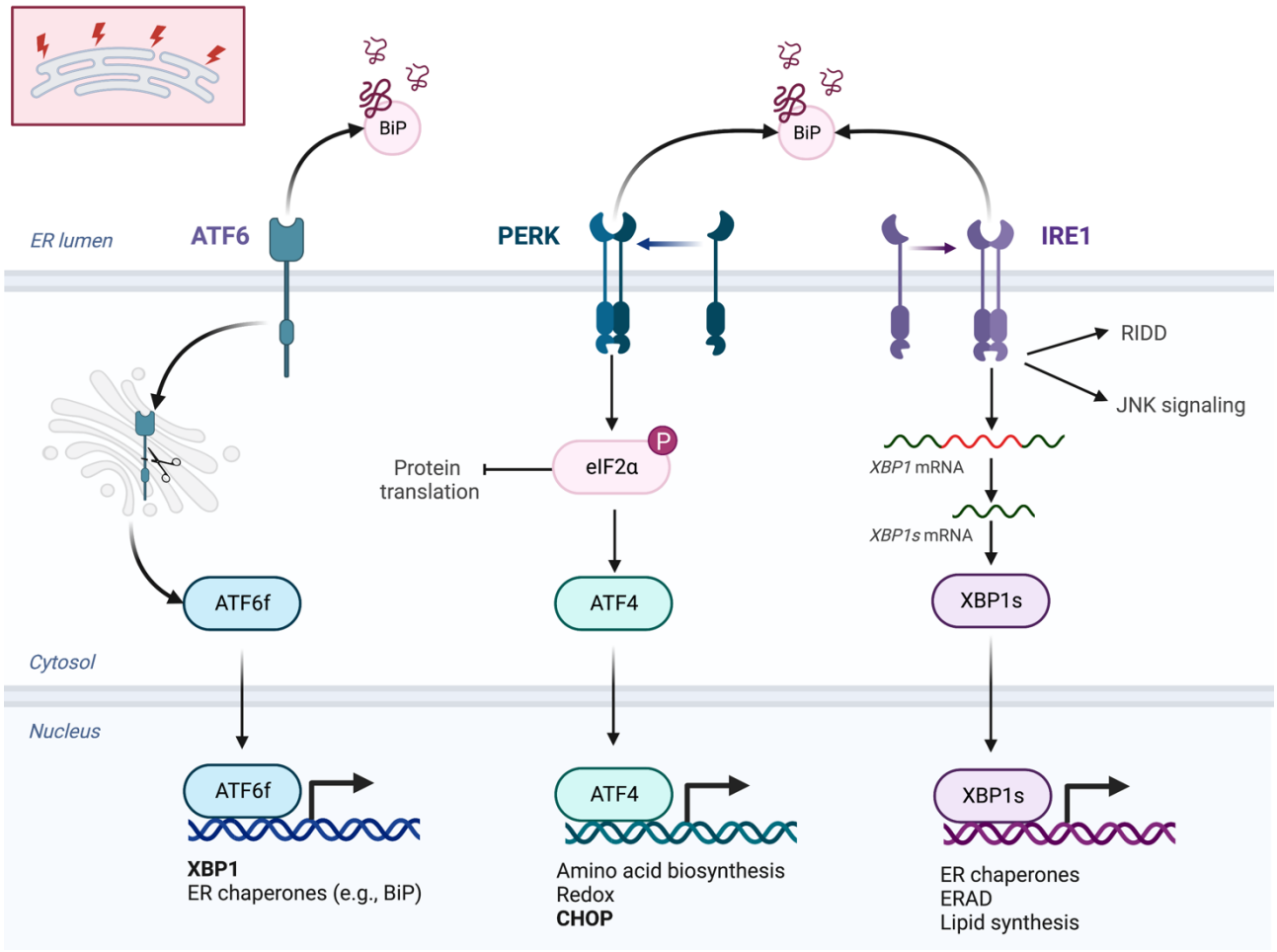

Figure 8: The endoplasmic reticulum unfolded protein response. When misfolded proteins bind to the binding immunoglobulin protein (BiP), it dissociates from activating transcription factor 6 alpha (ATF6α), a 90 kDa type II transmembrane glycoprotein, exposing two Golgi-localization sequences. The ATF6 is consequently translocated to the Golgi apparatus for its transmembrane anchor to be cleaved, and the resulting 50 kDa amino-terminal cytoplasmic fragment (ATF6f) becomes free to enter the nucleus and bind ER stress-response elements (158). On the other hand, the dissociation of BiP from protein kinase RNA-like ER kinase (PERK) and inositol-requiring enzyme-1 (IRE1) induces their dimerization and trans autophosphorylation (159). Activation of PERK leads to the phosphorylation of eukaryotic translation initiation factor-2α (eIF2α), which briefly attenuates global protein translation while upregulating activation transcription factor 4 (ATF4). In turn, ATF4 directs antioxidant responses to reduce ER stress or CHOP-mediated apoptosis during long-term ER stress. Apart from initiating regulated IRE1 dependent decay (RIDD) to reduce protein load, IRE1 splices XBP1 mRNA with its endoribonuclease activity, promoting its potent transcriptional ability (159). In turn, spliced XBP1 (XBP1s) induces the transcription of ER chaperones and genes involved in phospholipid biosynthesis and ER biogenesis (160). Finally, XBP1 actions also contribute to endoplasmic reticulum-associated protein degradation (ERAD) to eliminate misfolded or unassembled proteins from the ER (161). Nevertheless, persistent activation of IRE1 activates the JNK signaling cascade to stimulate pro-apoptotic factors (e.g., CHOP) (159). CHOP, CCAAT/enhancer-binding protein homologous protein.

1.4. Current treatment for PCOS

The clinical management of PCOS is influenced by several factors, including the phenotype and severity of symptoms, patient's age, ethnicity, overall health, fertility desire and related comorbidities (e.g., obesity, and type 2 diabetes mellitus). The international guidelines for PCOS management (35) comprehensively outlines a total of 166 recommendations, including 31 evidence-based recommendations, 59 clinical consensus recommendations and 76 clinical practice points. Notably, lifestyle-related changes (e.g., implementing more physical activity and a healthy diet) were advised as first-line therapeutic approaches, to improve general health, weight, hormonal balance, hyperandrogenism, IR, and inflammatory states. Medical treatment was proposed as second-line therapy for patients that needed improvements in reproductive outcomes and/or metabolic functions, or those with hyperandrogenic-related skin disorders. At the time of writing this dissertation, there are nine FDA-approved drugs indicated for PCOS (**Table 2**), and their administration depends on the patient's desires for pregnancy or to mitigate other symptoms (e.g., ache and hair growth). For patients that want to get pregnant, clinicians may recommend they begin to lose weight to reduce PCOS-related symptoms, increase insulin sensitivity, lower blood glucose levels, in addition to taking medications that promote ovulation (e.g., clomiphene or gonadotrophins). Along with the similar lifestyle changes, patients that have no desire for pregnancy are given oral contraceptives to regulate their menstrual cycles, reduce circulating androgen and acne, and/or diabetes medication to mitigate IR, reduce androgen, slow hirsutism, and promote regular ovulation. Notably, the exclusive administration of combined contraceptives treats acne, hirsutism and/or oligomenorrhea more effectively than progestin-only preparations (36). Further, different combinations of contraceptives treat hirsutism with similar efficacy, so the choice will depend on the adverse effects experienced by the patient and their preference for dosage.

Table 2: FDA-approved drugs currently indicated for PCOS.

ID	DRUG NAME	DRUG DESCRIPTION
DB00823	**Ethynodiol diacetate**	An oral contraceptive used to prevent pregnancy.
DB01216	**Finasteride**	An antiandrogenic compound that is used for the treatment of symptomatic benign prostatic hyperplasia (BPH) and male pattern hair loss in adult males by inhibiting Type II 5-alpha reductase.
DB01006	**Letrozole**	An aromatase inhibitor used to treat breast cancer in postmenopausal women.
DB14741	**Luteinizing hormone**	Not Annotated
DB01357	**Mestranol**	A synthetic estradiol found in oral contraceptive pills for contraception and the treatment of other conditions in the female reproductive system, such as dysmenorrhea and dysfunctional uterine bleeding.
DB00331	**Metformin**	A biguanide antihyperglycemic used in conjunction with diet and exercise for glycemic control in type 2 diabetes mellitus. It is also used off-label for insulin resistance in polycystic ovary syndrome (PCOS).
DB06713	**Norelgestromin**	A progestin used for the prevention of pregnancy in women who elect to use a transdermal patch as a method of contraception.
DB00957	**Norgestimate**	A progesterone used as a contraceptive and to treat acne vulgaris.
DB09389	**Norgestrel**	A progestin used in combination with ethinyl estradiol for oral contraception and prevention of pregnancy in women.

These drugs and their descriptions were exported from the DrugBank database (162) on January 5, 2023.

CHAPTER 2: OBJECTIVES

Mauro Cozzolino

The global objective of this PhD dissertation thesis was to characterize mechanisms of mitochondrial dysfunction in women with polycystic ovarian syndrome (PCOS).

This aim was addressed with three studies, where the specific goals were to:

1. Compile evidence on mitochondrial dysfunction in women with PCOS by conducting a review of the literature. (Study I)
2. Characterize the transcriptomic differences in granulosa cells and peripheral blood mononuclear cells between women with PCOS, young normoresponders and young poor responders, to reveal the molecules and mechanisms related to aberrant follicle development in PCOS. (Study II)
3. Evaluate how insulin resistance impacts the unfolded protein response in the mitochondria and endoplasmic reticulum of the granulosa cells of women with PCOS. (Study III)

CHAPTER 3: MATERIAL AND METHODS

3.1. Bibliographic review

For **Study I**, the PubMed database was queried using the following search terms: polycystic ovarian syndrome, PCOS, oxidative stress, mitochondrial dysfunction, unfolded protein response.

3.2. Ethical approval

For **Study II,** all study procedures were approved by and conducted according to the Institutional Review Board of Hospital Universitario y Politécnico La Fe, Valencia, Spain (520/2018). For **Study III,** All study procedures were approved by, and conducted in accordance with, the Institutional Review Board of the Hospital Universitario Puerta de Hierro, Madrid, Spain (1812-MAD-099-MC). In both cases, written informed consent was obtained from all patients prior to the collection of samples.

3.3. Study participants

For **Study II**, a total of 60 patients were recruited from the Hospital Universitario y Politécnico La Fe, in Valencia, Spain, including 20 women with PCOS, 20 young poor responders (YPRs), and 20 young normoresponders (YNRs). PCOS was defined by the revised Rotterdam diagnostic criteria (163), which includes (i) oligo- or anovulation, (ii) clinical and/or biochemical signs of hyperandrogenism, and (iii) polycystic ovaries and exclusion of other etiologies. In this regard, patients presenting with other causes of oligomenorrhea, hyperandrogenism (i.e., congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome), ovarian cancer, endometriosis, or other severe systemic disease were excluded from the study. In accordance with internal criteria and the Poseidon group 1a classification (164)(www.groupposeidon.com), a threshold of <5 mature follicles was established to represent the bottom 15th percentile of ART patients. Among these patients, women ≤35 years old, without PCOS, with <4 mature follicles (≥15 mm on transvaginal ultrasound) on the day of the trigger were selected for the YPR group. Alternatively, the YNR control group was selected from the top 25% of patients, with >14 mature follicles. Patients were included in

the control group if they were <35 years old, did not present with PCOS or anovulation, and developed >14 mature follicles (≥15 mm on transvaginal ultrasound).

In **Study III**, 40 women with PCOS were recruited from the Acibadem University IVF Center (Istanbul, Turkey). Patients were eligible for the study if they were aged 18–37 years, presented no family history of hereditary or chromosomal diseases, a normal karyotype, and a BMI between 18–29 kg/m^2. Diagnosis with PCOS was based on the revised Rotterdam criteria, as described above. For this study, patients with PCOS were divided according to whether they presented with or without insulin resistance [IR-PCOS (n=20) or nIR-PCOS (n=20), respectively], based on their HOMA values (80), that determines IR using the concentration of fasting plasma glucose and fasting insulin. Specifically, the participants with a HOMA value of ≥2 were considered to have IR-PCOS, while women with a value <2 were categorized as nIR-PCOS. Patients with other causes of oligomenorrhea or hyperandrogenism (such as congenital adrenal hyperplasia, androgen-secreting tumors, or Cushing's syndrome) were excluded from the study for clinical reasons.

For **Study III**, the YNRs were recruited from the oocyte donor program at the IVI Madrid (Madrid, Spain). These control patients were healthy women, aged 18–35 years, who met the following criteria to be oocyte donors: regular menstrual cycles, no family history of hereditary or chromosomal diseases, normal karyotype, BMI of 18–29 kg/m^2, no more than two previous miscarriages, no gynecological diseases or medical disorders, and a negative screening result for sexually transmitted infections (165). Patients with PCOS were excluded from this group. Finally, donors were expected to have ≥6 antral follicles per ovary at the beginning of the cycle.

3.4. Ovarian stimulation protocols, oocyte retrieval, and granulosa cell collection

In both **Study II** and **Study III**, the participants underwent controlled ovarian stimulation (COS) using the standard long GnRH agonist or antagonist protocol. COS was initiated after ultrasound-based confirmation of the absence of follicle dominance and serum estradiol <30 mIU/mL (**Study II**) or <50 mIU/mL (**Study III**). Recombinant FSH (150-300 IU) was administrated daily, alone, or in

combination with highly purified human menopausal gonadotropin (HP-HMG). When follicular diameter reached >18-19 mm, final follicular maturation was achieved using 5,000-10,000 IU human chorionic gonadotropin (hCG) and/or GnRH agonist. Specifically, in **Study II**, oocyte maturation was primarily achieved using GnRH agonists (GnRHa) alone for patients with PCOS, to decrease the risk of ovarian hyperstimulation syndrome (OHSS), while 10,000 IU hCG was primarily used for the YPR group, and the strategy used for the YNR group depended on the physician's preference. In **Study III**, only 10,000 IU hCG was for both patients with IR-PCOS and nIR-PCOS. Oocyte retrieval was scheduled 36 hours after the trigger administration. Follicular fluid samples were carefully collected from the first aspirated follicle of each ovary, and samples contaminated with blood were excluded. The GCs were isolated from each follicular fluid sample by centrifugation at 450 xg for 5 min, washed with 1 mL of phosphate buffered saline (PBS), and purified using a second centrifugation at maximum speed for another 5 min. Finally, the GCs were resuspended in 10 µL of PBS, and 50 µL of RNAlater™ (Sigma) was added prior to storin samples at -20ºC. Only the GC samples with >95% of purity were used for transcriptomic analyses in **Study II**.

3.5. Collection of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected in BD Vacutainer® tubes with EDTA (BD Diagnostics, Spain) to isolate PBMCs by standard Ficoll-based centrifugation protocols. Briefly, 4 mL of each blood sample was diluted to a final volume of 7 mL and added to 3 mL of Ficoll-Paque (GE Healthcare, Uppsala, Sweden), prior to centrifugation at 2500 rpm for 30 min at room temperature. The buffy coat was collected, and the PBMCs were resuspended in PBS and washed twice, followed by a second centrifugation. Finally, the pellet was resuspended in 100 µL of PBS, and 500 µL of RNAlater™ was added prior to storage at -20ºC until RNA extraction.

3.6. RNA extraction

Total RNA was extracted from PBMCs using the RNeasy Micro kit (Qiagen). RNA integrity and concentration were measured using the Agilent High Sensitivity RNA screen tape

system and Qubit2, respectively. All samples used for RNA-seq had an RNA integrity number (RIN) of ≥7. mRNA was purified from approximately 200 ng of total RNA, using oligo-dT beads, and sheared by incubation at 94ºC, in the presence of Mg (Kapa mRNA Hyper Prep).

3.7. Reverse transcription, amplification of cDNA, library generation, and RNA sequencing

Following first-strand synthesis with random primers, the second-strand synthesis and A-tailing were performed with deoxyuridine triphosphate (dUTP), to generate strand-specific sequencing libraries. Adapters were ligated to the 3' dTMP overhangs of the complementary DNA (cDNA) fragments, to provide sequence primer binding sites during amplification. Second-strand cDNAs marked with dUTP were digested, to prevent amplification and confer single-stranded specificity. Indexed libraries that met the appropriate cut-offs were quantified by quantitative real-time polymerase chain reaction (qRT-PCR), using a commercially available kit (KAPA Library Quantification, Roche) for accuracy and reproducibility. Amplicon size and DNA concentration were determined using the LabChip GX or Agilent Bioanalyzer, and only samples with a yield of ≥ 0.5 ng/ µL were used for RNA sequencing.

On the other hand, total RNA was extracted from purified GCs using the RNeasy kit (Qiagen). The cDNA was generated and amplified using the SMART-Seq® v4 Ultra®-Low Input RNA Kit for Sequencing (Takara Bio, USA), following the manufacturer's protocol. The dsDNA High Sensitivity kit on Qubit 2.0 (Life Technologies, Carlsbad, CA) and Tapestation 2200 (Agilent Technologies) were used to quantify the concentration and size distribution of the cDNA fragments, respectively. RNA sequencing libraries were constructed using the Nextera® XT DNA Library Preparation Kit (Illumina, San Diego, CA) and multiplexed using Nextera® XT Index Kit (Illumina). Indexed libraries were then pooled and sequenced on Illumina's Hiseq 2500 platform with 75 bp pair-end reads. Approximately 50 M reads were achieved for each of the fifteen samples.

3.8. Transcriptomic analysis

Illumina's HiSeq Control Software v2.0 (NCS) and Real-Time Analysis Software (RTA) were used for image analysis, base calling, and generation of sequence reads. Raw sequencing data were converted to FASTQ files using the bcl2fastq2 v1.8.4 software (Illumina) for quality assessment. Low-quality reads were excluded, and the remaining reads were aligned to the reference human genome (hg38) with gencode annotation (166), which has >100K annotated regions on the genome, using HiSAT2 for alignment. StringTie and Ballgown were then used to estimate transcript abundance, according to an established protocol (167). DESEq2 (168) was used for differential gene expression analysis. Gene expression values were calculated as fragments per kilobase of exon per million fragments mapped (FPKM) using the Cufflinks package v2.1.1. The genes were considered as differentially expressed if the adjusted p-value was ≤0.05. Downstream processing and visualization of the data was carried out in R.

3.8.1. Ingenuity Pathway Analysis

QIAGEN Ingenuity Pathway Analysis (IPA) software (version 45865156, 2018) was used to perform Gene Ontology (GO) enrichment analysis on the gene set, and identify significantly enriched pathways. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. The top five significantly differentially expressed genes (DEGs) among the different comparisons (i.e., PBMCs from YNR vs. PCOS; PBMCs from PCOS vs. YPR; GCs from YNR vs. PCOS; GCs from PCOS vs. YPR) were identified with Log2FC≥0.584 and false discovery rate (FDR)≤0.05.

3.9. qRT-PCR validation of transcriptomic findings

An independent set of four PBMC and four GC samples were used to experimentally validate eight selected DEGs (i.e., *MT-ATP6, MT-RNRI, MFRP, MTMR9, PXDN, RAB4B, TERC,* and *ZFP57*), using qRT-PCR. Total RNA was extracted from the PBMCs using RNAqueous™-Micro Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA), and was treated with DNase I

(Thermo Fisher Scientific) to remove any possible genomic DNA contamination. RNA was reverse transcribed in two steps, using the RETROscript™ kit (Invitrogen™). First, the template RNA and random primers were incubated at 85ºC for 3 min to eliminate any secondary structures. Then, the buffer and enzyme were added, and the reaction was carried out at 42ºC for 1 h. On the other hand, total RNA was extracted from the GCs using the RNeasy kit (Qiagen). The cDNA was generated and amplified using the SMART-Seq® v4 Ultra®-Low Input RNA Kit for Sequencing (Takara Bio, USA), following the manufacturer's protocol.

The PCR reactions were prepared with a final volume of 10 µL, using 5 µL PowerUp™ SYBR™ Green Master Mix (2x), 1µL forward and reverse primers, 1µL of DNA template, and 3µL of nuclease-free water. The specific primer sequences of the target genes are listed in ***Table 3***. PCR reactions were carried out in technical triplicates, on a ViiA™ 7 RT-PCR system (Applied Biosystems). Nuclease-free water was used as a negative control, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Thermocycling parameters were 95°C for 5 min followed by 45-cycle run with annealing temperature at 60ºC. Melting curve analysis was used to verify specificity of the amplification by confirming the absence of primer dimers and contamination. The ΔΔCt method was used to calculate relative gene expression, with respect to GAPDH.

Table 3: Specific primer sequences used for qRT-PCR validation of DEGs in Study II.

Gene	*Forward sequence*	*Reverse sequence*
MT-ATP6	TCAACCAATAGCCCTGGCCG	GCTTGGATTAAGGCGACAGCG
MT-RNR1	GCAAGCATCCCCGTTCCAGT	TGGGGGTGTGGCTAGGCTAA
MFRP	AGCCCTAACTACCCAGACCCT	CCCTTCCACAAACCCTGAGGA
MTMR9	GCTCAGTGCATCGACAGGGAAG	AGCCTGCAGCCACTCTCTTTC
PXDN	GTCTCCAGCTCGACCCACTT	CGCCAGAAGGCGTGATGTTC
RAB4B	CCCGCTTTGCCCAGGAGAAT	CGGGTCTAGCTCGCCTGAGT
TERC	AACTGAGAAGGGCGTAGGCG	GAATGAACGGTGGAAGGCGG
ZFP57	ATGCCAGCCAGAGGGTCCTT	AAGCTCTTTCTTCTTGCCTTCTGA

Adapted from Supplemental Table S1 in (169). MFRP, membrane frizzled-related protein; MT-ATP6, mitochondrially encoded ATP synthase membrane subunit 6; MTMR9, myotubularin-related protein 9; MT-RNR1, mitochondrially encoded 12S RRNA; PXDN, peroxidasin; RAB4B, member RAS oncogene family; TERC, telomerase RNA component; ZFP57, Zinc finger protein 57 homolog.

3.10. Granulosa cell RNA extraction, reverse transcription, and qRT-PCR

In **Study III**, the total RNA was extracted from the GCs of women with IR-PCOS and nIR-PCOS using the Quick-RNA™ Microprep Kit (Zymo Research) and the concentration was measured using the Qubit™ 3.0 fluorometer (Life Technologies). RNA samples were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific). Each qRT-PCR reaction included 10 µL iQ™ SYBR® Green Supermix (Bio-Rad), 500 nM of each of the forward and reverse primers (**Table 4**), 100 ng cDNA, and enough double-distilled water to yield a final volume of 20 µL. The qRT-PCR was carried out using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) to analyze the expression of UPR^{er} genes (i.e., *IRE1*, *ATF4*, *ATF6*, *XBP1*, *BiP*, and *CHOP*), and UPR^{mt} genes (i.e., *HSP10*, *HSP14*, *HSP60*, and *CLPP*). All samples were run in triplicate, using GAPDH as a housekeeping gene, and the ΔΔCt method was used to calculate relative gene expression. Data are presented as a mean of three replicates ± standard deviation.

Table 4: Specific primer sequences used to analyze gene expression of specific UPR^{er} and UPR^{mt} genes by qRT-PCR in Study III.

	Gene	*Forward sequence*	*Reverse sequence*
UPR^{mt}	**CLPP**	CATCTACGCCAAGCACACCA	TGGCTTAGAGCAACGCCTG
	Hsp10	GACCGACAACGACCCCTAAC	GTTCCACTGCAGCTCCCATTT
	Hsp40	GGGTTTCCACTTCGGGTTCT	AATACATGCCGCCTGAACCA
	Hsp60	TGTTTTGGGAGGGGGTTGTG	ATTGTTGATGGGCACAAGAGC
UPR^{er}	**BiP (GRP78)**	GTATGGTGCTGCTGTCCAGG	GGTGTCAGGCGATTCTGGTC
	XBP1	GGATGGATGCCCTGGTTGCT	CTTGGCTCTCTGTCTCAGAG
	CHOP	GACGAGAGAGGCGAGTACTGA	TCTCGGACGGTCCCTAACTT
	IRE1	GACCTGCGTAAATTCAGGACC	GGTCTCCTCCCCAGCATTTA
	ATF4	CATGGGTTCTCCAGCGACAA	GGAGGCCCCTAACCCTAGAT
	ATF6	TCAGTCTCGTCTCCTCGGT	AGACTGGCTACTCACAGGAAC
	GAPDH	TGGACCTGACCTGCCGTCTA	CTGCTTCACCACCTTCTTGA

Adapted from Supplemental Table 1 in (170). ATF4/6, activating transcription factor 4/6; BiP (GRP78), 78 KDa Glucose-Regulated Protein; CHOP, CCAAT/enhancer-binding protein homologous protein; CLPP, Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit; HSP, heat shock protein; IRE1, inositol-requiring enzyme-1; XBP1, X-box binding protein 1.

3.11. Statistical analysis

For **Study II**, the characteristics of PCOS, YPR, and YNR are presented as mean ± standard deviation. In **Study III**, the patient characteristics were analyzed by chi-square test. In both studies, the gene expression data obtained by qRT-PCR was analysed using univariate ANOVA tests followed by two-tailed t-tests, using GraphPad Prism version 5.00 software (GraphPad, San Diego, CA, USA). In all cases, $p < 0.05$ was considered statistically significant.

CHAPTER 4: STUDY I

Mitochondrial dysfunction in women with polycystic ovary syndrome

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CURRENT OPINION

Mitochondrial function in women with polycystic ovary syndrome

Mauro Cozzolino[a,b,c] *and Emre Seli*[a,d]

Purpose of review
To provide an overview of mitochondrial functional alterations in women with polycystic ovary syndrome (PCOS).

Recent findings
Although numerous studies have focused on PCOS, the pathophysiological mechanisms that cause this common disease remain unclear. Mitochondria play a central role in energy production, and mitochondrial dysfunction may underlie several abnormalities observed in women with PCOS. Recent studies associated mtDNA mutations and low mtDNA copy number with PCOS, and set out to characterize the potential protective role of mitochondrial and endoplasmic reticulum unfolded protein responses (UPR^{mt} and UPR^{er}).

Summary
Mitochondrial dysfunction likely plays a role in the pathogenesis of PCOS by increasing reactive oxygen (ROS) and oxidative stress. This occurs in a metabolic milieu often affected by insulin resistance, which is a common finding in women with PCOS, especially in those who are overweight or obese. Mutations in mtDNA and low mtDNA copy number are found in these patients and may have potential as diagnostic modalities for specific PCOS phenotypes. More recently, UPR^{er} and UPR^{mt} are being investigated as potential cellular rescue mechanisms in PCOS, the failure of which may lead to apoptosis, and contribute to decreased reproductive potential.

Keywords
insulin resistance, mitochondrial dysfunction, oxidative stress, polycystic ovary syndrome, unfolded protein response

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most prevalent benign gynecological disorder; it affects approximately 5% of women of childbearing age and causes infertility [1,2]. The main features of PCOS according to NIH criteria of 1990 include all of the following: hyperandrogenism and/or hyperandrogenemia, oligo-anovulation, and exclusion of related disorders [3]. In 2003, the European Society of Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM) (Rotterdam) consensus group [4] added a new clinical parameter, the antral follicle count (AFC) as a diagnostic criterion. According to the Rotterdam criteria, two of the following three criteria, in addition to exclusion of related disorders results in the diagnosis of PCOS: oligo-anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries. Subsequently, Androgen Excess PCOS (AE-PCOS) Society in 2006 suggested that one of the findings among the Rotterdam criteria should be hirsutism and/or hyperandrogenism – therefore oligo-anovulation and polycystic ovaries do not lead to a PCOS diagnosis [5]. Most recent international guidelines concurred with the previous parameters considered for the diagnosis of PCOS, and emphasized that serum AMH levels should not yet be used as an alternative to ultrasound or as a single test for the diagnosis of PCOS [6▪]. Similarly they recognized insulin resistance as a key feature of PCOS, but did not recommend clinical testing for insulin resistance at the current time. All these proposed definitions identify different phenotypes of PCOS, in which excessive

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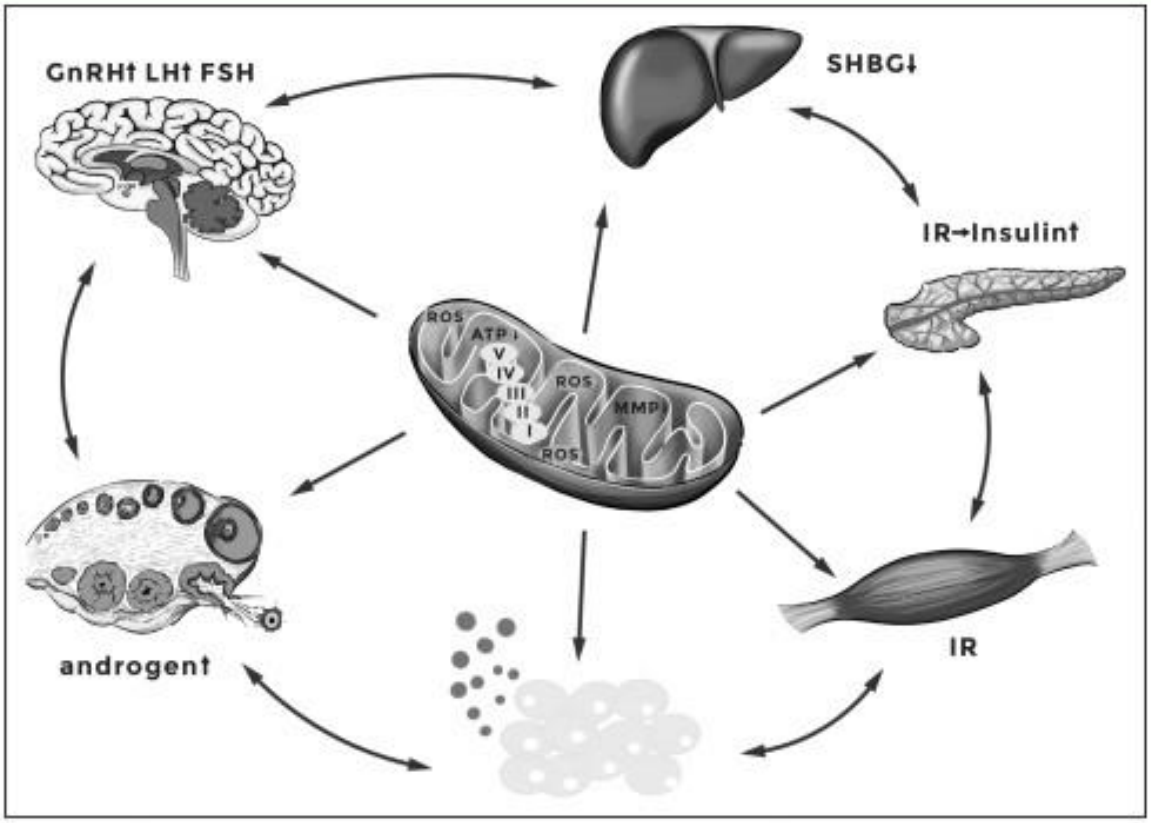

FIGURE 1. The putative role of mitochondria in the metabolic activity of organ systems in women with polycystic ovary syndrome.

hyperinsulinemia. Moreover, normal-weight women with PCOS also have increased adipose insulin resistance [31].

In addition to its effect on ovulatory dysfunction, obesity is associated with altered metabolism and mitochondrial dysfunction in oocytes, culminating in impaired oocyte quality [33]. Oocytes of obese mice fed a high-fat diet have abnormal morphologies, lower ATP production, and higher levels of detrimental reactive oxygen (ROS). Indeed, diet-induced obesity in mice can intensify the production of ROS, causing increased oxidative stress in oocytes and embryos. Conversely, weight loss resulting from dietary restriction can significantly reduce ROS production and oxidative damage to lipids, proteins, and amino acids [34].

EFFECT OF OXIDATIVE STRESS ON GRANULOSA CUMULUS CELLS OF WOMEN WITH POLYCYSTIC OVARY SYNDROME

In recent years, an increasing number of studies found higher oxidative and metabolic stress in women with PCOS [35,36], and suggested that these factors may play a role in the pathogenesis of this disorder [37]. In women with PCOS who have high free testosterone levels, increased susceptibility to DNA damage may be induced by oxidative stress [38]. The increase in oxidative stress seems to also affect granulosa cells, which play an important role in oocyte development, ovulation, fertilization, and subsequent embryo implantation [39]. Granulosa cells' metabolic activity has important implications for oocyte quality [40], as metabolic dysfunction in granulosa cells may lead to abnormal folliculogenesis in women with PCOS [41]. Different markers of oxidative stress, including lipid peroxidation and carbonyl protein content, are elevated in women with PCOS [42,43]. Complementary to the increase in oxidative stress, women with PCOS also have reduced total antioxidant status [43,44], reduced glutathione [38], as well as a decrease in the level of haptoglobin, a protein with antioxidant properties [45].

The follicular fluid contains proteins, sugar, ROS, antioxidants, and hormones. The concentration of these substances directly affects the maturation and quality of the oocytes. The imbalance between antioxidant factors and ROS in the follicular fluid affects oocyte quality, fertilization, and

embryonic development [46]. Follicular fluid analysis has shown that women with PCOS have increased total malondialdehyde (MDA) levels and lower total antioxidant capacity (TAC) and thiol concentrations compared with healthy women. Consistent with these findings, multiple clinical studies have reported that women with PCOS often exhibit increased ROS production and decreased antioxidant concentrations in the granulosa cells [47] and leukocytes [24,48].

Mitochondria represent the primary site of cellular ROS generation, leading to the damage of mitochondrial components, such as mtDNA [49]. Lai *et al*. found that granulosa cells in PCOS patients have an intracellular ROS level almost four times higher than in the control group, and a significant increase in apoptosis [47]. Thus, the accumulation of ROS in granulosa cells could compromise the function of mitochondrial oxidative metabolism and lead to abnormal morphology, programmed cell death of granulosa cells and follicular atresia [50]. Moreover, impairment of oxidative phosphorylation has been associated with the development of insulin resistance, growing insulin secretion from beta cells and dysregulation of fatty acid metabolism in mice and humans [51]. These mechanisms likely contribute to high ROS production, granulosa cells injury, and impaired follicle development.

MITOCHONDRIAL DNA IN WOMEN WITH POLYCYSTIC OVARY SYNDROME

Mitochondria are unique organelles that contain DNA [mitochondrial DNA (mtDNA)], which is a circular 15–17 kb molecule in most mammals, and contain 37 genes: 22 are for transfer RNAs (tRNAs), 2 are for the small and large subunits of ribosomal RNA (rRNAs), and 13 are for proteins.

Mutations of mitochondrial DNA in polycystic ovary syndrome

The mitochondrial genome is believed to be more vulnerable to oxidative damage and have a higher mutation rate compared with nuclear DNA. This is because of the absence of protective histones, the lack of efficient DNA repair and proximity to the electron transport chain (ETC), where free radicals derived from oxygen are produced [52,53].

The mtDNA displacement ring (D-loop) is the noncoding region of the mitochondrial genome. Mutations in this region could affect the replication, recombination, or membrane association of mtDNA [54**]. Mitochondrial DNA mutations in the D-loop region and as well as in genes that encode OXPHOS proteins, tRNAs and rRNAs were identified in women with PCOS (Table 1). Zhuo *et al*. assessed peripheral blood cells in 57 women with PCOS and 38 controls and identified 16 variants in the D-Loop area, 7 in 12S rRNA, 3 in 16S rRNA. In addition, they detected other sequence variants that occurred in mitochondrial polypeptide-coding genes, suggesting that mitochondrial dysfunction might be involved in the pathogenesis of PCOS [55]. In addition, they also identified six tRNA polymorphisms in PCOS patients, which were absent in the control group [55]. These tRNA mutations included $tRNA^{Gln}$ T4395C, $tRNA^{Cys}$ G5821A, $tRNA^{Asp}$ A7543G, $tRNA^{Lys}$ A8343G, $tRNA^{Arg}$ T10454C, and $tRNA^{Glu}$ A14693G [55]. As most of the mitochondrial proteins are encoded by nuclear DNA and

Table 1. Reported variants and single nucleotide polymorphisms in mitochondrial DNA in women with polycystic ovary syndrome

Gene	Abnormalities
$tRNA^{Gln}$	Mutation
$tRNA^{Cys}$	Mutation
$tRNA^{Asp}$	Mutation
$tRNA^{Lys}$	Mutation
$tRNA^{Arg}$	Mutation
$tRNA^{Glu}$	Mutation
$tRNA^{Gln}$	Mutation
C3275T mutation in $tRNA^{Leu}$	Mutation
T4363C mutation in $tRNA^{Gln}$	Mutation
A8343G mutation in $tRNA^{Lys}$	Mutation
D310 in the mtDNA D-loop	SNPs
A189G in the mtDNA D-loop	SNPs
PGC-1α rs8192678 'Ser' allele	Mutation
A3302G in mt-$tRNA^{Leu\ (UUR)}$	Mutation
C7492T in mt-$tRNA^{Ser\ (UCN)}$	Mutation
T12338C in *ND5*	Mutation
ND4 11,719 G to A	Mutation
ND5 13,928 G to C	Mutation
D-loop 73 A to G 150 T to C 328 C to T 16 142 T to C 16 189 T to C 16 224 T to C	SNPs
12S rRNA 750 A to G 12S rRNA 827 A to G 12S rRNA 1438 A to G	SNPs
16S rRNA 2706 A to G 16S rRNA 3109 T to C	SNPs
CytB 14766 C to T 15 326 A to G 15 535 C to T	SNPs

According to references [55–58]. SNPs, single nucleotide polymorphisms.

mitochondrial tRNAs act as key effectors in translation and are linked to metabolic activity, authors suggested that these tRNA variants could contribute to the pathogenesis of PCOS. Moreover, additional tRNA variants were detected in PCOS patients with insulin resistance [56–58].

In women with PCOS, a decrease in the expression of genes involved in OXPHOS was demonstrated in skeletal muscle, leading to the hypothesis that alterations in mitochondrial function could underlie some of the pathological characteristics of insulin resistance [20••]. To support this hypothesis, the function of mitochondrial complex I in the electron transport chain (ETC) was found to be reduced in PCOS patients compared with controls matched for age and BMI, indicating that in some women with PCOS, oxygen consumption is impaired [59]. These observations suggest a potential pathway through which mtDNA mutations could contribute to the pathogenesis of PCOS.

Mitochondrial DNA copy number in polycystic ovary syndrome

mtDNA copy number is considered to be a relative measure of the number or mass of mitochondria. mtDNA copy number negatively correlates with ROS levels in the cell and is significantly lower in women with PCOS [60,61]. In addition, PCOS women with variants of tRNA also have lower mtDNA copy number, supporting a correlation between PCOS and tRNA variants [57]. In women with PCOS, mtDNA quantity negatively correlates with insulin resistance, whereas the association between lower mtDNA copy number and PCOS persists after being corrected for insulin resistance [25]. Similarly, in PCOS women with insulin resistance, mtDNA copy number positively correlates with SHBG [25], which does not only regulate androgen bioactivity [62] but also correlates inversely with fasting glucose, insulin, and insulin resistance [63].

The maintenance of an adequate amount of mtDNA is essential for the preservation of mitochondrial function and cell growth [64]. Damage to mtDNA is associated with mitochondrial dysfunction leading to a reduction of cellular metabolic activity and activation of apoptotic cell death [21]. Therefore, lower mtDNA content in women with PCOS may cause an increase in ROS and oxidative stress in the cell, ultimately resulting in DNA and RNA damage. In mice models of insulin resistance, mtDNA copy number is decreased in MII oocytes, which display impaired ATP biosynthesis and low fertilization rates [65]. Therefore, insulin resistance in women with PCOS may further disrupt mitochondrial function, suppress both oxidative and antioxidative capacities, increasing ROS and reducing ATP biosynthesis [66••].

THE ROLE OF UNFOLDED PROTEIN RESPONSE IN METABOLIC ASPECTS OF POLYCYSTIC OVARY SYNDROME

Insulin resistance and ensuing hyperinsulinemia in women with PCOS may lead to an increase in ROS and mitochondrial dysfunction, which, in turn, may cause activation of the mitochondrial unfolded protein response (UPR^{mt}) and endoplasmic reticulum-unfolded protein response (UPR^{er}).

Mitochondrial unfolded protein response in women with polycystic ovary syndrome

Mitochondrial chaperone system has an important effect on systemic energy homeostasis in obesity and metabolic diseases. UPR^{mt} is a transcriptional response that is activated by multiple forms of mitochondrial dysfunction and regulated by mitochondrial-to-nuclear communication [67–69]. Except for the 13 essential proteins of electron transport (ETC) that are encoded by mitochondrial DNA, the vast majority of mitochondrial proteins are encoded in the nucleus and are imported from the cytoplasm. As the mitochondrial matrix is enclosed by internal and external membranes, the import of proteins is complicated, requiring several specific molecular machineries [70]. Cells use a variety of mechanisms to monitor the health of the intracellular mitochondrial network [71]. If mitochondrial function declines, the UPR^{mt} is activated to promote the repair and recovery of this network and to maintain cellular function [72]. UPR^{mt} signalling originates in the matrix of the mitochondria when the misfolded or unassembled proteins accumulate and overload the capacity of the chaperones. In response to unfolded protein stress in mammalian cells, the expression of the nuclear-encoded mitochondrial chaperones and the protease is induced transiently, the extent of which correlates with the level of unfolded proteins in mitochondria. Although it is unclear how mitochondrial chaperones contribute to the improvement of mitochondrial homeostasis in obesity and insulin resistance, recent works indicate a role in the recovery of mitochondrial function and biogenesis in mice.

Endoplasmic reticulum unfolded protein response in women with polycystic ovary syndrome

The endoplasmic reticulum (ER) stress is induced by the accumulation of incorrectly folded protein in

the ER, which can be caused by several physiological and pathological conditions. Indeed, multiple factors may induce ER stress, including hyperglycemia, oxidative stress, cytotoxicity, inflammation, pharmacological agents, pathogens, altered Ca^{++} homeostasis, nutrient deprivation, and genetic mutations that ultimately activate cell death pathways. In general, these conditions increase the demand for protein folding or attenuate the protein folding capacity of the ER [73]. The stress induced by this failure to adequately fold proteins causes activation of various signal transduction pathways in the ER. The protein response deployed, collectively called the UPR^{er}, plays a primary role in stress adaptation and affects a wide range of cellular functions [74,75]. Previous studies have demonstrated that ER stress is activated in granulosa cells of follicles in later stages [76], and to a greater extent in patients with PCOS compared with non-PCOS controls [74]. When ER stress cannot be resolved, apoptosis mechanisms become activated. Azhary *et al.* showed that hyperandrogenism may cause ER stress in granulosa cells of antral follicles and induce apoptosis if UPR cannot restore cellular hemostasis. The expression of pro-apoptotic proteins then becomes upregulated in granulosa cells of antral follicles and contribute to apoptotic loss of these cells [77**]. ER stress activated by hyperandrogenism in PCOS contributes to the growth arrest of antral follicles by promoting apoptosis of granulosa cells.

CONCLUSION

Women with PCOS are often overweight or obese and display a number of metabolic aberrations associated with mitochondrial dysfunction. Increased ROS and oxidative stress are observed in somatic cells and oocytes of women with PCOS and are associated with mutations in mtDNA regions encoding *OXPHOS* genes and tRNAs, as well as a decrease in mtDNA copy number. These metabolic abnormalities and the failure of UPR^{er} and UPR^{mt} to restore homeostasis may result in the activation of apoptotic pathways in several cell types, including granulosa cells, and hasten the growth of follicles and generation of healthy oocytes. Further characterization of the role of mitochondrial dysfunction in the pathogenesis of PCOS may provide novel mechanistic insights, which can then be exploited for the reproductive success of affected subjects.

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Conflicts of interest

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REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. Endocr Rev 2012; 33:981–1030.
2. Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr Rev 2016; 37:467–520.
3. Zawadzki JK, D A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif A, Givens JR, Haseltine FP, Merriam GR, editors. Polycystic ovary syndrome. Boston: Blackwell Scientific; 1992 . pp. 377–384.
4. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004; 81:19–25.
5. Azziz R, Carmina E, Dewailly D, *et al.* Position statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab 2006; 91:4237–4245.
6. • Teede HJ, Misso ML, Costello MF, *et al.* Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Hum Reprod 2018; 33:1602–1618.

The first international and most recent guidelines on the assessment and management of PCOS.

7. Wood JR, Nelson VL, Ho C, *et al.* The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. J Biol Chem 2003; 278:26380–26390.
8. Duleba AJ, Spaczynski RZ, Olive DL. Insulin and insulin-like growth factor I stimulate the proliferation of human ovarian theca-interstitial cells. Fertil Steril 1998; 69:335–340.
9. Christakou CD, Diamanti-Kandarakis E. Role of androgen excess on metabolic aberrations and cardiovascular risk in women with polycystic ovary syndrome. Womens Health (Lond) 2008; 4:583–594.
10. Rajendran S, Willoughby SR, Chan WP, *et al.* Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects. Atherosclerosis 2009; 204:509–514.
11. Broekmans FJ, Knauff EA, Valkenburg O, *et al.* PCOS according to the Rotterdam consensus criteria: change in prevalence among WHO-II anovulation and association with metabolic factors. BJOG 2006; 113:1210–1217.
12. Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. J Clin Endocrinol Metab 2015; 100:911–919.
13. Siristatidis CS, Maheshwari A, Vaidakis D, Bhattacharya S. In vitro maturation in subfertile women with polycystic ovarian syndrome undergoing assisted reproduction. Cochrane Database Syst Rev 2018; 11:CD006606.
14. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. Fertil Steril 2001; 76:936–942.
15. Heijnen EM, Eijkemans MJ, Hughes EG, *et al.* A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome. Hum Reprod Update 2006; 12:13–21.
16. Homburg R. Pregnancy complications in PCOS. Best Pract Res Clin Endocrinol Metab 2006; 20:281–292.
17. Kollmann M, Martins WP, Lima ML, *et al.* Strategies for improving outcome of assisted reproduction in women with polycystic ovary syndrome: systematic review and meta-analysis. Ultrasound Obstet Gynecol 2016; 48:709–718.
18. Weghofer A, Munne S, Chen S, *et al.* Lack of association between polycystic ovary syndrome and embryonic aneuploidy. Fertil Steril 2007; 88:900–905.
19. Qiao J, Feng HL. Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. Hum Reprod Update 2011; 17:17–33.

20. Li Y, Wang L, Xu J, *et al.* Higher chromosomal aberration rate in miscarried ▪▪ conceptus from polycystic ovary syndrome women undergoing assisted reproductive treatment. Fertil Steril 2019; 111:936.e2–943.e2.

The study show in women with PCOS an increased risk of chromosomally aberrant miscarriage compared with non-PCOS controls during ART

21. Serra D, Mera P, Malandrino MI, *et al.* Mitochondrial fatty acid oxidation in obesity. Antioxid Redox Signal 2013; 19:269–284.
22. Iager AE, Kocabas AM, Otu HH, *et al.* Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. Fertil Steril 2013; 99:745.e5–752.e6.
23. Wang Q, Frolova AI, Purcell S, *et al.* Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. PLoS One 2010; 5:e15901.
24. Victor VM, Rocha M, Banuls C, *et al.* Induction of oxidative stress and human leukocyte/endothelial cell interactions in polycystic ovary syndrome patients with insulin resistance. J Clin Endocrinol Metab 2011; 96:3115–3122.
25. Lee SH, Chung DJ, Lee HS, *et al.* Mitochondrial DNA copy number in peripheral blood in polycystic ovary syndrome. Metabolism 2011; 60:1677–1682.
26. Skov V, Glintborg D, Knudsen S, *et al.* Reduced expression of nuclear-encoded genes involved in mitochondrial oxidative metabolism in skeletal muscle of insulin-resistant women with polycystic ovary syndrome. Diabetes 2007; 56:2349–2355.
27. Azziz R, Woods KS, Reyna R, *et al.* The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 2004; 89:2745–2749.
28. Dahan MH, Reaven G. Relationship among obesity, insulin resistance, and hyperinsulinemia in the polycystic ovary syndrome. Endocrine 2019; 64:685–689.
29. Shahin L, Hyassat D, Batieha A, *et al.* Insulin sensitivity indices in patients with ▪ polycystic ovary syndrome with different body mass index categories. Curr Diabetes Rev 2019. doi: 10.2174/1573399815666190823151222.

This study showed that insulin resistance is prevalent among women with PCOS.

30. Gayoso-Diz P, Otero-Gonzalez A, Rodriguez-Alvarez MX, *et al.* Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: effect of gender and age: EPIRCE cross-sectional study. BMC Endocr Disord 2013; 13:47.
31. Dumesic DA, Phan JD, Leung KL, *et al.* Adipose insulin resistance in normal-weight women with polycystic ovary syndrome. J Clin Endocrinol Metab 2019; 104:2171–2183.
32. Vrbikova J, Cibula D, Dvorakova K, *et al.* Insulin sensitivity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2004; 89:2942–2945.
33. Igosheva N, Abramov AY, Poston L, *et al.* Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. PLoS One 2010; 5:e10074.
34. Dandona P, Mohanty P, Ghanim H, *et al.* The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. J Clin Endocrinol Metab 2001; 86:355–362.
35. Sabuncu T, Vural H, Harma M, Harma M. Oxidative stress in polycystic ovary syndrome and its contribution to the risk of cardiovascular disease. Clin Biochem 2001; 34:407–413.
36. Gonzalez F, Rote NS, Minium J, Kirwan JP. Reactive oxygen species-induced oxidative stress in the development of insulin resistance and hyperandrogenism in polycystic ovary syndrome. J Clin Endocrinol Metab 2006; 91:336–340.
37. Murri M, Luque-Ramirez M, Insenser M, *et al.* Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update 2013; 19:268–288.
38. Dinger Y, Akcay T, Erdem T, *et al.* DNA damage, DNA susceptibility to oxidation and glutathione level in women with polycystic ovary syndrome. Scand J Clin Lab Invest 2005; 65:721–728.
39. Adashi EY. Endocrinology of the ovary. Hum Reprod 1994; 9:815–827.
40. Uyar A, Torrealday S, Seli E. Cumulus and granulosa cell markers of oocyte and embryo quality. Fertil Steril 2013; 99:979–997.
41. Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. 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 2001; 86:1318–1323.
42. Kuşçu NK, VA. Oxidative stress but not endothelial dysfunction exists in nonobese, young group of patients with polycystic ovary syndrome. Acta Obstet Gynecol Scand 2009; 88:612–617.
43. Fenkci V, Fenkci S, Yilmazer M, Serteser M. Decreased total antioxidant status and increased oxidative stress in women with polycystic ovary syndrome may contribute to the risk of cardiovascular disease. Fertil Steril 2003; 80:123–127.
44. Palacio JR, Iborra A, Ulcova-Gallova Z, *et al.* The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients. Clin Exp Immunol 2006; 144:217–222.
45. Insenser M, Martinez-Garcia MA, Montes R, *et al.* Proteomic analysis of plasma in the polycystic ovary syndrome identifies novel markers involved in iron metabolism, acute-phase response, and inflammation. J Clin Endocrinol Metab 2010; 95:3863–3870.
46. Nunez-Calonge R, Cortes S, Gutierrez Gonzalez LM, *et al.* Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors. Reprod Biomed Online 2016; 32:446–456.
47. Lai Q, Xiang W, Li Q, *et al.* Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. Front Med 2018; 12:518–524.
48. Banuls C, Rovira-Llopis S, Martinez de Maranon A, *et al.* Metabolic syndrome enhances endoplasmic reticulum, oxidative stress and leukocyte-endothelium interactions in PCOS. Metabolism 2017; 71:153–162.
49. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417:1–13.
50. Zhang J, Xu Y, Liu H, Pan Z. MicroRNAs in ovarian follicular atresia and granulosa cell apoptosis. Reprod Biol Endocrinol 2019; 17:9.
51. Yi HS, Chang JY, Shong M. The mitochondrial unfolded protein response and mitohormesis: a perspective on metabolic diseases. J Mol Endocrinol 2018; 61:R91–R105.
52. Seli E. Mitochondrial DNA as a biomarker for in-vitro fertilization outcome. Curr Opin Obstet Gynecol 2016; 28:158–163.
53. Cozzolino M, Marin D, Sisti G. New Frontiers in IVF: mtDNA and autologous germline mitochondrial energy transfer. Reprod Biol Endocrinol 2019; 17:;. 55.
54. Reddy TV, Govatati S, Deenadayal M, *et al.* Impact of mitochondrial DNA copy ▪▪ number and displacement loop alterations on polycystic ovary syndrome risk in south Indian women. Mitochondrion 2019; 44:35–40.

The study analysis showed significantly diminished mtDNA copy number in PCOS patients compared with controls.

55. Zhuo G, Ding Y, Feng G, *et al.* Analysis of mitochondrial DNA sequence variants in patients with polycystic ovary syndrome. Arch Gynecol Obstet 2012; 286:653–659.
56. Ding Y, Zhuo G, Zhang C, Leng J. Point mutation in mitochondrial tRNA gene is associated with polycystic ovary syndrome and insulin resistance. Mol Med Rep 2016; 13:3169–3172.
57. Ding Y, Zhuo G, Zhang C. The mitochondrial tRNALeu(UUR) A3302G mutation may be associated with insulin resistance in woman with polycystic ovary syndrome. Reprod Sci 2016; 23:228–233.
58. Ding Y, Xia BH, Zhang CJ, Zhuo GC. Mitochondrial tRNA(Leu(UUR)) C3275T, tRNA(Gln) T4363C and tRNA(Lys) A8343G mutations may be associated with PCOS and metabolic syndrome. Gene 2018; 642: 299–306.
59. Victor VM, Rocha M, Banuls C, *et al.* Mitochondrial complex I impairment in leukocytes from polycystic ovary syndrome patients with insulin resistance. J Clin Endocrinol Metab 2009; 94:3505–3512.
60. Hutter E, Unterluggauer H, Garedew A, *et al.* High-resolution respirometry–a modern tool in aging research. Exp Gerontol 2006; 41:103–109.
61. Yowe DL, Ames BN. Quantitation of age-related mitochondrial DNA deletions in rat tissues shows that their pattern of accumulation differs from that of humans. Gene 1998; 209:23–30.
62. Ibanez L, Potau N, Zampolli M, *et al.* Hyperinsulinemia and decreased insulin-like growth factor-binding protein-1 are common features in prepubertal and pubertal girls with a history of premature pubarche. J Clin Endocrinol Metab 1997; 82:2283–2288.
63. Lewis JG, Borowski KK, Shand BI, *et al.* Plasma sex hormone-binding globulin, corticosteroid-binding globulin, cortisol, and free cortisol levels in outpatients attending a lipid disorders clinic: a cross-sectional study of 1137 subjects. Horm Metab Res 2010; 42:274–279.
64. Jeng JY, Yeh TS, Lee JW, *et al.* Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. J Cell Biochem 2008; 103:347–357.
65. Ou XH, Li S, Wang ZB, *et al.* Maternal insulin resistance causes oxidative stress and mitochondrial dysfunction in mouse oocytes. Hum Reprod 2012; 27:2130–2145.
66. Hu M, Zhang Y, Guo X, *et al.* Hyperandrogenism and insulin resistance induce ▪▪ gravid uterine defects in association with mitochondrial dysfunction and aberrant reactive oxygen species production. Am J Physiol Endocrinol Metab 2019; 316:E794–E809.

According to Li *et al.* in 2019, PCOS women have an increased risk of miscarriage, this study revealed that hyperandrogenism and insulin resistance could induce mitochondria damage resulting imbalance between oxidative and antioxidative stress responses.

67. Nargund AM, Pellegrino MW, Fiorese CJ, *et al.* Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. Science 2012; 337:587–590.
68. Yoneda T, Benedetti C, Urano F, *et al.* Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. J Cell Sci 2004; 117(Pt 18):4055–4066.
69. Schulz AM, Haynes CM. UPR(mt)-mediated cytoprotection and organismal aging. Biochim Biophys Acta 2015; 1847:1448–1456.
70. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. Annu Rev Biochem 2007; 76:723–749.
71. Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annu Rev Genet 2012; 46:265–287.

72. Fiorese CJ, Haynes CM. Integrating the UPR(mt) into the mitochondrial maintenance network. Crit Rev Biochem Mol Biol 2017; 52:304–313.
73. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science 2011; 334:1081–1086.
74. Takahashi N, Harada M, Hirota Y, *et al.* Activation of endoplasmic reticulum stress in granulosa cells from patients with polycystic ovary syndrome contributes to ovarian fibrosis. Sci Rep 2017; 7:10824.
75. Takahashi N, Harada M, Hirota Y, *et al.* A potential role of endoplasmic reticulum stress in development of ovarian hyperstimulation syndrome. Mol Cell Endocrinol 2016; 428:161–169.
76. Harada M, Nose E, Takahashi N, *et al.* Evidence of the activation of unfolded protein response in granulosa and cumulus cells during follicular growth and maturation. Gynecol Endocrinol 2015; 31:783–787.
77. Azhary JMK, Harada M, Takahashi N, *et al.* Endoplasmic reticulum
•• stress activated by androgen enhances apoptosis of granulosa cells via induction of death receptor 5 in PCOS. Endocrinology 2019; 160:119–132.

In this study was showed that androgens could active ER stress in granulosa cells of antral follicles which consequently results in the activation of apoptosis pathway.

CHAPTER 5: STUDY II

Transcriptomic landscape of granulosa cells and peripheral blood mononuclear cells in women with PCOS compared to young poor responders and women with normal response

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Transcriptomic landscape of granulosa cells and peripheral blood mononuclear cells in women with PCOS compared to young poor responders and women with normal response

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STUDY QUESTION: Are transcriptomic profiles altered in ovarian granulosa cells (GCs) and peripheral blood mononuclear cells (PBMNCs) of women with polycystic ovary syndrome (PCOS) compared to young poor responders (YPR) and women with normal response to ovarian stimulation?

SUMMARY ANSWER: RNA expression profiles in ovarian GCs and PBMNCs were significantly altered in patients with PCOS compared with normoresponder controls (CONT) and YPR.

WHAT IS KNOWN ALREADY: PCOS is characterised by a higher number of follicles at all developmental stages. During controlled ovarian hyperstimulation, PCOS women develop a larger number of follicles as a result of an exacerbated response, with an increased risk of ovarian hyperstimulation syndrome. Despite the number of developing follicles, they are often heterogeneous in both size and maturation stage, with compromised quality and retrieval of immature oocytes. Women with PCOS appear to have a longer reproductive lifespan, with a slightly higher menopausal age than the general population, in addition to having a higher antral follicular count. As a result, the ovarian follicular dynamics appear to differ significantly from those observed in women with poor ovarian response (POR) or diminished ovarian reserve.

STUDY DESIGN, SIZE, DURATION: Transcriptomic profiling with RNA-sequencing and validation using quantitative reverse transcription PCR (qRT-PCR). Women with PCOS (N = 20), YPR (N = 20) and CONT (N = 20). Five patients for each group were used for sequencing and 15 samples per group were used for validation.

PARTICIPANTS/MATERIALS, SETTING, METHODS: PCOS was defined using the revised Rotterdam diagnostic criteria for PCOS. The YPR group included women <35 years old with <4 mature follicles (at least 15 mm) on the day of the trigger. According to internal data, this group represented the bottom 15th percentile of patients' responses in this age group. It was consistent with Patient-Oriented Strategies Encompassing Individualize D Oocyte Number (POSEIDON) criteria for POR (Group 3). The young CONT group included women <35 years without PCOS or anovulation, who developed >14 mature follicles (at least 15 mm on transvaginal ultrasound). According to internal data, a threshold of >14 mature follicles was established to represent the top 25% of patients in this age group in this clinic.

Overall, n = 60 GCs and PBMNCs samples were collected and processed for total RNA extraction. To define the transcriptomic cargo of GCs and PBMNCs, RNA-seq libraries were successfully prepared from samples and analysed by RNA-seq analysis. Differential gene expression analysis was used to compare RNA-seq results between different groups of samples. Ingenuity pathway analysis was used to perform Gene Ontology and pathways analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: In PBMNCs of PCOS, there were 65 differentially expressed genes (DEGs) compared to CONT, and 16 compared to YPR. In GCs of PCOS, 4 genes showed decreased expression compared to CONT, while 58 genes were differentially expressed compared to YPR. qRT-PCR analysis confirmed the findings of the RNA-seq. The functional enrichment analysis performed revealed that DEGs in GCs of PCOS compared to CONT and YPR were prevalently involved in protein ubiquitination, oxidative phosphorylation, mitochondrial dysfunction and sirtuin signaling pathways.

LARGE SCALE DATA: The data used in this study is partially available at Gene Ontology database.

LIMITATIONS, REASONS FOR CAUTION: The analysis in PBMNCs could be uninformative due to inter-individual variability among patients in the same study groups. Despite the fact that we considered this was the best approach for our study's novel, exploratory nature.

WIDER IMPLICATIONS OF THE FINDINGS: RNA expression profiles in ovarian GCs and PBMNCs were altered in patients with PCOS compared with CONT and YPR. GCs of PCOS patients showed altered expression of several genes involved in oxidative phosphorylation, mitochondrial function and sirtuin signaling pathways. This is the first study to show that the transcriptomic landscape in GCs is altered in PCOS compared to CONT and YPR.

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Introduction

After being recognized as a clinical entity more than 85 years ago by Stein and Leventhal (Azziz, 2006), there has been several attempts to refine the clinical diagnostic criteria for polycystic ovary syndrome (PCOS). Among the widely accepted definitions, the first one was the 1991 National Institute of Health (NIH) consensus definition which required oligo/amenorrhea and hyperandrogenemia/hyperandorenism (symptoms associated with response to androgens) in the absence of other causative factors. The Rotterdam criteria, which were later proposed in 2004 (Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a), added the presence of polycystic ovaries to the two previously described factors, and required two out of these three factors to be present for a PCOS diagnosis. The Androgen Excess and PCOS Society (PCOS-AE) definition recently proposed a revision of the Rotterdam criteria; accepting the two out of three-diagnostic paradigm, but requiring hyperandrogenism to be present (Azziz et al., 2009). Polycystic ovary is defined as an ovary containing 12 or more follicles (or 25 or more follicles using new ultrasound technology) measuring 2 to 9 mm in diameter or alternatively as an ovary that has a volume of >10 ml on ultrasonography. A single ovary meeting either of these definitions is sufficient for diagnosis of polycystic ovaries (Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004b). A high number of antral follicles is among the most important characteristics of women with this complex and potentially multi-factorial syndrome.

As expected, due to the increased antral follicle count (AFC) in women with PCOS (Webber et al., 2003; Maciel et al., 2004; Bhide et al., 2019), these patients tend to respond to controlled ovarian stimulation (COS) with gonadotropins by producing a higher number of oocytes and achieving higher levels of maximum serum estradiol (Sun et al., 2020). They are also at a higher risk of developing ovarian hyperstimulation syndrome (OHSS). In addition to having a higher AFC, women with PCOS seem to have a prolonged reproductive lifespan (Carroll et al., 2012), with slightly higher menopausal age than the general population (Ramezani Tehrani et al., 2010). Therefore, the ovarian follicular dynamics seem to show a significant contrast to that observed in women with poor ovarian response (POR) or diminished ovarian reserve (DOR).

POR/DOR represents the opposite end of a spectrum regarding follicular pool, and reaching a consensus on the definition of POR has also been challenging. A commonly used definition is the ESHRE (European Society for Human Reproduction and Embryology) Bologna criteria (Younis et al., 2015). It requires two out of this three criteria to be present: a maternal age >40 years (or any other risk factor for low response); a previous poor response cycle with ≤3 oocytes retrieved; and an abnormal ovarian reserve test (AFC < 5–7 or anti-mullerian hormone (AMH) < 0.5–1.1 ng/ml). A more recent and detailed definition has come from the Patient-Oriented Strategies Encompassing Individualize D Oocyte Number (POSEIDON) group, considering age, AFC, serum AMH levels and past response to ovarian stimulation (Esteves et al., 2019). POSEIDON Group 1 refers to young infertile women (<35 years old), with acceptable ovarian reserve markers (AFC ≥ 5; AMH ≥ 1.2 ng/ml), and unexpected poor (<4 oocytes retrieved) or suboptimal (4–9 oocytes retrieved) response to conventional ovarian stimulation, while POSEIDON Group 3 includes women <35 years old with low ovarian reserve markers (AFC < 5; AMH < 1.2 ng/ml). POSEIDON Groups 2 and 4 include

women AMH ≥35 years old, with similar characteristics to Groups 1 and 3, respectively. Regardless of the diagnostic criteria used, it is widely accepted that women with POR/DOR tend to have lower AFC and respond poorly to COS, in contrast to women with PCOS.

Granulosa cells (GCs) surround the oocyte, support follicular development and are essential in the transition from the primordial into a mature follicle. The interactions between GCs and oocytes are critical for coordinated oocyte maturation (Thomas and Vanderhyden, 2006). Therefore, investigating the molecular pathways involved in the proliferation, differentiation and functional transformation of GCs is crucial for the understanding of mechanisms that regulate folliculogenesis. GCs not only play a critical role in normal folliculogenesis, but they are also involved in the abnormal folliculogenesis observed in disorders such as PCOS (Yilmaz *et al.*, 2018), and premature ovarian insufficiency (Collins *et al.*, 2017). Small antral follices in PCOS patients' ovaries, failed to develop into larger dominant follicles. PCOS in the murine is characterised by an increase in the follicle number and GC proliferation (Cozzolino and Seli, 2020). Furthermore, increased GC proliferation in smaller follicles was observed in the ovaries of women with PCOS (Yildiz *et al.*, 2012; Liu *et al.*, 2016). In contrast, there is an increase in GC apoptosis in young poor responders (YPR). Therefore, GC dysfunction and/or proliferative dysregulation seems to be among the pathological features of women with PCOS and POR, and GCs constitute a useful cellular model to investigate these complex conditions.

In this study, we hypothesized that characterising the differences between women with PCOS and those with POR/DOR could be useful to identify clinically relevant molecular mechanisms that promote follicular growth and biomarkers of aberrant follicle development. To this end, we characterised the transcriptomic landscape of women with PCOS, comparing and contrasting it to women with POR and normoresponder controls (CONT). To achieve a detailed understanding of the differences between these opposite entities, we analysed both ovarian somatic cells (GCs) and non-reproductive somatic cells (peripheral blood mononuclear cells [PBMNCs]).

Materials and methods

Study patients

This was a prospective study and the samples were prospectively collected. All study procedures were approved by and conducted according to the Institutional Review Board of Hospital Universitario y Politécnico La Fe, Valencia, Spain (520/2018). Prior to collecting samples, all patients were informed, and written consent was obtained

PCOS was defined using the revised Rotterdam diagnostic criteria for PCOS (Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a). Other causes of oligomenorrhea or hyperandrogenism congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome were excluded on clinical grounds for the study. The YPR group included women <35 years old with <4 mature follicles (at least 15 mm) on the day of the trigger. According to internal data, this group represented the bottom 15th percentile of patients' responses in this age group and was consistent with POSEIDON criteria for POR (Group 3). Women with ovarian cancer, endometriosis or severe systemic disease were excluded. The young normoresponder (CONT) group included women <35 years without PCOS or anovulation, who developed >14 mature follicles (at least 15 mm on transvaginal ultrasound). According to internal data a threshold of >14 mature follicles was established to represent the top 25% of patients in this age group. A total of 20 women with PCOS, 20 YPR and 20 CONT were recruited for the study at the Hospital La Fe (Valencia, Spain).

Controlled ovarian stimulation protocols, oocyte retrieval and granulosa cell collection

The patients included in the study received COS using the GnRH agonist standard long protocol or antagonist protocol. Ovarian stimulation was achieved using recombinant FSH (rFSH) and highly purified HMG (HP-HMG). COS was started after confirming the absence of dominant follicular development by ultrasound. Serum estradiol (E2) <30 mIU/ml. rFSH was administrated daily, with dosages ranging from 150 to 300 IU, alone or in combination with hMG. When the lead follicular diameter was greater than 18–19 mm, 5000–10 000 IU hCG and/or GnRH agonist (GnRHa) were used to achieve final follicular maturation. To reduce the risk of OHSS in PCOS patients, oocyte maturation was primarily achieved using GnRHa alone, whereas those in the YPR group used 10 000 IU hCG. Patients in the CONT group used one of these strategies based on their physician's preference. Oocyte retrieval was scheduled 36 h after trigger administration.

Follicular fluid samples were carefully collected from the first aspirated follicle of each ovary. Samples containing blood contamination were excluded. Ovarian GCs were isolated and purified from each follicular fluid sample by centrifugation at 450 *g* for 5 min. Recovered GCs were then washed with 1 ml of phosphate buffer saline (PBS) and centrifuged at maximum speed for 5 min. The GCs were resuspended in 10 µl of PBS. After that, 50 µl of RNA were added and samples were stored at −20°C. Over 95% of purity was achieved for all GC samples before being used in subsequent experiments. The GCs were then processed for RNA extraction.

Collection of peripheral blood mononuclear cell samples

Blood was collected in EDTA BD Vacutainer® tubes (BD Diagnostics, Spain) to isolate PBMNCs by standard Ficoll-based centrifugation protocols. Briefly, 4 ml of blood was diluted to a final volume of 7 ml and added to 3 ml of Ficoll-Paque (GE Healthcare, Uppsala, Sweden) to undergo a 2500 rpm centrifugation for 30 min at RT. Buffy coat containing PBMNCs was then collected and PBMNCs were resuspended in PBS and washed twice. The pellet was resuspended in 100 µl of PBS, with 500 µl of RNA later and stored at −20°C until ready for RNA extraction.

RNA extraction and sequencing

Total RNA was extracted from PBMNCs using the RNeasy Micro kit (Qiagen). RNA integrity and concentration were measured using the Agilent High Sensitivity RNA screen tape system and Qubit2, respectively. All samples used for RNA-seq had an RNA integrity number (RIN) of 7 or higher. mRNA was purified from approximately 200 ng of total RNA with oligo-dT beads and sheared by incubation at 94°C in

the presence of Mg (Kapa mRNA Hyper Prep). Following first-strand synthesis with random primers, second-strand synthesis and A-tailing were performed with dUTP for generating strand-specific sequencing libraries. Adapter ligation with 3′ dTMP overhangs was ligated to library insert fragments. Library amplification amplified fragments carrying the appropriate adapter sequences at both ends. Strands marked with dUTP were not amplified. Indexed libraries that meet appropriate cut-offs for both were quantified by quantitative reverse transcription PCR (qRT-PCR) using a commercially available kit (KAPA Biosystems) and insert size distribution determined with the LabChip GX or Agilent Bioanalyzer. Samples with a yield of ≥0.5 ng/µl were used for sequencing.

RNA was extracted from purified GCs and cDNA was amplified using Smart-Seq v4 ultra-low input RNA kit for sequencing following the manufacturer's protocol. cDNA was amplified and measured using dsDNA High Sensitivity Kit on Qubit 2.0 (Life Technologies, Carlsbad, CA, USA) for concentration and Tapestation 2200 (Agilent Technologies, Santa Clara, CA, USA) for size distribution. RNA-sequencing libraries were constructed using the Nextera® XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and multiplexed using Nextera® XT Index Kit (Illumina). Libraries were quantified by Qubit2.0 and Tapestation 2200 (Agilent technologies). Indexed libraries were then pooled and sequenced on Illumina's Hiseq 2500 platform with 75 bp pair-end reads. We analysed 15 samples in total, and approximately 50 million paired-end reads were achieved for each sample.

RNA-sequencing analysis

Image analysis, base calling and generation of sequence reads were produced using the HiSeq Control Software v2.0 (NCS) and Real-Time Analysis Software (RTA). Data were converted to FASTQ files using the bcl2fastq2 v1.8.4 software (Illumina Inc.). The reads were trimmed for quality and aligned with the reference human genome hg38 with gencode annotation (Frankish *et al.*, 2019). The normal annotation has ~50K entries, but the gencode annotation has over 100K annotated regions on the genome. We used HiSAT2 for alignment, and StringTie and BallGown for transcript abundance estimation (transcript level expression analysis of RNA-seq experiments with HISAT, String Tie and Ball gown). We used DESEq2 for differential gene expression. Gene expression values were calculated as FPKM using Cufflinks 2.1.1. The genes were called as differentially expressed if the adjusted *P*-value was ≤0.05. We used R for downstream processing and visualization of the data.

Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) Ingenuity Systems QIAGEN (Content version: 45865156, 2018, www.qiagen.com/ingenuity, Redwood City, CA, USA) software was used to perform Gene Ontology (GO) and pathways analyses. A differential expression Log2 fold change (FC) ≥0.584 false discovery rate (FDR) ≤0.05 was assigned as differentially expressed in different comparisons. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Differentially expressed genes (DEGs) used in pathway analysis were determined between treated and control groups by using a filtering criteria FC >0.5 and Benjamini–Hochberg (B-H FDR) ≤0.05 (Benjamini and Hochberg,1995). IPA was used to identify pathways overrepresented in DEGs (Benjamini and Hochberg, 1995).

Quantitative reverse transcription PCR

Another set of PBMNC samples were used for the validation of selected genes that were seen to be differentially regulated in the RNA-sequencing studies. The qRT-PCR screening was performed on genes involved in the relevant clinical pathway. The primer sequences of the genes used for the validation are listed in Supplementary Table SI.

Total RNA was obtained from PBMNCs using RNAqueous Microkit (Thermo Fisher Scientific, Waltham, MA, USA) and was treated with DNase I (Thermo Fisher Scientific) for genomic DNA contamination. Reverse transcription was performed using the RETROscript kit (Thermo Fisher Scientific) in two steps: first, template RNA and random primers were incubated at 85°C for 3 min to eliminate any secondary structures, and then the buffer and enzyme were added, and the reaction was carried out at 42°C for 1 h. Gene expression analysis was done by real-time PCR using Syber Green on the ViiA7 real-time PCR machine from Applied Biosystems. To check reproducibility, each qPCR reaction was carried out in triplicate, and water was used as a negative control. The amplification was a 45-cycle run with annealing temperature at 60°C. GAPDH was used as the housekeeping gene. Moreover, at the end of the amplification, a melting curve for each sample was generated to assess the absence of primer dimers or contamination.

Real-time PCR (RT-PCR) was also performed to confirm differential expression of genes identified as altered in the cumulus cells. RNA was amplified, and cDNA was prepared using established protocol of SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing. Gene expression analysis was done by RT-PCR using Syber Green on the ViiA7 real-time PCR machine by Applied Biosystems. The PCR reaction was prepared using 5 µl PowerUp™ SYBR™ Green Master Mix (2×), 1 µl forward and reverse primers, 1 µl of DNA template and 3 µl of nuclease-free water. The components were mixed thoroughly and briefly centrifuged. PCR cycling conditions were 95°C for 5 min followed by 45-cycle run with annealing temperature at 60°C. GAPDH was used for normalization. ΔΔCt method was used for the calculation of the difference in the expression of genes A melt curve analysis was subsequently performed to confirm the specificity of products.

Statistical analysis

All the characteristics of PCOS, YPR and healthy controls were given as mean ± SD. The expression data for GCs and PBMNCs obtained by RT-qPCR was analysed with the two-tailed *t*-tests using the GraphPad Prism version 5.00 software (GraphPad, San Diego, CA, USA; www.graphpad.com). Differences between groups were considered significant when the *P*-value was <0.05.

Results

RNAseq analyses were performed to compare mRNA expression levels in ovarian GCs and PBMNCs between the three experimental groups of women: (i) PCOS (n = 5) who were young (age 32.5 ± 0.7 years) with a robust response to ovarian stimulation during ART; (ii) YPR (n = 5; age 33.1 ± 1.4 years) with poor response to ovarian stimulation; (iii) CONT (n = 5; age 31.0 ± 2.1 years) who were women undergoing ART treatment. They did not have PCOS or

anovulation, and showed a robust response to COS. Baseline characteristics and the number of oocytes retrieved are reported in Table I.

To identify the genes and pathways differentially expressed in PCOS, YPR and CONT, an unbiased approach was adopted with comprehensive genome-wide transcriptomic analysis. The most significant genes for each comparison are reported in the Supplementary Table SII.

Differential gene expression in the PBMNCs of women with PCOS

Differentially expressed genes in PBMNCs of women with PCOS compared to CONT

Hierarchal clustering of the DEGs partitioned into two distinct clusters showed differential gene expression in PBMNCs of women with PCOS when compared with the CONT (Fig. 1a), suggesting high reproducibility of the sequencing data. A total of 65 genes showed changes in the expression between these two groups. 10 genes showed decreased gene expression in the PCOS samples when compared with controls and 55 genes showed increased expression (Fig. 1b). For genes that were over-expressed the FC ranged from 1.8 to 22.9. For under-expressed genes, the FC ranged from −1.8 to −6.49 (Fig. 1c). Zinc finger protein 57 homolog (ZFP57) and Immunoglobulin Lambda Constant (IGLC1) were significantly downregulated in PBMNCs of women with PCOS compared to the controls, FC = 22.9 and 9.31, respectively. Telomerase RNA component (TERC) and Small Nucleolar RNA, C/D Box 14D (SNORD14D) were overexpressed in PCOS compared to controls, FC = −4.08 and −3.23, respectively (Fig. 1c). The functional enrichment analysis based on data from IPA software were used to perform GO, and revealed that DEGs in PBMNCs of women with PCOS were mainly involved in

Table I General characteristics of patients in the three groups.

	CONT	PCOS	YPR
Age (years)	31.0±2.1	32.5 ±0.7	33.1 ±1.4
Number of oocytes	22 ± 3.9	28 ± 6.4	5 ± 0.7
Number of metaphases II	21 ± 2.2	16 ± 3.8	4 ± 0.6
Infertility reason			
Tubal	6	–	0
Endometriosis	1	–	4
Male infertility	9	–	3
Idiopathic	4	–	–
Diminished ovarian reserve	–	–	13
PCOS	–	20	–
Duration of infertility	3.3 ± 1.6	3.0 ± 1.3	3.1 ± 1.2
Tests for insulin resistance	1.7 ± 0.5	3.0 ± 2.1	0.9 ± 0.5
Antral follicle count	17.7 ± 9.8	26.3 ± 13.8	3.1 ± 1.8
AMH (pmol/l)	17.7 ± 10.3	37.7 ± 18.3	5.4 ± 2.7
Ovarian stimulation protocol	GnRH antagonist protocol	GnRH antagonist protocol	GnRH antagonist protocol
Duration of stimulation	10.0 ± 1.5	10.6 ± 1.8	10.9 ± 1.7
Total dose of gonadotropin	2183 ± 926	1798 ± 912	3911 ± 1526
Type of triggering			
hCG	10	8	18
Agonist GnRH	9	9	1
Dual triggering	1	3	1
Estradiol trigger day	2238 ± 848	3335 ± 1863	1873 ± 696
Progesterone trigger day	1.26 ± 0.86	1.13 ± 0.57	0.83 ± 0.44
Rate/number OHSS*	2/20 (10%)	8/20 (40%)	0/20 (0%)
OTC therapy rate	6/20	14/20	0/20
PCOS sub-phenotype	0 (0%)		0 (0%)
Phenotype A with hyperandrogenism +oligomenorrhea +PCO		2(10%)	
Phenotype B with hyperandrogenism +oligomenorrhea		4 (20%)	
Phenotype C with hyperandrogenism+PCO		10 (50%)	
Phenotype D with oligomenorrhea +PCO		4 (20%)	

CONT, control group of normal responders; OHSS, ovarian hyperstimulation syndrome; OTC, over the counter; PCO, polycystic ovary; YPR, young poor responder.
*Only cases of mild OHSS are reported in the study, no cases of moderate or severe OHSS.

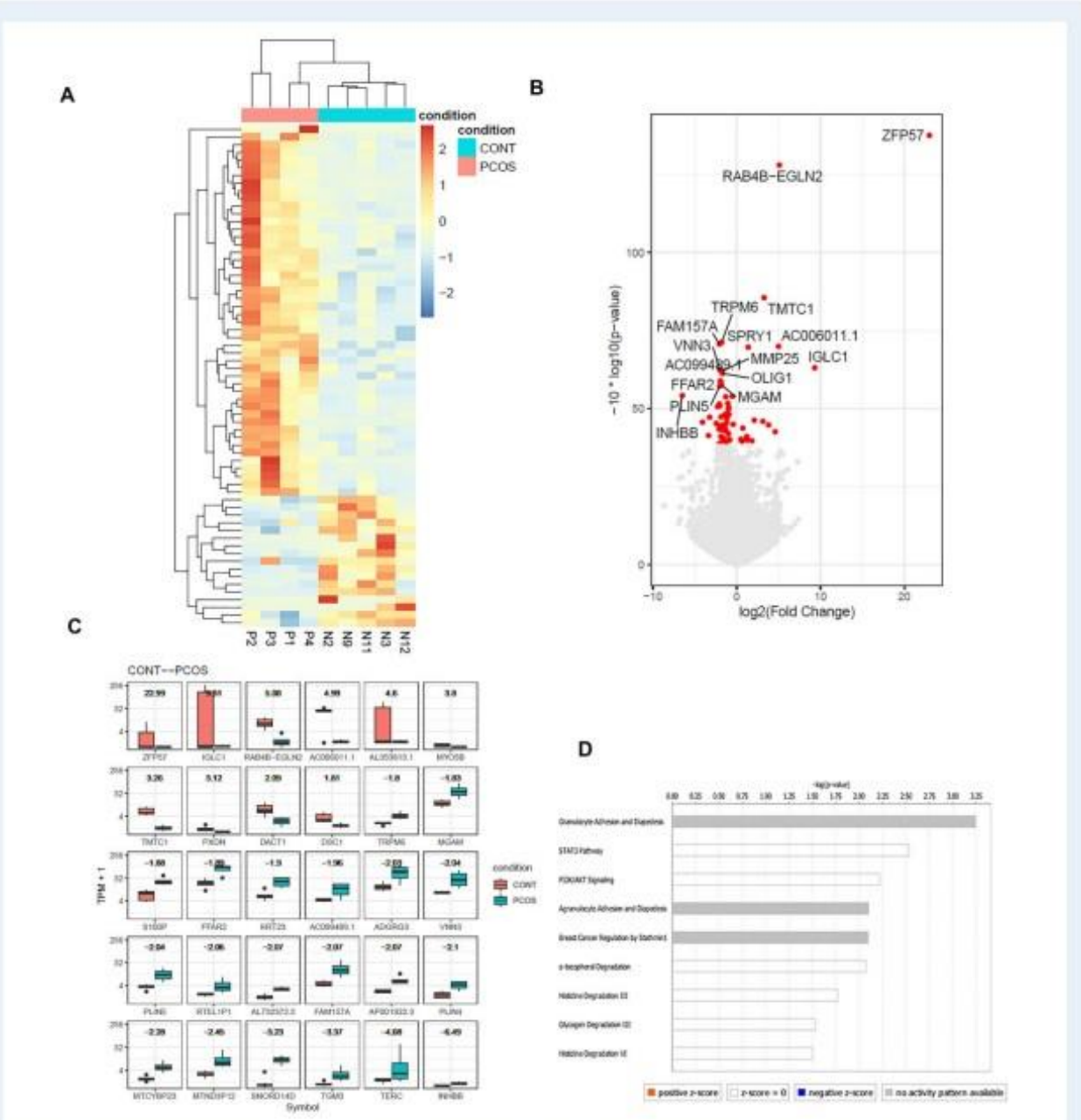

Figure 1. Gene expression is altered in PCOS women's peripheral blood mononuclear cells (PBMNCs) compared to normoresponder controls (CONT). (**a**) The heat map illustration shows differentially expressed genes in CONT and PCOS in PBMNCs. The colour spectrum ranging from red to blue indicates normalized levels of gene expression from high to low. (**b**) Volcano plots for RNA-seq comparing CONT and PCOS. Red spots on the left upper box represent –log10 (*P*-value) ≥2; red spots on the right upper box represent the –log10 (*P*-value) <2. (**c**) Selected differentially expressed genes in CONT and PCOS, $P < 0.05$ for each. The transcripts per million (TPM) value represents the relative expression level comparable between samples. For the box plots, the bottom and top whiskers denote 5 and 95 percentile values, the bottom and top bounds of the rectangle denote the 25 and 75 percentile values, and the line in between denotes the median (50 percentile) value of the distribution. (**d**) Pathway analysis was evaluated using the Gene Ontology bioinformatics tool in PBMNCs. Log2 fold change (FC) ≥0.584 false discovery rate (FDR) ≤0.05.

the pathway of granulocyte adhesion and breast cancer regulation. All affected pathways are reported in Fig. 1d.

Differentially expressed genes in PBMNCs of women with PCOS compared to YPR

Gene expression was found to be altered in PBMNCs of women with PCOS compared to YPR. The heat map showed the hierarchical cluster of the DEGs partitioned into two distinct clusters PCOS and YPR (Fig. 2a). Sixteen genes showed significant changes in the expression. Seven of them were upregulated in the PCOS when compared with the YPR samples, while nine genes showed decreased expression (Fig. 2b). FC ranged from 3.51 to 22.91 for upregulated and −0.6 to −22.94 for downregulated genes (Fig. 2c). The pathway analysis did not assign the genes to any specific pathways.

Differential gene expression in the granulosa cells of women with PCOS

Differentially expressed genes in granulosa cells of women with PCOS compared to CONT

Hierarchal clustering of the DEGs partitioned into two distinct clusters in the heat map of significant genes. It showed differential GC gene expression between women with PCOS and control (Fig. 3a). Four genes showed decreased expression in the PCOS group (Fig. 3b and c). There were no genes found to be overexpressed in PCOS compared to controls. For the overexpressed genes, FC ranged from 1.77 to 5.1 as reported in Fig. 3c. The functional enrichment analysis performed GO revealed that DEGs in PCOS compared to controls were prevalently included in the protein ubiquitination pathway (Fig. 3d).

Differential expression was seen in granulosa cells of PCOS patients compared to the YPR

Differential gene expression analysis in GCs showed a significant change in the expression of mRNAs in PCOS compared to YPR, as demonstrated by the heat map illustration (Fig. 4a). In total, 58 genes were differentially expressed between PCOS and YPR (Fig. 4b). Figure 4c shows the box plot representation of the top overexpressed genes with FC ranging from 2.07 to 2.33, and the underexpressed genes with FC ranging from −2.08 to −2.76. The IPA showed that the principal cluster of genes were involved in the pathways of oxidative phosphorylation, mitochondrial dysfunction and sirtuin signaling. The clusters of genes involved in oxidative phosphorylation were overexpressed in PCOS compared to YPR. In contrast, the genes involved in the pathway of sirtuin signaling were downregulated in PCOS compared to YPR (Fig. 4d).

Validation of differentially expressed selected genes by RT-qPCR

For validation of the RNA-sequencing data by qRT-PCR, two genes from each set were chosen for further analysis. qRT-PCRs were performed in independent samples (n = 15 for each group), and results showed a similar expression pattern to that observed in the RNA-sequencing.

In PBMNCs of women with PCOS compared to controls, there was a trend toward a decreased expression of RAB4 B ($P = 0.30$) and increased expression of TERC ($P = 0.1$) (Supplementary Fig. S1a). Similarly, a trend toward a decreased expression of peroxidasin (PXDN) ($P = 0.3$) and ZFP57 ($P = 0.1$) in the PBMNCs of women with PCOS compared to YPR was observed (Supplementary Fig. S1b).

When samples from women with PCOS were compared to controls, there was a decrease in the expression of membrane frizzled-related protein (MFRP) ($P = 0.03$) and a trend toward decreased expression of myotubularin-related protein 9 (MTMR9) ($P = 0.3$) in the GCs (Supplementary Fig. S2a). When GCs samples from women with PCOS were compared to those from YPR, there was an increase in the expression mitochondrially encoded 12S RRNA (MT-RNR1) ($P = 0.03$) and a trend toward increased expression of mitochondrially encoded ATP synthase membrane subunit 6 (MT-ATP6) ($P = 0.1$) (Supplementary Fig. S2b).

Discussion

This study describes the transcriptomic analysis of PBMNCs and GCs in women with PCOS compared to controls and young women with DOR, showing differences in gene expression landscapes and pathway alterations following ovarian stimulation.

In PBMNCs of women with PCOS, RNA-seq analysis found several genes to be altered compared to controls. Some of these genes were involved in the mechanisms of granulocyte and agranulocyte adhesion and diapedesis. In addition, TERC expression was increased in PBMNCs of patients with PCOS. There have been several studies suggesting that somatic cell telomere length may be affected in women with PCOS. Some suggested shortened telomeres in leukocytes in women with PCOS (Li *et al.*, 2014). Others did not find a difference in the leukocyte telomere length but described a negative correlation between inflammatory biomarkers and telomere length (Pedroso *et al.*, 2015) or detected altered telomere length in their GCs (Wei *et al.*, 2017).

The granulosa cumulus cells play an important role in follicular development, oocyte maturation, and ovulation. The crucial impact of follicular somatic cells on peri-ovulatory events is in large part mediated by the bidirectional communication between cumulus cells (CCs) and the oocyte through specialized gap junctions. Defective communication between the CCs and the oocyte may impair oocyte quality and result in poor embryo development and decreased fertility. Similarly, altered gene expression and thus function of GCs and CCs that affect extracellular matrix formation, cellular proliferation, differentiation or apoptosis could be associated with lower oocyte quality.

We found an increased expression of genes involved in oxidative phosphorylation and mitochondrial dysfunction in GCs of women with PCOS compared to YPR. This suggests that oxidative stress plays a more dominant role in the dysfunction of follicles from women with PCOS compared to similar age women with poor ovarian reserve. In contrast, the genes involved in the pathway of sirtuin signaling were downregulated in PCOS compared to YPR.

Sirtuins are complex proteins implicated in longevity. They take part in pathways that regulate oxidative stress, maintenance of metabolic homeostasis, DNA repair and mitochondrial function. The downregulation of sirtuin pathway in GCs of women with PCOS compared to YPR is quite interesting. It suggests that the metabolic dysfunction inherent to PCOS may negatively impact the sirtuin pathway and may downregulate longevity-promoting gene expression.

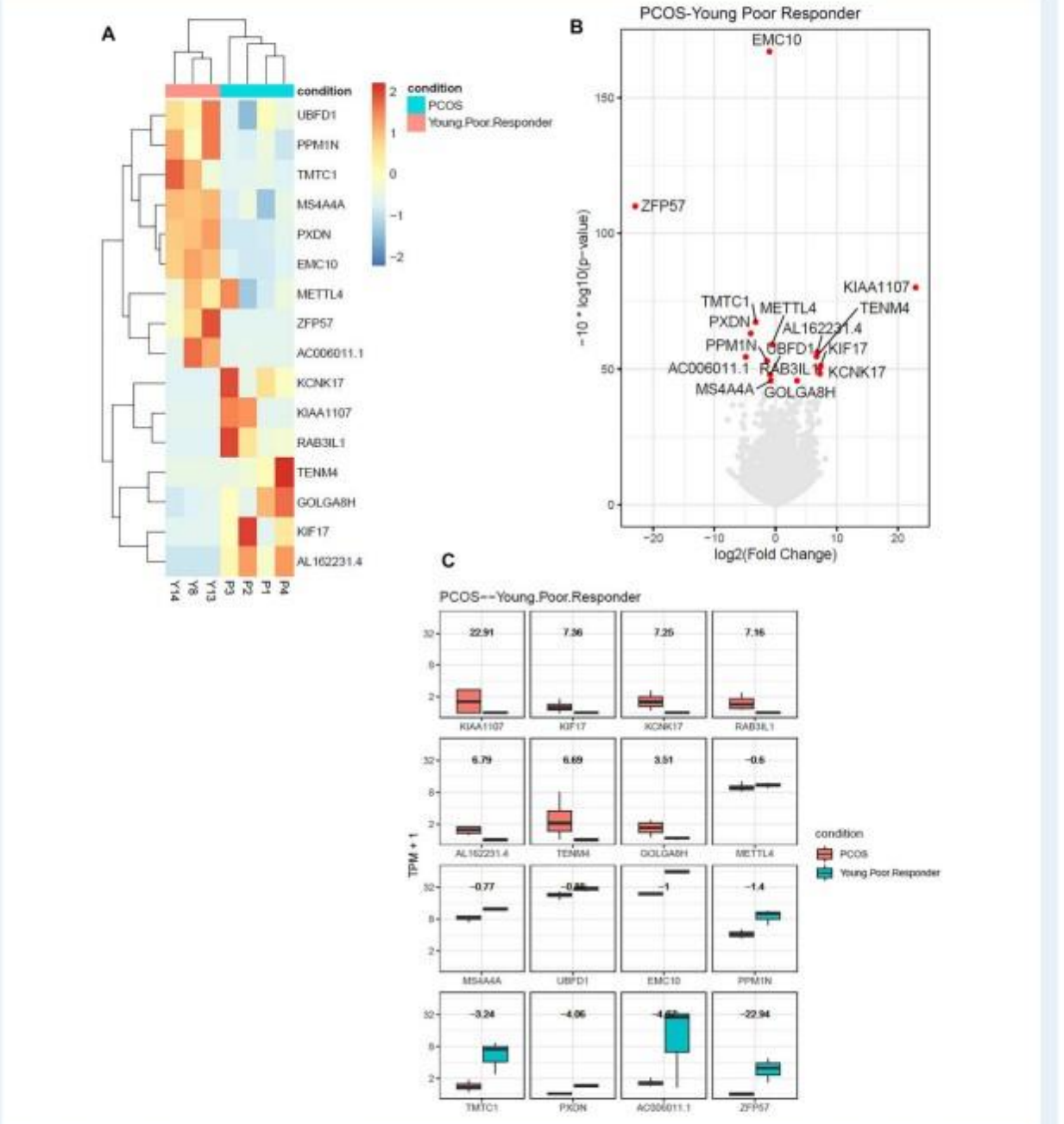

Figure 2. Comparison in gene expression between PCOS and young poor responder (YPR) in peripheral blood mononuclear cells (PBMNCs). (**a**) The heat map illustration shows differentially expressed genes in PCOS and YPR in PBMNCs. The colour spectrum ranging from red to blue indicates normalized levels of gene expression from high to low. (**b**) Volcano plots for RNA-seq comparing PCOS and YPR. Red spots on the left upper box represent –log10 (*P*-value) ≥2; red spots on the right upper box represent the –log10 (*P*-value) <2. (**c**) Selected differentially expressed genes in PCOS and YPR, $P < 0.05$ for each. The transcripts per million (TPM) value represents the relative expression level comparable between samples. For the box plots, the bottom and top whiskers denote 5 and 95 percentile values, the bottom and top bounds of the rectangle denote the 25 and 75 percentile values, and the line in between denotes the median (50 percentile) value of the distribution.

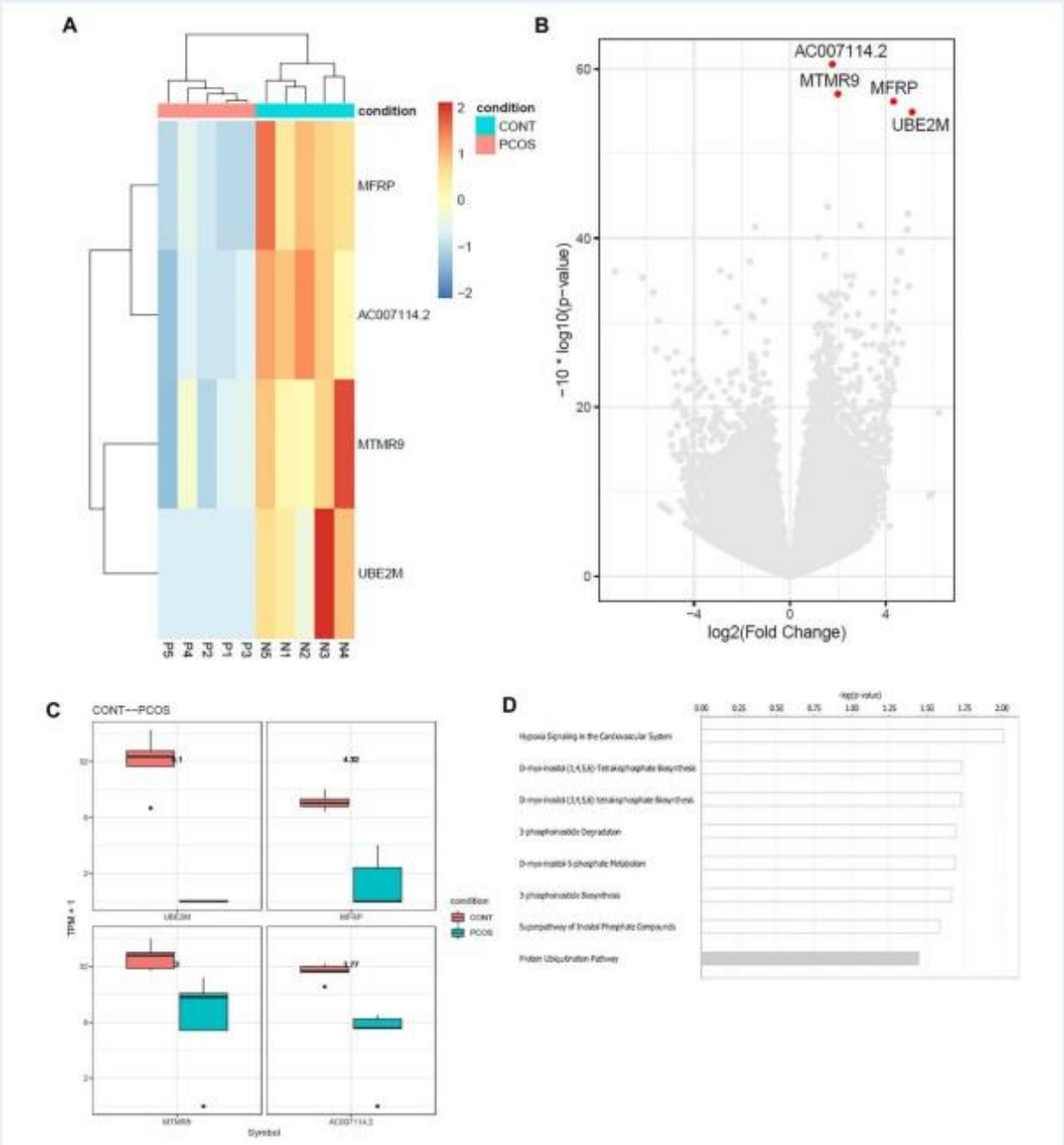

Figure 3. Gene expression is altered in PCOS women's granulosa cumulus cells (GCs) compared to normoresponder controls (CONT). (**a**) The heat map illustration shows differentially expressed genes in CONT and PCOS in GCs. The colour spectrum ranging from red to blue indicates normalized levels of gene expression from high to low. (**b**) Volcano plots for RNA-seq comparing CONT and PCOS. Red spots on the left upper box represent –log10 (*P*-value) ≥2; red spots on the right upper box represent the –log10 (*P*-value) <2. (**c**) Selected differentially expressed genes in CONT and PCOS, $P < 0.05$ for each. The transcripts per million (TPM) value represents the relative expression level comparable between samples. For the box plots, the bottom and top whiskers denote 5 and 95 percentile values, the bottom and top bounds of the rectangle denote the 25 and 75 percentile values, and the line in between denotes the median (50 percentile) value of the distribution. (**d**) Pathway analysis was evaluated using the Gene Ontology bioinformatics tool in GCs. Log2 fold change (FC) ≥0.584 false discovery rate (FDR) ≤0.05.

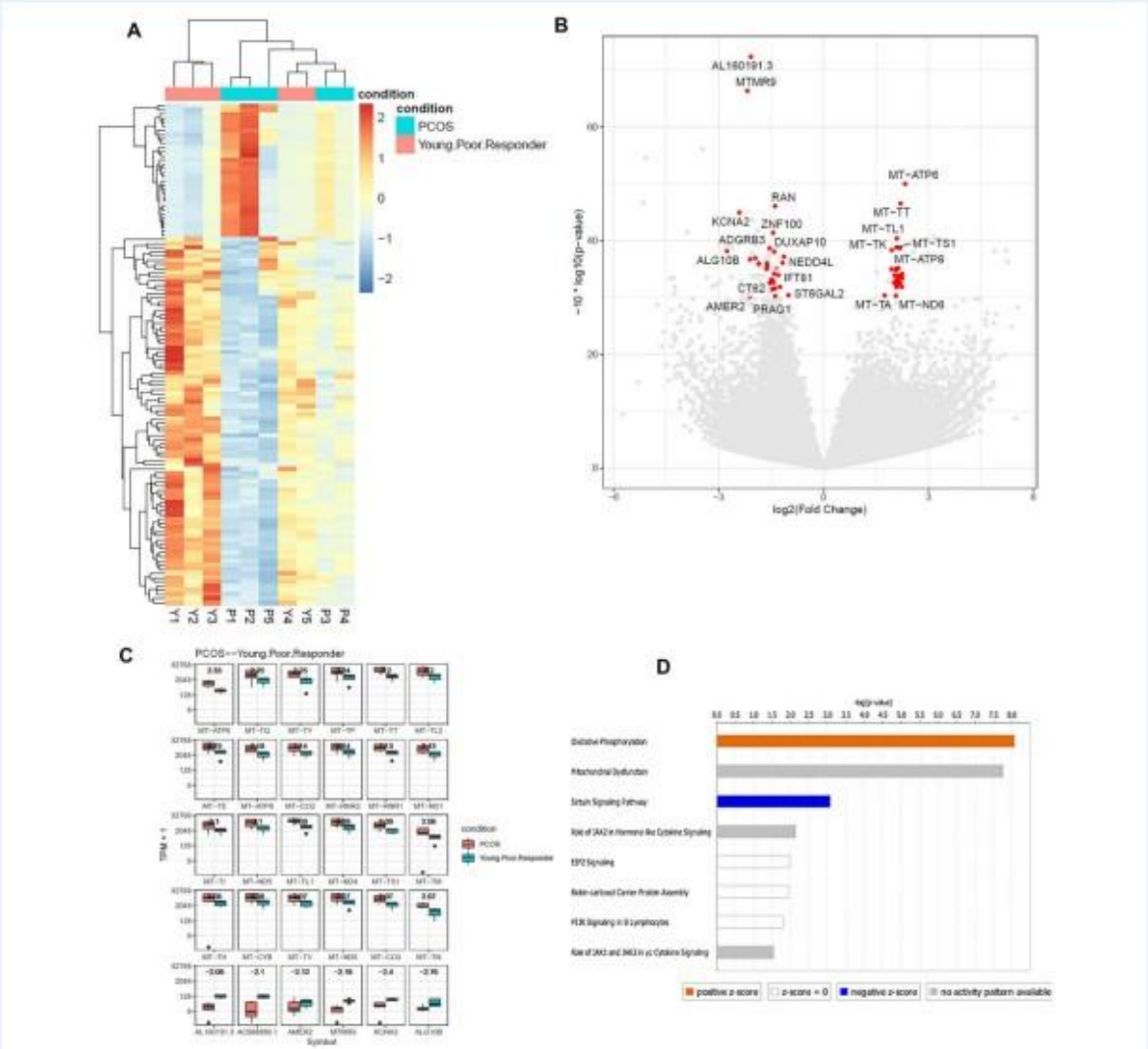

Figure 4. Gene expression is altered in granulosa cumulus cells (GCs) of women with PCOS and young poor responder (YPR). (**a**) The heat map illustration showing differentially expressed genes in PCOS and YPR in GCs. The colour spectrum ranging from red to blue indicates normalized levels of gene expression from high to low. (**b**) Volcano plots for RNA-seq comparing PCOS and YPR. Red spots on the left upper box represent –log10 (*P*-value) ≥2; red spots on the right upper box represent the –log10 (*P*-value) <2. (**c**) Selected differentially expressed genes in PCOS and YPR, $P < 0.01$ for each. The transcripts per million (TPM) value represents the relative expression level comparable between samples. For the box plots, the bottom and top whiskers denote 5 and 95 percentile values, the bottom and top bounds of the rectangle denote the 25 and 75 percentile values, and the line in between denotes the median (50 percentile) value of the distribution. (**d**) Pathway analysis was evaluated using the Gene Ontology bioinformatics tool in GCs. Log2 fold change (FC) ≥0.584 false discovery rate (FDR) ≤0.05.

Sirtuin signaling responds to metabolic challenges, inflammatory signals or hypoxic/oxidative stress (Tatone *et al.*, 2015) and has emerged as a critical player in the regulation of key processes in oogenesis. In GCs, sirtuins may play a role in the regulation of proliferation and hormonal metabolism, as they are responsible for the activation of steroidogenesis associated with luteinization (Tatone *et al.*, 2018). Data suggest that sirtuin 1 (SIRT1) is involved in the maintenance of cellular redox balance with an important role in sensing and modulating oxidative stress in GCs (Tatone *et al.*, 2015). Furthermore, the mitochondrial sirtuins have been implicated as sensors of metabolic state in human GCs. Specific alterations to the mitochondrial sirtuins might affect mitochondrial proteins, resulting in metabolic alterations in the

ovarian follicle (Pacella-Ince et al., 2014), potentially contributing to follicular dysfunction in women with PCOS.

Several genes involved in the mechanism of oxidative phosphorylation were upregulated in the GCs of women with PCOS, showing a link to a possible increase of oxidative stress in their ovaries. Increased oxidative stress has been widely accepted as a pivotal pathological feature of PCOS. However, the regulatory mechanisms of oxidative stress in the ovaries have not been fully clarified. Oxidative phosphorylation followed by electron transport chain reaction represented the most important metabolic pathway related to energy production in cells. Nevertheless, it is associated with a high cost due to the production of reactive oxygen species (ROS) (Halliwell, 2007). Although ROS are pivotal in the biological processes and act as signaling molecules, ROS may interact with several biomolecules such as lipids and nucleic acids, causing damage in the CCs and GCs.

The normal redox homeostasis in the endoplasmic reticulum (ER), may be altered by the excessive ROS production. The imbalance in redox homeostasis induces ER stress, which causes the unfolded protein response by activating genes encoding factors involved in protein folding and antioxidative machinery to restore ER homeostasis (Zhang et al., 2019). Altered ROS production in PCOS could be a causative factor reducing the functionality of GCs (Tarin, 1995) with consequent compromise follicle quality and aberrant reproductive endocrinology in the ovary (Qiao et al., 2014). Our results showed that several genes associated with complex IV deficiency of the mitochondrial respiratory chain such as MT-RNR1, MT-RNR2 (Mitochondrially Encoded 12S RRNA) and COX2 (Mitochondrially Encoded Cytochrome C Oxidase II) were dysregulated in GCs from PCOS patients. These specific genes are considered essential components of the respiratory chain that catalyses the reduction of oxygen to water. In fact, decreased mitochondrial biogenesis and mtDNA have been described in GCs from PCOS women (Zhao et al., 2015). Altogether, these results support the hypothesis that mitochondrial dysfunction of human GCs in women with PCOS may contribute to impaired steroidogenesis, fertilization, oocyte maturation and oocyte quality (Sreerangaraja Urs et al., 2020).

The results of qRT-PCR analyses showed a similar expression pattern to that observed in the RNA-sequencing. In PBMNCs of women with PCOS compared to controls, there was a trend toward decreased expression of RAB4 B ($P = 0.30$) and increased expression of TERC ($P = 0.1$) (Supplementary Fig. S1a). Similarly, a trend toward a decreased expression of PXDN ($P = 0.3$) and ZFP57 ($P = 0.1$) in the PBMNCs of women with PCOS compared to YPR was observed (Supplementary Fig. S1b). In the GCs, a decrease in the expression of MFRP ($P = 0.03$) and a trend toward a decreased expression of MTMR9 ($P = 0.3$) was observed when samples of women with PCOS were compared to controls (Supplementary Fig. S2a). Comparing GC samples from women with PCOS to those from YPR, we also observed an increase in the expression MT-RNR1 ($P = 0.03$) and a trend toward increased expression of MT-ATP6 ($P = 0.1$) (Supplementary Fig. S2b).

In the current study, oocyte maturation was achieved using hCG, GnRHa or a combination of both. A study by Miller et al. (2015) previously demonstrated that patients who receive hCG alone or as part of a double trigger (together with GnRHa) have higher levels of vascular endothelial growth factor and lower levels of pigment epithelium-derived growth factor. Others found increased epidermal growth factor-like proteins (amphiregulin and epiregulin) and decreased gap junction protein Connexin 43 in women triggered with a combination of hCG and GnRHa compared to hCG alone (Haas et al., 2016). Therefore, in the current study, we cannot exclude the possibility that different trigger methods might contribute to differential gene expression between the groups. It is however noteworthy that none of the previous studies identified the genes implicated in our study to be affected by the medications used to induce oocyte maturation and ovulatory changes.

In many respects, follicular numbers and patterns of response to COS of women with PCOS and POR seem to be at the opposite ends of a spectrum. Therefore, it is worth investigating whether these two important fertility-related disorders develop as a consequence of a perturbation in a single pathway (i.e. up- vs downregulation of similar gene groups). Our study is a step in this direction and our preliminary findings suggest specific differences between women with PCOS and POR. Future studies with adequate sample size directed at well-defined subgroups of these extremely heterogeneous disorders are needed to delineate shared gene pathways that can be exploited to identify therapeutic targets.

Conclusion

RNA expression profiles in ovarian GCs and PBMNCs were significantly altered in patients with PCOS compared with CONT and YPR. GCs of PCOS patients showed altered expression of several genes involved in oxidative phosphorylation, mitochondrial function and sirtuin signaling pathways. These findings are consistent with prior proposed mechanisms involved in the known features of PCOS.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Authors' roles

M.C. and E.S. take primary responsibility for the paper. S.T., S.H., I.P. and M.R. collected the data. M.C. and S.T. did the statistical analyses. M.C. and E.S. coordinated the research. S.H., L.R., R.S. and A.P. participated in the data analyses. M.C., S.T. and E.S. drafted the manuscript.

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Conflict of interest

M.C., S.H., S.T., L.R., M.R., I.R., A.P. and R.S. declare no conflict of interests concerning this research. E.S. is a consultant for and receives research funding from the Foundation for Embryonic Competence.

References

Azziz R. Controversy in clinical endocrinology: diagnosis of polycystic ovarian syndrome: the Rotterdam criteria are premature. *J Clin Endocrinol Metab* 2006;**91**:781–785.

Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE *et al.*; Task Force on the Phenotype of the Polycystic Ovary Syndrome of The Androgen Excess and PCOS Society. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. *Fertil Steril* 2009; **91**:456–488.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 1995;**57**:289–300.

Bhide P, Pundir J, Gudi A, Shah A, Homburg R, Acharya G. The effect of myo-inositol/di-chiro-inositol on markers of ovarian reserve in women with PCOS undergoing IVF/ICSI: a systematic review and meta-analysis. *Acta Obstet Gynecol Scand* 2019;**98**:1235–1244.

Carroll J, Saxena R, Welt CK. Environmental and genetic factors influence age at menarche in women with polycystic ovary syndrome. *J Pediatr Endocrinol Metab* 2012;**25**:459–466.

Collins G, Patel B, Thakore S, Liu J. Primary ovarian insufficiency: current concepts. *South Med J* 2017;**110**:147–153.

Cozzolino M, Seli E. Mitochondrial function in women with polycystic ovary syndrome. *Curr Opin Obstet Gynecol* 2020;**32**:205–212.

Esteves SC, Alviggi C, Humaidan P, Fischer R, Andersen CY, Conforti A, Bühler K, Sunkara SK, Polyzos NP, Galliano D *et al.* The POSEIDON criteria and its measure of success through the eyes of clinicians and embryologists. *Front Endocrinol (Lausanne)* 2019;**10**:814.

Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, Mudge JM, Sisu C, Wright J, Armstrong J *et al.* GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res* 2019;**47**:D766–D773.

Haas J, Ophir L, Barzilay E, Machtinger R, Yung Y, Orvieto R, Hourvitz A. Standard human chorionic gonadotropin versus double trigger for final oocyte maturation results in different granulosa cells gene expressions: a pilot study. *Fertil Steril* 2016;**106**: 653–659.

Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans* 2007; **35**:1147–1150.

Li Q, Du J, Feng R, Xu Y, Wang H, Sang Q, Xing Q, Zhao X, Jin L, He L *et al.* A possible new mechanism in the pathophysiology of polycystic ovary syndrome (PCOS): the discovery that leukocyte telomere length is strongly associated with PCOS. *J Clin Endocrinol Metab* 2014;**99**:E234–E240.

Liu T, Li Q, Wang S, Chen C, Zheng J. Transplantation of ovarian granulosa-like cells derived from human induced pluripotent stem cells for the treatment of murine premature ovarian failure. *Mol Med Rep* 2016;**13**:5053–5058.

Maciel GAR, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ, Erickson GF. Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2004;**89**:5321–5327.

Miller I, Chuderland D, Ron-El R, Shalgi R, Ben-Ami I. GnRH agonist triggering modulates PEDF to VEGF ratio inversely to hCG in granulosa cells. *J Clin Endocrinol Metab* 2015;**100**: E1428–E1436.

Pacella-Ince L, Zander-Fox DL, Lan M. Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age. *Hum Reprod* 2014;**29**:1490–1499.

Pedroso DCC, Miranda-Furtado CL, Kogure GS, Meola J, Okuka M, Silva C, Calado RT, Ferriani RA, Keefe DL, dos Reis RM. Inflammatory biomarkers and telomere length in women with polycystic ovary syndrome. *Fertil Steril* 2015;**103**:542–547.e2.

Qiao J, Wang Z, Feng H, Miao Y, Wang Q, Yu Y, Wei Y, Yan J, Wang W, Shen W *et al.* The root of reduced fertility in aged women and possible therapentic options: Current status and future perspects. *Mol Aspects Med* 2014;**38**:54–85.

Ramezani Tehrani F, Solaymani-Dodaran M, Hedayati M, Azizi F. Is polycystic ovary syndrome an exception for reproductive aging? *Hum Reprod* 2010;**25**:1775–1781.

Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod* 2004a;**19**:41–47.

Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* 2004b;**81**:19–25.

Sreerangaraja Urs DB, Wu W, Komrskova K, Postlerova P, Lin Y, Tzeng C, Kao S. Mitochondrial function in modulating human granulosa cell steroidogenesis and female fertility. *Int J Mol Sci* 2020;**21**: 3592.

Sun B, Ma Y, Li L, Hu L, Wang F, Zhang Y, Dai S, Sun Y. Factors associated with ovarian hyperstimulation syndrome (OHSS) severity in women with polycystic ovary syndrome undergoing IVF/ICSI. *Front Endocrinol (Lausanne)* 2020;**11**:615957.

Tarin JJ. Aetiology of age-associated aneuploidy: a mechanism based on the 'free radical theory of ageing'. *Hum Reprod* 1995;**10**: 1563–1565.

Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, Amicarelli F. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Hum Reprod Update* 2018;**24**:267–289.

Tatone C, Di Emidio G, Vitti M, Di Carlo M, Santini S, D'Alessandro AM, Falone S, Amicarelli F. Sirtuin functions in female fertility: possible role in oxidative stress and aging. *Oxid Med Cell Longev* 2015; **2015**:659687–659611.

Thomas FH, Vanderhyden BC. Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reprod Biol Endocrinol* 2006; **4**:19.

Webber L, Stubbs S, Stark J, Trew G, Margara R, Hardy K, Franks S. Formation and early development of follicles in the polycystic ovary. *Lancet* 2003;**362**:1017–1021.

Wei D, Xie J, Yin B, Hao H, Song X, Liu Q, Zhang C, Sun Y. Significantly lengthened telomere in granulosa cells from women with polycystic ovarian syndrome (PCOS). *J Assist Reprod Genet* 2017;**34**:861–866.

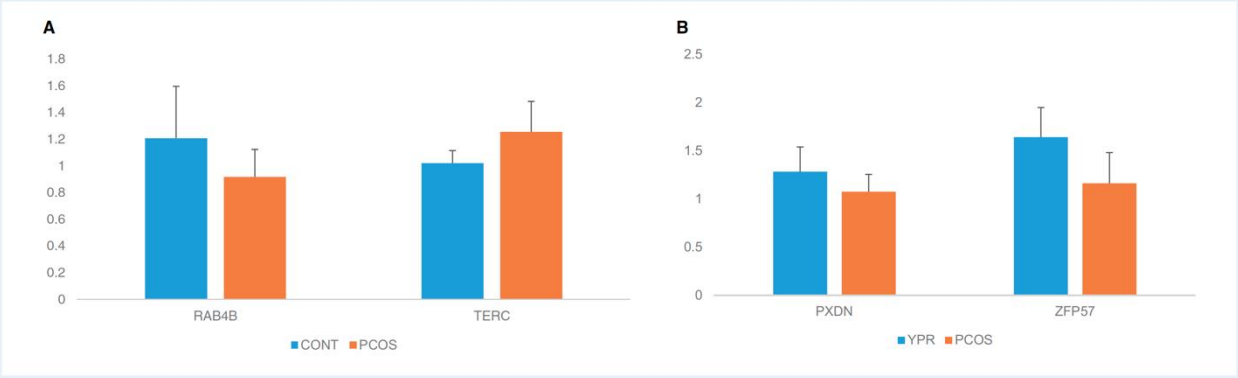
Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. *Hum Reprod* 2012;**27**:3067–3073.

Yilmaz B, Vellanki P, Ata B, Yildiz BO. Metabolic syndrome, hypertension, and hyperlipidemia in mothers, fathers, sisters, and brothers of women with polycystic ovary syndrome: a systematic review and meta-analysis. *Fertil Steril* 2018;**109**:356–364.

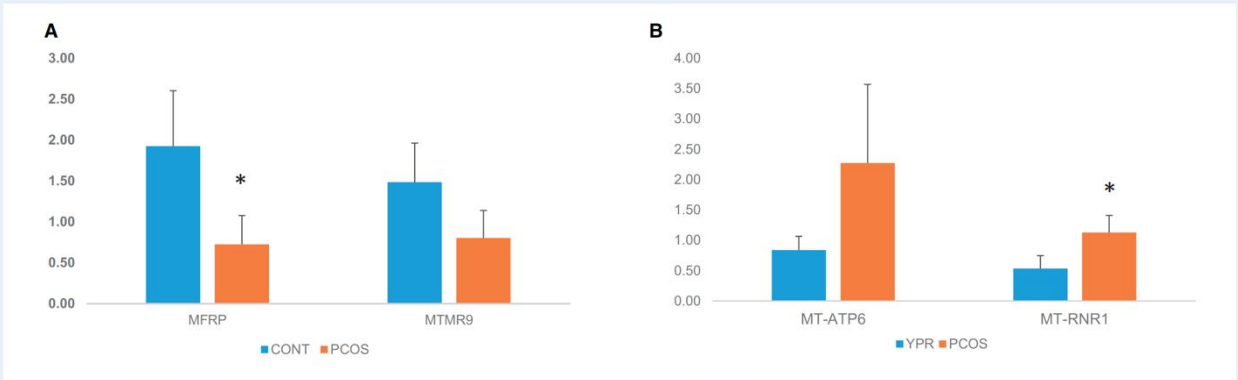
Younis JS, Ben-Ami M, Ben-Shlomo I. The Bologna criteria for poor ovarian response: a contemporary critical appraisal. *J Ovarian Res* 2015;**8**:76.

Zhang Z, Zhang L, Zhou L, Lei Y, Zhang Y, Huang C. Redox signaling and unfolded protein response coordinate cell fate decisions under ER stress. *Redox Biol* 2019;**25**:101047.

Zhao H, Zhao Y, Li T, Li M, Li J, Li R, Liu P, Yu Y, Qiao J. Metabolism alteration in follicular niche: the nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. *Free Radic Biol Med* 2015;**86**:295–307.



Supplementary Figure S1. Relative mRNA levels of genes that were differentially regulated in peripheral blood mononuclear cells (PBMNCs) were assessed by RT-qPCR. Graphs show the differential expression of selected transcripts in PCOS compared to CONT (**a**), and PCOS compared to YPR (**b**). Data are plotted as the fold change ($2^{-\Delta\Delta Ct}$).



Supplementary Figure S2. Relative mRNA levels of genes that were differentially regulated in GCs were assessed by RT-qPCR. Graphs show the differential expression of selected transcripts in CONT compared to PCOS (**a**) and PCOS compared to YPR (**b**). Data are plotted as the fold change ($2^{-\Delta\Delta Ct}$), $^{*}P < 0.05$.

*Note: Supplementary Table SI was presented as **Table 3** herein.

Supplementary Table SII Top five upregulated/downregulated genes for each comparison.

RNA-seq						
PBMNCs CONT vs PCOS		**PBMNCs PCOS vs YPR**		**GCs PCOS vs CONT**	**GCs PCOS vs YPR**	
Upregulated/FC	**Downregulated/FC**	**Upregulated/FC**	**Downregulated/FC**	**Downregulated/FC**	**Upregulated/FC**	**Downregulated/FC**
ZFP57 22.9	*INHBR* −6.49	*KIAA1107* 22.91	*ZFP57* −22.94	*UBE2M* 5.1	*MT-ATP6* 2.33	*ALG10B* −2.76
IGLC1 9.31	*TERC* −4.08	*KIF17* 7.36	*AC006011.1* −4.87	*MFRP* 4.33	*MT-TQ* 2.25	*KCNA2* −2.4
RAB4B-EGNL2 5.08	*TGM3* −3.37	*KCNK17* 7.25	*PXDN* −4.06	*MTMR9* 2.86	*MT-TY* 2.25	*MTMR9* −2.18
AC006011.1 4.99	*SNORD14D* −3.21	*RAB3IL1* 7.16	*TMTC1* −3.24	*AC007114.2* 1.77	*MT-TP* 2.24	*AMER* −2.12
AL353013.1 4.66	*MTND3P12* −2.45	*AL162231.4* 6.71	*PPM1N* −1.4		*MT-TT* 2.22	*AC0068880.1* −2.1

CONT, control group of normal responders; FC, fold change; GCs, granulosa cells; PBMNCs, peripheral blood mononuclear cells; PCOS, polycystic ovarian syndrome; YPR, young poor responder.

CHAPTER 6: STUDY III

Distress response in granulosa cells of women affected by PCOS with or without insulin resistance

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ORIGINAL ARTICLE

Distress response in granulosa cells of women affected by PCOS with or without insulin resistance

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Abstract

Purpose In this study, we investigated whether metabolic dysfunction in women with Polycystic ovarian syndrome (PCOS) induces granulosa cell (GC) stress and activates in the endoplamatic reticulum and the mitochondria (UPR^{er} and UPR^{mt}, respectively).

Methods Women who were diagnosed with PCOS (based on the Rotterdam criteria), were divided into two groups, PCOS with insulin resistance (PCOS-IR; $n = 20$) and PCOS with no insulin resistance (PCOS-nIR; $n = 20$), and compared to healthy oocyte donors (CONT; $n = 20$). Insulin resistance (IR) was assessed on the results of homeostasis model assessment (HOMA) that determines IR using the concentration of fasting plasma glucose and fasting insuline. Expression of UPR^{er} genes (i.e., *IRE1, ATF4, ATF6, XBP1, BIP*, and *CHOP*), and UPR^{mt} genes (i.e., *HSP60, HSP10, CLPP*, and *HSP40*) was assessed in cumulus GCs by qRT-PCR.

Results We found that several genes involved in UPR^{er} and UPR^{mt} were overexpressed in the GCs of PCOS-IR and PCOS-nIR compared to CONT. *IRE1*, *ATF4* and *XBP1*, that are activated by ER stress, were significantly overexpressed in PCOS-IR compared to CONT. *BIP* and *CHOP* were overexpressed in PCOS groups compared to CONT. *HSP10* and *HSP40* were upregulated in PCOS-IR and PCOS-nIR groups compared to the CONT. *HSP60* and *CLPP* showed no statistical different expression in PCOS-IR and PCOS-nIR compared to CONT group.

Conclusion Our findings suggest that the GCs of women with PCOS (with or without IR) are metabolically distressed and upregulate UPR^{er} and UPR^{mt} genes. Our study contributes to the understanding of the molecular mechanisms underlying the pathological changes that occur in the follicular microenvironment of women with PCOS.

Keywords PCOS · Hyperinsulinemia · Unfolded protein response · Endoplasmic reticulum · Mitochondria · Metabolic distress

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, with a prevalence of 6–10%, and is the leading cause of anovulatory infertility [1]. The pathophysiology of PCOS is

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complex and involves disorderly gonadotropin secretion, androgen excess, insulin resistance (IR), ovarian dysfunction, and follicular arrest [2]. While the most widely used definitions of PCOS are based on hyperandrogenaemia (elevated serum androgens) and its associated conditions (e.g., hirsutism and alopecia) along with polycystic ovaries [3–5], many other clinical and biochemical aspects have been associated with PCOS (including an elevated luteinizing hormone [LH], a decreased follicle-stimulating hormone [FSH] to LH ratio, hyperinsulinaemia, IR, hyperlipidaemia, and obesity) [6]. As such, we postulate this complex clinical syndrome has a heterogeneous etiology that may be caused by several pathophysiologic pathways, many of which remain to be defined.

Over the past two decades, numerous studies have revealed intra-ovarian processes, such as chronic inflammation and oxidative stress, that may play key roles in the pathogenesis of PCOS [2, 7–10]. In the United States, 50–80% of women with PCOS are overweight or obese; and 50–70% have IR [11–13]. Insulin resistance and consequent hyperinsulinemia in women with PCOS may contribute to an altered redox balance that promotes oxidative stress within granulosa cells (GCs). The hyperandrogenism in women with PCOS further increases levels of pro-fibrotic stimuli, aggravating cellular stress in the GCs of antral follicles [14], and promoting apoptosis [15]. Thus, PCOS may enhance production of reactive oxygen species (ROS) and mitochondrial dysfunction, which, in turn, may activate intracellular responses to stress, such as the mitochondrial unfolded protein response (UPR^{mt}) and the endoplasmic reticulum unfolded protein response (UPR^{er}).

The functions of the rough endoplasmic reticulum (ER) (characterized by the ribosomes that stud its membrane), include protein synthesis, folding, modification, and transport to the cytoplasm. Meanwhile, the smooth ER is responsible for the production and metabolism of lipids and steroids. When the protein-folding workload exceeds the capacity of the ER, the resulting accumulation of unfolded or misfolded proteins in the lumen leads to ER stress [16–19], which elicits the UPR^{er}. This response then activates several signal transduction pathways, that promote correct folding or degradation of the unfolded proteins [20, 21] and modulate a variety of cellular functions [20]. Notably, the UPR^{er} primarily seeks to restore homeostasis, however, if the ER stress cannot be resolved, it may induce programmed cell death [22]. The four main actions of the UPR^{er} include: (i) attenuating mRNA translation to reduce protein synthesis, (ii) amplifying the synthesis of ER chaperones to increase protein-folding capacity, (iii) producing ER-associated degradation factors to remove the irreparably misfolded proteins, and (iv) inducing apoptosis [20, 21].

On the other hand, mitochondria primarily generate energy from available substrates, and have additional roles in calcium homeostasis, fatty acid oxidation, and apoptosis. As the majority of mitochondrial proteins are encoded by nuclear genes, and imported into the mitochondria unfolded, the mitochondrial chaperones are essential for maintaining homeostasis, by mediating protein folding, and quality control, the intermembrane redox balance, and ultimately, regulating metabolic pathways [23]. When mitochondrial proteotoxic stress increases, cells activate the UPR^{mt}, which upregulates genes that encode the mitochondrial chaperone system [24]. In turn, the chaperones promote protein folding within the mitochondria, and activate newly synthesized proinflammatory signal transducers [25]. Similar to the UPR^{er}, when the UPR^{mt} fails to re-establish homeostasis, cell death ensues. This phenomenon was detected in Clpp knockout mouse models that exhibit female and male infertility [26]. $Clpp^{-/-}$ female mice generate a lower number of mature oocytes and two-cell embryos, and no blastocysts. Clpp mediates degradation of unfolded mitochondrial proteins and activates UPR^{mt} to maintain protein homeostasis.

Folliculogenesis is a highly ordered process, with multiple factors being expressed to varying degrees throughout follicle development. The GCs (surrounding oocytes) secrete unique factors throughout folliculogenesis that mediate somatic cell growth, influence oocyte maturation through bi-directional communication, and ultimately, regulate follicule development. Accordingly, the status of oocytes reflects that of the GCs, which are vulnerable to changes in their microenvironment during folliculogenesis [27, 28]. Particularly, as growing follicles undergo dynamic changes in their microenvironment, such as cell proliferation and hypoxia, cellular stress can intermittently occur in follicles [27, 28]. Therefore, the stress induced in the mitochondria and ER of GCs of women with PCOS may play a role in the pathophysiology of follicular growth and maturation [29]. Based on these premises, the goal of the present study was to assess the expression of UPR-related genes in the ER and mitochondria in GCs of women with PCOS (with and without IR).

Materials and methods

Study design

A prospective cohort study was conducted using women diagnosed with PCOS in addition to IR (PCOS-IR; $n = 20$) and non-insulin-resistance (PCOS-nIR; $n = 20$) compared to young normoresponder oocyte donors (CONT; $n = 20$). On the day of oocyte retrieval, GCs were collected, and total RNA was extracted. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to determine the expression of select UPR^{er} genes (i.e., *BIP*,

ATF4, *ATF6*, *IRE1*, *CHOP*, and *XBP*), and UPR^{mt} genes (i.e., *HSP60*, *HSP10*, *CLPP*, and *HSP40*) in the GCs.

Patient cohorts

All study procedures were approved by, and conducted in accordance with, the Institutional Review Board of the Hospital Universitario Puerta de Hierro, Madrid, Spain (1812-MAD-099-MC). Written informed consent was obtained from all patients prior to collecting human samples.

Forty women with PCOS were recruited from the Acibadem University IVF Center (Istanbul, Turkey), aged 18–37 years, no family history of hereditary or chromosomal diseases, normal karyotype, body mass index (BMI) of 18–29 kg/m^2. Diagnosis with PCOS was based on the revised Rotterdam criteria, that includes (i) oligo- or anovulation, (ii) clinical and/or biochemical signs of hyperandrogenism, (iii) polycystic ovaries and exclusion of other etiologies [30]. Patients with PCOS were then divided into those with IR and those without IR (non-insulin-resistance [nIR]), based on the results of the homeostasis model assessment (HOMA) that determines IR using the concentration of fasting plasma glucose and fasting insulin [31]. The PCOS women with a HOMA value of ≥2 were considered to have IR, while women with a value <2 were categorized as nIR. Patients with other causes of oligomenorrhea or hyperandrogenism (such as congenital adrenal hyperplasia, androgen-secreting tumors or Cushing's syndrome) were excluded from the study for clinical reasons.

Meanwhile, the young normoresponder controls were recruited from the oocyte donor program at the IVI Madrid (Madrid, Spain). These control patients were healthy women, aged 18–35 years, who met the following criteria to be oocyte donors: regular menstrual cycles, no family history of hereditary or chromosomal diseases, normal karyotype, BMI of 18–29 kg/m^2, no more than two previous miscarriages, no gynaecological diseases or medical disorders, and a negative screening result for sexually transmitted infections [32]. Patients with PCOS were excluded. Finally, donors were expected to have at least six antral follicles per ovary at the beginning of the cycle.

Controlled ovarian stimulation protocols, oocyte retrieval, and GC isolation

Following ultrasound-based confirmation of the absence of follicle dominance, and detection of serum estradiol (E2) < 50 mIU/mL, participants underwent controlled ovarian stimulation (COS) using the standard long gonadotrophin-releasing hormone agonist protocol or the antagonist protocol. Briefly, COS was carried out with daily administration of 150–300 IU recombinant human follicle-stimulating hormone alone or in combination with the highly effective human menopausal gonadotropin (HP-HMG), depending on the physician's preference. Final follicular maturation was triggered with 10,000 IU human chorionic gonadotropin when the lead follicular diameter was >18–19 mm, and oocyte retrieval was scheduled 36 h thereafter. The follicular fluid (FF) was carefully aspirated from the antral follicles of each ovary by laparoscopy. The ovarian GCs were isolated from each sample of FF by centrifugation at 450 × *g* for 5 min. The recovered GCs were washed with 1 mL of phosphate-buffered saline (PBS) and centrifuged at maximum speed for 5 min for purification. Finally, the GCs were resuspended in 10 µL of PBS, and 50 µL of RNAlater® (Sigma) was added prior to storing samples at −20 °C until further use.

RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted from the GCs using the QuickRNA™ Microprep Kit (Zymo Research) and the concentration was measured using the Qubit™ 3.0 fluorometer (Life Technologies). RNA samples were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific). Each qRT-PCR reaction included 10 µL iQ™ SYBR® Green Supermix (Bio-Rad), 500 nM of each of the forward and reverse primers (presented in Supplemental Table 1), 100 ng cDNA, and enough double-distilled water to yield a final volume of 20 µL. The qRT-PCR was carried out using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) to analyze the following genes: *IRE1, ATF4, ATF6, XBP1, BIP, CHOP, HSP60, HSP10, CLPP*, and HSP40. All samples were run in triplicate, using GAPDH as a housekeeping gene, and the ΔΔCt method was used to calculate relative gene expression. Data are presented as a mean of three replicates ± SD.

Statistical analysis

The patient characteristics were analyzed by chi-square test. The gene expression data were analyzed using univariate ANOVA tests followed by two-tailed *t*-tests, using GraphPad Prism version 5.00 software (GraphPad, San Diego, CA, USA; www.graphpad.com). In all cases, $p < 0.05$ was considered statistically significant.

Results

Patient characteristics and biochemical analyses

Characteristics of the study participants, including mean age, BMI, number of oocytes retrieved, serum estradiol, fasting blood glucose, fasting insulin, and HOMA results are presented in Table 1. Eeven older patients with PCOS

Table 1 Baseline, hormonal and metabolic characteristics of the patients included in the study

	CONT	PCOS-IR	PCOS-nIR	*p* value
Age (years)	25.12 ± 2.26	30.65 ± 5.25	29.94 ± 6.21	$p < 0.001$; $p < 0.001$
BMI (kg/m^2)	23.76 ± 1.86	27.64 ± 6.92	24.58 ± 3.64	$p < 0.01$; $p < 0.05$
Number of oocytes	18.12 ± 9.84	26.45 ± 15.68	22.66 ± 6.92*	$p < 0.01$; $p < 0.05$
Estradiol (mg/dl)	2981 ± 1556.89	5262.34 ± 3719.52	4675.65 ± 2553.21	$p < 0.001$; $p < 0.001$
Fasting Blood Glucose (mg/dL)	80.89 ± 6.31	95.27 ± 23.41	85.89 ± 10.51*	$p < 0.001$; $p < 0.01$
Fasting Insulin (IU)	4.12 ± 1.22	24.15 ± 27.24	6.82 ± 1.33	$p < 0.001$, $p < 0.01$
HOMA	1.11 ± 0.40	6.74 ± 7.80	1.51 ± 0.40	$p < 0.001$; $p < 0.05$

p value is referred to in the comparison PCOS-IR vs CONT and PCOS-nIR vs CONT

presented highly oestradiol and number of oocyte. Women categorized as PCOS-IR and PCOS-nIR were significantly older than those in the CONT group (30.65 ± 5.25 and 29.94 ± 6.2 vs 25.12 ± 2.26 years, respectively; $p < 0.001$), however, no statistically significant difference was detected between the PCOS-IR and PCOS–nIR groups. Women in the PCOS-IR group had a significantly higher BMI (27.64 ± 6.92) compared to the PCOS-nIR (24.58 ± 3.64; $p = 0.032$) and CONT (23.76 ± 1.86; $p = 0.012$) groups.

Altered expression of genes involved with the UPR^{er} in women with PCOS

The UPR^{er}-related transcription factors *IRE1*, *ATF4*, and *XBP1*, that are activated by ER stress, were significantly overexpressed in the GCs of PCOS-IR compared to CONT ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively) (Figs. 1a, b, 2a). In the GCs of the PCOS-nIR group, *IRE1* was also significantly enhanced compared to the CONT group ($p < 0.05$; Fig. 1a), however *ATF4* and *XBP1* were not differentially regulated (Figs. 1b, 2a). Nevertheless, when PCOS-IR and PCOS-nIR groups were compared, *ATF4* and *XBP1* were found to be significantly upregulated in GCs of the PCOS-IR women ($p < 0.001$ in both cases; Figs. 1b, 2a). No difference was found in *ATF6* gene expression among study groups (Fig. 1c).

The chaperone *BIP*, involved with protein folding in the ER, was specifically increased in GCs of PCOS-IR compared to CONT and PCOS-nIR ($p < 0.01$ and $p < 0.05$, respectively; Fig. 2b). The transcription factor *CHOP*, which initiates apoptosis in response to organelle stress, was overexpressed in GCs of both PCOS-IR and PCOS-nIR compared to the CONT group ($p < 0.05$; Fig. 2c).

Altered expression of genes involved with the UPR^{mt} in women with PCOS

The GCs of women with PCOS (both -IR and -nIR) showed an upregulation of the UPR^{mt} genes compared to the CONT group. Among the genes mediating the UPR^{mt}, the chaperone proteins *HSP10* and *HSP40*, involved with the correct folding of proteins transported into the mitochondria, were upregulated in the PCOS-IR ($p < 0.001$ and $p < 0.0001$, respectively) and PCOS-nIR ($p < 0.05$ and $p < 0.05$, respectively) groups compared to CONT (Fig. 3a, b). Further, in GCs of the PCOS-IR group, expression of *HSP40* was also significantly higher compared to GCs of the PCOS-nIR group ($p < 0.01$), while HSP10 levels were not differentially expressed between the groups (Fig. 3a, b). The two other important genes involved in the UPR^{mt}, *HSP60* and *CLPP*, that respectively promote protein folding and degradation, did not show differential expression in PCOS-IR or PCOS-nIR compared to the CONT group (Fig. 3c, d).

Discussion

Polycystic ovary syndrome (PCOS) is associated with metabolic and endocrine abnormalities [33]. In corroboration, we found that GCs from women with PCOS (with or without IR) show signs of metabolic distress and altered expression of UPR^{er} and UPR^{mt} genes. These findings suggest that hyperinsulinaemia-induced ER stress alters GC gene expression in PCOS patients, leading to an altered follicular microenvironment that may have negative repercussions on oocyte maturation and competence. We hypothesize that in the future, this ER stress may be alleviated with medications that increase insulin sensitivity, such as metformin. Although metformin has been used in the treatment regimens of infertile women with PCOS since 1994, its role in follicle development was postulated to be involved with the sensitization to FSH [34] and additional preclinical research, ideally using a PCOS animal model, would be required to determine the effective doses and appropriate duration of treatment to alleviate ER stress without compromising oocyte maturation and subsequent embryo and fetal development.

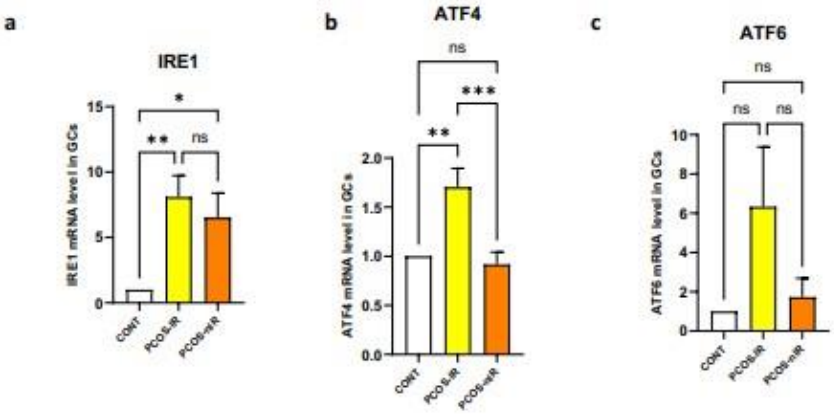

Fig. 1 Endoplasmic Reticulum Unfolded Protein Response (UPR^{er}). Relative mRNA levels of genes that are involved in UPR^{er} were assessed by RT-qPCR in GCs. Graphs show the differential expression of selected transcripts in *IRE1* (**a**), *ATF4* (**b**), *ATF6* (**c**). Data are plotted as the fold change ($2^{-\Delta\Delta Ct}$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

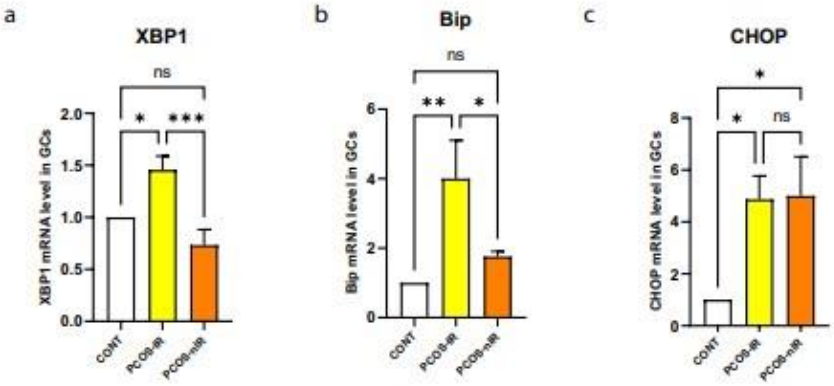

Fig. 2 Endoplasmic Reticulum Unfolded Protein Response (UPR^{er}). Relative mRNA levels of genes that are involved in UPR^{er} were assessed by RT-qPCR in GCs. Graphs show the differential expression of selected transcripts in *XBP1* (**a**), *BIP* (**b**), *CHOP* (**c**). Data are plotted as the fold change ($2^{-\Delta\Delta Ct}$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

A principal factor in the initiation of the UPR^{er} is the 78 kDa glucose-regulated protein (GRP78) [35, 36]. GRP78 relays signals from three ER transmembrane molecules, inositol-requiring enzyme 1 (IRE1), RNA-dependent protein kinase-like ER kinase (PERK; also known as Eukaryotic Translation Factor 2 Alpha Kinase 3 [EIF2AK3]), and activating transcription factor 6 (ATF6) [37], to initiate the downstream UPR. Since we found higher expression of *IRE1* in GCs from PCOS patients compared with controls, we suspect the impact of PCOS on ER stress could be mediated by IRE1. The main function of IRE1 is to activate the genes involved in the UPR, through X-box-binding protein 1 (XBP1), a transcription factor essential for the transcription of genes encoding ER chaperones. In our study, *XBP1* was enhanced in GCs of women with PCOS-IR, indicating the cells began synthesizing chaperones in attempts to restore homeostasis in the ER, reduce stress, and ultimately avoid cell death. Nevertheless, severe or prolonged ER stress triggers the apoptotic pathway [38], and the CCAAT/enhancer-binding protein homologous protein (CHOP) is involved in ER stress-induced cell death. In the GCs of women with PCOS (with and without IR), the augmented cell stress seems to cause overexpression of *CHOP* that may promote apoptosis and suggests that the cellular homeostasis in PCOS could not be restored, at least in some of the GCs.

The general upregulation patterns found in the PCOS-IR group suggest that hyperinsulinaemia is associated with ER stress in the GCs. Thus, we hypothesized that hyperinsulinaemia might lead to GC dysfunction that indirectly impairs oocyte competence in PCOS patients. This corroborates previously reported analyses of ER stress in the GCs of metformin-treated ovaries of PCOS mice, which showed reduced expression of *CHOP*, *XBP1*, and *ATF4* [39]. In mice with PCOS, the administration of metformin increased insulin sensitivity, improved metabolism, and mitigated polycystic symptoms by activating the adenosine monophosphate-activated protein kinase signalling pathway to reduce ER stress [35]. Future studies will be needed to verify whether treatment with metformin could alleviate GCs stress by improving insulin sensitivity in PCOS patients.

Signal transduction through ER stress and the UPR have important implications in ovarian follicle development [40]. Suppressing the ER stress in cumulus cells improved the

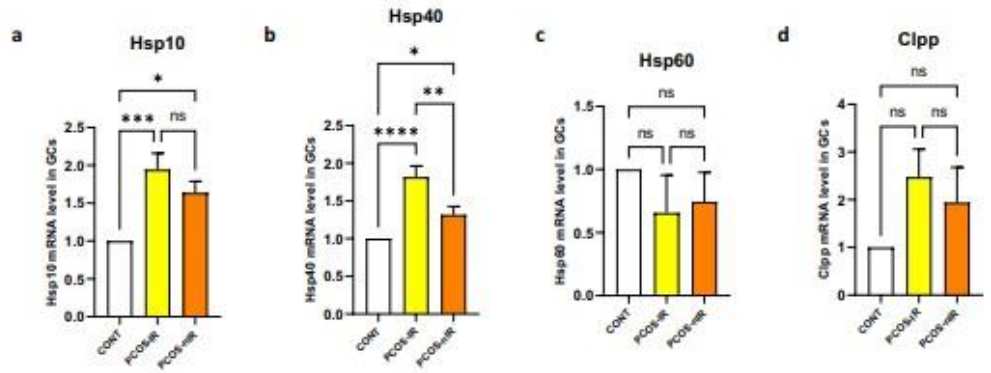

Fig. 3 Mitochondrial Unfolded Protein Response (UPR^{mt}). Relative mRNA levels of genes that are involved in UPR^{mt} were assessed by RT-qPCR in GCs. Graphs show the differential expression of selected transcripts in *HSP10* (**a**), *HSP40* (**b**), *HSP60* (**c**), *CLPP* (**d**). Data are plotted as the fold change ($2^{-\Delta\Delta Ct}$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

meiotic maturation of porcine oocytes [41]. Meanwhile, ER stress in murine cumulus cells impaired protein secretion, mitochondrial activity, and oocyte developmental competence [42]. Therefore, ER stress in ovarian somatic cells, affects oocyte maturation, follicle development, and ovulation [41–46]. On the other hand, increased expression of UPR-related genes, such as *ATF4*, *ATF6*, *XBP1*, and *CHOP*, have been reported in GCs of patients with PCOS [34]. In corroboration, another study reported that *ATF4* and *ATF6* mRNA were overexpressed in women with PCOS, and treatment with the antioxidant resveratrol effectively reduced transcription of *CHOP* and *XBP1* [47].

Heat shock proteins (HSPs) promote cell survival by acting as molecular chaperones that aid in the proper folding of nascent and stress-accumulated misfolded proteins, to avoid their accumulation. Indeed, mitochondrial HSPs play a protective role, allowing cells to survive in otherwise lethal conditions [48]. Remarkably, HSPs can counteract both the intrinsic and the extrinsic apoptotic pathways, by (i) modulating several signalling pathways outside of the mitochondria, (ii) controlling the release of pro-apoptotic molecules from the mitochondria, and (iii) preventing the activation of caspases and consequent apoptosis [48]. In this study, we found *HSP10* and *HSP40* overexpressed in GCs of patients with PCOS-IR. These two important members of the HSP family regulate apoptotic signalling pathways to prevent apoptosis and thus play a role in cell protection. As with ER stress, the overexpression of *HSP10* and *HSP40* in the mitochondria could be related to the elevated apoptosis of GCs during follicular atresia. The activation of UPR^{mt} also promotes apoptosis via induction of the UPR transcription factor CHOP.

The main limitation of this study may be the age difference between the PCOS and control patients. As recently reviewed, age-related ovarian ageing leads to the accumulation of ROS in oocytes, and the consequential oxidative stress negatively impacts reproductive outcomes [49] (through GC apoptosis, chromosomal non-disjunction, failed implantation, and reduced embryo competence). In this regard, the overexpression of UPR-related genes may partially be due to age, however, the elevated number of recovered oocytes and serum estradiol in the PCOS-IR group may counteract this effect and highlights the efficacy of individualizing COS protocols. Finally, *GAPDH* was used as a housekeeping gene based on previous studies, however, a recent report argued that beta-actin may be a more appropriate reference gene, due to the increased expression of GAPDH in placental tissue of PCOS patients [50]. The authors cannot rule out the effect of different factors (such as stress or different hormonal environments) on *GAPDH* transcription, and due to the limited quantity of cDNA obtained from the GCs, it was not possible to evaluate the expression of beta-actin in our samples.

PCOS patients showed altered expression of several genes involved in oxidative phosphorylation and mitochondrial function [51]. Our study confirmed an altered expression in ER and mitochondrial genes of PCOS patients with IR.

In conclusion, ER and mitochondrial stress responses are activated in the GCs of women with PCOS.

Despite the study's limitations and the need for additional research in accordance with the new available data regarding the use of adequate house-keeping gene when working with samples derived from PCOS patients, this study contributes to the understanding the molecular mechanisms underlying the pathological changes that occur in the follicular microenvironment of women with PCOS.

Data availability

All the data are included in the paper.

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Author contributions M.C. take primary responsibility for the paper, collected data, made statistical analysis, and write the paper; S.H. edited the final version of the paper; Y.C., J.A.G.V., B.T., A.P., S.R., G.K., AB, recruited patients and collected data and E.S. reviewed the final version of the paper.

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Compliance with ethical standards

Conflict of interest M.C., S.H., Y.C., J.A.G.V., B.T., A.P., S.R., G.K. A.B., A.P. declares no competing interests concerning this research. E.S. is a consultant for and receives research funding from the Foundation for Embryonic Competence.

Consent for publication All patients were informed and gave written consent.

Ethical approval and consent to participate All the procedures performed in the studies that involved human participants were made under the ethical standards of the Institutional and National Research Committee, and also with the 1964 Declaration of Helsinki as well as its later amendments, or comparable ethical standards. All study procedures were approved and conducted according to the Institutional Review Board of Hospital Universitario Puerta de Hierro, Madrid, Spain (1812-MAD-099-MC). Patients signed an informed consense.

References

1. M. Cozzolino, E. Seli, Mitochondrial function in women with polycystic ovary syndrome. Curr. Opin. Obstet. Gynecol. **32**, 205–12. (2020)
2. D.A. Dumesic, S.E. Oberfield, E. Stener-Victorin, J.C. Marshall, J.S. Laven, R.S. Legro, Scientific Statement on the Diagnostic Criteria, Epidemiology, Pathophysiology, and Molecular Genetics of Polycystic Ovary Syndrome. Endocr. Rev. **36**, 487–525 (2015)
3. J.K. Zawadski, A. Dunaif. Diagnostic Criteria for Polycystic Ovary Syndrome: Towards a Rational Approach. In: Dunaif, A., Givens, J.R. and Haseltine, F., Eds., Polycystic Ovary Syndrome, Blackwell Scientific, Boston, 377–384 (1992)
4. R. Azziz, E. Carmina, D. Dewailly, E. Diamanti-Kandarakis, H.F. Escobar-Morreale, W. Futterweit et al. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertil. Steril. **91**, 456–488 (2009)
5. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil. Steril. **81**, 19–25 (2004)
6. F.J. Broekmans, E.A. Knauff, O. Valkenburg, J.S. Laven, M.J. Eijkemans, B.C. Fauser, PCOS according to the Rotterdam consensus criteria: Change in prevalence among WHO-II anovulation and association with metabolic factors. BJOG **113**, 1210–1217 (2006)
7. J. Adams, Z. Liu, Y.A. Ren, W.S. Wun, W. Zhou, S. Kenigsberg et al. Enhanced Inflammatory Transcriptome in the Granulosa Cells of Women With Polycystic Ovarian Syndrome. J. Clin. Endocrinol. Metab. **101**, 3459–3468 (2016)
8. N.B. Karuputhula, R. Chattopadhyay, B. Chakravarty, K. Chaudhury, Oxidative status in granulosa cells of infertile women undergoing IVF. Syst. Biol. Reprod. Med. **59**, 91–98 (2013)
9. J. Schmidt, B. Weijdegard, A.L. Mikkelsen, S. Lindenberg, L. Nilsson, M. Brannstrom, Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women. Mol. Hum. Reprod. **20**, 49–58 (2014)
10. M. Cozzolino, A. Vitagliano, L. Pellegrini, M. Chiurazzi, A. Andriasani, G. Ambrosini, N. Garrido, Therapy with probiotics and synbiotics for polycystic ovarian syndrome: a systematic review and meta-analysis. Eur. J. Nutr. **59**(7), 2841–2856 (2020).
11. J. Kataoka, I. Larsson, S. Bjorkman, B. Eliasson, J. Schmidt, E. Stener-Victorin, Prevalence of polycystic ovary syndrome in women with severe obesity - Effects of a structured weight loss programme. Clin. Endocrinol. (Oxf.) **91**, 750–758 (2019)
12. S. Sam, Obesity and Polycystic Ovary Syndrome. Obes Manag. **3**(2), 69–73 (2007)
13. B.O. Yildiz, E.S. Knochenhauer, R. Azziz, Impact of obesity on the risk for polycystic ovary syndrome. J. Clin. Endocrinol. Metab. **93**, 162–168 (2008)
14. N. Takahashi, M. Harada, Y. Hirota, E. Nose, J.M. Azhary, H. Koike et al. Activation of Endoplasmic Reticulum Stress in Granulosa Cells from Patients with Polycystic Ovary Syndrome Contributes to Ovarian Fibrosis. Sci. Rep. **7**(10824), 017–11252-7 (2017)
15. J.M.K. Azhary, M. Harada, N. Takahashi, E. Nose, C. Kunitomi, H. Koike et al. Endoplasmic Reticulum Stress Activated by Androgen Enhances Apoptosis of Granulosa Cells via Induction of Death Receptor 5 in PCOS. Endocrinology **160**, 119–132 (2019)
16. P. Walter, D. Ron, The unfolded protein response: from stress pathway to homeostatic regulation. Science **334**, 1081–1086 (2011)
17. S.Z. Hasnain, R. Lourie, I. Das, A.C. Chen, M.A. McGuckin, The interplay between endoplasmic reticulum stress and inflammation. Immunol. Cell Biol. **90**, 260–70. (2012)
18. J. Grootjans, A. Kaser, R.J. Kaufman, R.S. Blumberg, The unfolded protein response in immunity and inflammation. Nat. Rev. Immunol. **16**, 469–484 (2016)
19. D.T. Rutkowski, R.J. Kaufman, A trip to the ER: coping with stress. Trends Cell Biol. **14**, 20–28 (2004)
20. D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. **8**, 519–529 (2007)
21. M. Schroder, R.J. Kaufman, The mammalian unfolded protein response. Annu Rev. Biochem **74**, 739–789 (2005)
22. C. Xu, B. Bailly-Maitre, J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions. J. Clin. Investig. **115**, 2656–2664 (2005)
23. Y.C. Chae, A. Angelin, S. Lisanti, A.V. Kossenkov, K.D. Speicher, H. Wang et al. Landscape of the mitochondrial Hsp90 metabolome in tumours. Nat. Commun. **4**, 2139 (2013)
24. J.E. Aldridge, T. Horibe, N.J. Hoogenraad, Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. PLoS ONE **2**, e874 (2007)
25. A.J. Caplan, A.K. Mandal, M.A. Theodoraki, Molecular chaperones and protein kinase quality control. Trends Cell Biol. **17**, 87–92 (2007)
26. T. Wang, E. Babayev, Z. Jiang, G. Li, M. Zhang, E. Esencan, et al. Mitochondrial unfolded protein response gene Clpp is required to maintain ovarian follicular reserve during aging, for oocyte competence, and development of pre-implantation embryos. Aging Cell. **17**(4), e12784 (2018)

27. Z. Huang, D. Wells, The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Mol. Hum. Reprod. **16**, 715–725 (2010)
28. A. Uyar, S. Torrealday, E. Seli, Cumulus and granulosa cell markers of oocyte and embryo quality. Fertil. Steril. **99**, 979–97. (2013)
29. M. Harada, E. Nose, N. Takahashi, Y. Hirota, T. Hirata, O. Yoshino et al. Evidence of the activation of unfolded protein response in granulosa and cumulus cells during follicular growth and maturation. Gynecol. Endocrinol. **31**, 783–787 (2015)
30. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil. Steril. **81**, 19–25 (2004)
31. D.R. Matthews, J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, R.C. Turner, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia **28**, 412–419 (1985)
32. M. Cozzolino, M. Cruz, A. Patel, J. Patel, A. Pacheco, J.A. Garcia-Velasco, Serum and follicular fluid Stem Cell Factor assay in IVF poor responder and normal responder patients: a predictive biomarker of oocyte retrieval. Arch. Gynecol. Obstet. **300**, 447–54. (2019)
33. R. Azziz, How polycystic ovary syndrome came into its own. F. S Sci. **2**, 2–10 (2021)
34. L. Harborne, R. Fleming, H. Lyall, J. Norman, N. Sattar, Descriptive review of the evidence for the use of metformin in polycystic ovary syndrome. Lancet **361**, 1894–1901 (2003)
35. A.S. Lee, Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. Nat. Rev. Cancer **14**(4), 263–76. (2004)
36. P. Walter, D. Ron, The unfolded protein response: from stress pathway to homeostatic regulation. Science **334**, 1081–1086 (2011)
37. C.M. Haynes, K. Petrova, C. Benedetti, Y. Yang, D. Ron, ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. Dev. Cell **13**, 467–480 (2007)
38. T. Nakagawa, H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature **403**, 98–103 (2000)
39. J. Jin, Y. Ma, X. Tong, W. Yang, Y. Dai, Y. Pan et al. Metformin inhibits testosterone-induced endoplasmic reticulum stress in ovarian granulosa cells via inactivation of p38 MAPK. Hum. Reprod. **35**, 1145–58. (2020)
40. M. Harada, E. Nose, N. Takahashi, Y. Hirota, T. Hirata, O. Yoshino et al. Evidence of the activation of unfolded protein response in granulosa and cumulus cells during follicular growth and maturation. Gynecol. Endocrinol. **31**, 783–787 (2015)
41. H.J. Park, J.Y. Park, J.W. Kim, S.G. Yang, J.M. Jung, M.J. Kim, et al. Melatonin improves the meiotic maturation of porcine oocytes by reducing endoplasmic reticulum stress during in vitro maturation. J. Pineal. Res. 2018;64:https://doi.org/10.1111/jpi.12458. Epub 2017 Dec 4.
42. L.L. Wu, D.L. Russell, R.J. Norman, R.L. Robker, Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DeltaPsi m), and embryo development. Mol. Endocrinol. **26**, 562–573 (2012)
43. L.M. Cree, E.R. Hammond, A.N. Shelling, M.C. Berg, J.C. Peek, M.P. Green, Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. Hum. Reprod. **30**, 1410–1420 (2015)
44. J. Liu, L.F. Luo, D.L. Wang, W.X. Wang, J.L. Zhu, Y.C. Li et al. Cadmium induces ovarian granulosa cell damage by activating PERK-eIF2alpha-ATF4 through endoplasmic reticulum stress. Biol. Reprod. **100**, 292–299 (2019)
45. K. Vasickova, L. Moran, D. Gurin, P. Vanhara, Alleviation of endoplasmic reticulum stress by tauroursodeoxycholic acid delays senescence of mouse ovarian surface epithelium. Cell Tissue Res. **374**, 643–52. (2018)
46. J. Lee, U. Ozcan, Unfolded protein response signaling and metabolic diseases. J. Biol. Chem. **289**, 1203–1211 (2014)
47. S. Brenjian, A. Moini, N. Yamini, L. Kashani, M. Faridmojtahedi, M. Bahramrezaie et al. Resveratrol treatment in patients with polycystic ovary syndrome decreased pro-inflammatory and endoplasmic reticulum stress markers. Am. J. Reprod. Immunol. **83**, e13186 (2020)
48. D. Lanneau, M. Brunet, E. Frisan, E. Solary, M. Fontenay, C. Garrido, Heat shock proteins: essential proteins for apoptosis regulation. J. Cell Mol. Med. **12**, 743–761 (2008)
49. L. Wang, J. Tang, L. Wang, F. Tan, H. Song, J. Zhou et al. Oxidative stress in oocyte aging and female reproduction. J. Cell Physiol. **236**, 7966–83. (2021)
50. E. Panagodimou, V. Koika, F. Markatos, A. Kaponis, G. Adonakis, N.A. Georgopoulos et al. Expression stability of ACTB, 18S, and GAPDH in human placental tissues from subjects with PCOS and controls: GAPDH expression is increased in PCOS. Hormones (Athens) **21**, 329–33. (2022)
51. M. Cozzolino, S. Herraiz, S. Titus, L. Roberts, M. Romeu, I. Peinado, R.T. Scott, A. Pellicer, E. Seli, Transcriptomic landscape of granulosa cells and peripheral blood mononuclear cells in women with PCOS compared to young poor responders and women with normal response. Hum. Reprod. **37**(6), 1274–1286 (2022)

CHAPTER 7: DISCUSSION

PCOS is a common endocrine disorder in women of reproductive age, associated with metabolic and endocrine abnormalities (171), that is often related to subfertility and infertility, due to poor oocyte and embryo quality (50-52). Despite its high prevalence, the exact etiology of PCOS remains poorly understood (172), and there is an international consensus that further research is required (35). This thesis dissertation aimed to address this research gap. First, a review of the literature was conducted to summarize the mounting evidence on mitochondrial dysfunction in women with PCOS. Next, a prospective study was carried out to determine if GC dysfunction and/or proliferative dysregulation underlie PCOS and poor ovarian response (POR)/diminished ovarian response (DOR) phenotypes. As expected, **Study II** found that the transcriptomic profiles of the GCs and PBMCs were altered in women with PCOS (compared to YPR and YNR controls). Finally, in **Study III**, women with IR-PCOS were compared to women with PCOS and nIR-PCOS and YNR oocyte donors. This prospective study demonstrated that both women with IR-PCOS and nIR-PCOS exhibit signs of metabolic distress and altered expression of UPR^{er} and UPR^{mt} genes in their GCs.

7.1. Interpretation of findings

Although elevated oxidative stress has been widely accepted as a pivotal pathological feature of PCOS, its regulatory mechanisms have not been identified in the ovaries. Oxidative phosphorylation is the process by which the ETC creates a proton gradient across the inner mitochondrial membrane, that is in turn used to make ATP (in a second process called chemiosmosis), the chemical energy used by eukaryotic cells (**Figure 4**). In this regard, dysregulation of mitochondrial function, mediated by mutations in mtDNA that can dysregulate the expression of genes encoding the respiratory chain complexes, excessive ROS levels, and reduced mtDNA copy number or mitochondrial biogenesis, can lead to GC damage and/or apoptosis, and ultimately contribute to the PCOM and/or PCOS-related pathogenesis. Since GC mitochondrial dysfunction was previously associated with reduced oocyte competence and poor pregnancy outcomes in diabetic women (83), and GCs are such a crucial determinant of oocyte development potential (14), **Study II**

aimed to ascertain if mitochondrial dysfunction could be underlying the reproductive challenges faced by women with PCOS.

Comparing the transcriptomic profiles of the GCs of YNRs and women with PCOS, we identified four DEGs in the PCOS group which were related to an inhibition of protein ubiquitination (i.e., *AC007114.2*, a long non-coding RNA [lncRNA]; Myotubularin Related Protein 9 [*MTMR9*]; Membrane Frizzled-Related Protein [*MFRP*]; and Ubiquitin Conjugating Enzyme E2 M [*UBE2M*]). Protein ubiquitination is a post-translational modification that marks intracellular proteins for lysosomal degradation, playing key roles in cell cycle progression and transcriptional regulation (173). Ubiquitination has been associated with DNA damage response, chromosome condensation, and cytoskeleton organization (i.e., spindle morphology and segregation) in addition to the deposition of extracellular matrix during cumulus expansion and follicular steroidogenesis (173), that all contribute to the acquisition of oocyte competence. Regarding our findings, the down-regulation of *MTMR9* may contribute to DNA-damage induced apoptosis (174) and autophagy (175), while the down-regulation of frizzled proteins may dysregulate Wnt signaling. Notably, at least four dysregulated Wnt-related genes were associated with PCOS in humans (176), and mice with attenuated Wnt signaling exhibited impaired GC differentiation and oocyte maturation (177). Further, in GCs of murine antral follicles, the interaction of FSH and Wnt signaling pathways activates β-catenin, enhancing FSH action and promoting the growth of preovulatory follicles, while in human GCs, canonical and non-canonical Wnt signaling molecules regulate the expression of apoptotic mediators (178). Interestingly, the silencing of *UBE2M* was recently demonstrated to inhibit proliferation and suppresses the G1/S cell cycle phase transition by attenuating β-catenin expression, and in turn, the transcription of cyclin D1 (179). Finally, mounting evidence is showing that lncRNAs can interact with DNA, RNA and proteins to modulate chromatin structure and function; regulate gene transcription; affect RNA splicing, stability, and translation; form/regulate organelles and nuclear condensates (180). Despite the elusive molecular mechanisms of *AC007114.2*, other

mitochondrially-encoded lncRNAs have been found to stabilize mitochondrial mRNAs (ND5, ND6, and CYT B) or regulate their gene expression (181). Thus, if *AC007114.2* hypothetically has similar functions, its down-regulation can adversely affect OXPHOS, by disrupting the formation of complex I (ND5 and ND6) and III (CYT B) of the respiratory chain (**Figure 4**), which can lead to ROS production in the GCs. Although ROS are endogenously produced through OXPHOS and are critical signaling molecules in cell proliferation and survival (182), excessive ROS induces oxidative stress, which can directly damage lipids and nucleic acids, and disrupt the redox homeostasis (in the ER), causing aberrant cellular responses (**Figure 5**). In this regard, excessive ROS may hamper GC function, compromise follicle quality, and ultimately, ovarian hormone production in patients with PCOS.

On the other hand, comparisons between the GCs of YPRs and women with PCOS revealed 58 DEGs related to excessive OXPHOS, mitochondrial dysfunction, and downregulated sirtuin signaling pathways (which hinders the UPR^{mt}). Specifically, the GCs from PCOS patients expressed dysregulated genes associated with a mitochondrial respiratory chain complex IV deficiency, such as mitochondrially-encoded 12S ribosomal RNAs (*MT-RNR1* and *MT-RNR2*) along with mitochondrially-encoded cytochrome C oxidase II (*COX2*). These findings align with those of previous studies describing that women with PCOS had impaired mitochondrial function in leukocytes and endothelial cells (84, 85), reduced expression of mitochondrial OXPHOS genes in the skeletal muscle (86), elevated markers of oxidative stress (i.e., lipid peroxidation and carbonyl protein content) (116, 183), and decreased mitochondrial biogenesis and mtDNA in their GCs (184). Further, sirtuin signaling is activated in response to metabolic challenges, inflammatory signals or hypoxic/oxidative stress (185) and has emerged as a critical player in the regulation of key processes in oogenesis. Specifically, sirtuin 1 (SIRT1) was associated to the maintenance of cellular redox balance by sensing and modulating oxidative stress in GCs (185), while other sirtuins activate luteinization-related steroidogenesis (186), and thus may regulate proliferation and hormonal

metabolism of GCs. In this context, dysregulated sirtuins might adversely affect the mitochondrial proteins and follicular metabolism (187), which potentially contributes to follicular dysfunction in women with PCOS. Altogether, these results support the hypothesis that the mitochondrial dysfunction in the GCs of women with PCOS may contribute to impaired steroidogenesis, fertilization, oocyte maturation and oocyte quality (188).

Our transcriptomic analysis of PBMCs revealed 16 DEGs between the PCOS and YPR groups, however, these genes were not linked to any specific pathways. Nevertheless, the expression of both zinc finger protein 57 (*ZPF57*) and peroxidasin (*PXDN*) was downregulated in the PCOS group (absolute fold change = -22.94 and -4.06, respectively). *ZFP57* was recently related to DNA methylation (189, 190), supporting the role of epigenetic regulation in PCOS (191), while *PXDN* is crucial for cell survival, growth, and extracellular matrix assembly (192) which is a major determinant of follicle recruitment and development in the ovary (193). On the other hand, among the 65 DEGs we identified between the PCOS and YNR groups (which were mainly related to granulocyte adhesion and breast cancer regulation), we found telomerase RNA component (*TERC*), whose expression was quadrupled in the PCOS group (absolute fold change = 4.08). Our findings corroborate those of Velazquez *et al.* (2021) (194) who found significantly longer telomerases in peripheral blood leukocytes of patients with hyperandrogenic PCOS compared to oocyte donor controls (p=0.001) and PCOS patients without hyperandrogenism (p=0.04). A slower telomerase shortening rate has also recently been observed in the leukocytes of Finnish women with IR-PCOS (195). Nevertheless, these findings are controversial because of the vulnerability of telomeric DNA to the oxidative damage that is often associated with PCOS. In fact, other groups have postulated that shorter telomere length (or accelerated telomerase shortening) contributes to PCOS pathogenesis, while some have found no association – possibly due to different study designs and or population cohorts (195). In addition, we highlight that member RAS oncogene family (*RAB4B)* was repressed

in the PCOS group (absolute fold change = -5.08), and its deficiency has been associated with the acquisition of insulin resistance due to its role in glucose import (196).

Study III highlighted that ER stress, and the subsequent UPR, have important implications in oocyte maturation, follicle development, and ovulation (153, 156, 197-201). Perturbations that alter ER homeostasis can lead to the accumulation of unfolded proteins (149) and activation of the UPR^{er}, which, as depicted in **Figure 8**, promotes the transcription of genes encoding factors involved in protein folding and antioxidative machinery to restore ER homeostasis (202). Deficiencies in ER chaperones [particularly the 78 kDa glucose-regulated protein (*GRP78*)–which has been associated to IR (203)] activates the UPR^{er} by relaying signals from three ER transmembrane molecular sensors (i.e., *ATF6*, *PERK* and *IRE1*) (148, 204, 205). Our study revealed that the GCs of women with IR-PCOS have a higher expression of *IRE1* than controls, suggesting downstream activation of X-box-binding protein 1 (*XBP1*), which in turn, promotes transcription of ER chaperones to reduce stress. Aligning with this hypothesis, the GCs of our patients with IR-PCOS overexpressed *XBP1*, however the concurrent overexpression of the CCAAT/enhancer-binding protein homologous protein (*CHOP*) indicated that the GCs' attempts to restore ER homeostasis were unsuccessful (155). These findings corroborate previous reports that describe the increased expression of UPR-related genes (i.e., *ATF4/6*, *XBP1* and *CHOP*) in the GCs of patients with PCOS (206, 207). Together with the evidence that ER stress impaired protein secretion, mitochondrial activity, and oocyte developmental competence in murine cumulus cells (198), the general upregulation of UPR-related genes observed in our IR-PCOS group suggest that hyperinsulinaemia might promote ER stress in the GCs, leading to their dysfunction, and ultimately, impaired oocyte competence.

Several groups have attenuated ER stress responses using the antioxidant resveratrol, which effectively reduced transcription of CHOP and XBP1 in patients with PCOS (207); melatonin, which ultimately improved the meiotic maturation of porcine oocytes (197); tauroursodeoxycholic acid (TUDCA), which decreased the frequency of ovarian surface epithelial cell replicative senescence

(201), inhibited testosterone-induced apoptosis and expression of death receptor 5 (DR5) and CHOP in GCs (157), and reduced the accumulation of advanced glycation end products (and expression of their receptors) in GCs, improving the estrous cycles of PCOS mice and reducing the number of atretic antral follicles (208); and metformin (204, 209), among others. Notably, hyperandrogenism-induced ER stress in the GCs of PCOS mice was alleviated with *in vitro* treatment of metformin, which reduced the expression of CHOP, XBP1, and ATF4 (209). Metformin also increased insulin sensitivity, improved metabolism, and mitigated PCOS symptoms in mice, by activating the adenosine monophosphate-activated protein kinase signaling pathway to reduce ER stress (204). Based on this evidence, future studies should be performed to verify whether treatment with metformin could alleviate GCs stress by improving insulin sensitivity in PCOS patients.

Similarly, in response to persisting mitochondrial stress, the UPR^{mt} can fail to restore homeostasis and lead to *CHOP*-mediated apoptosis. In **Study III**, we found *HSP10* and *HSP40* overexpressed in the GCs of patients with IR-PCOS, which may explain the elevated apoptosis of GCs during follicular atresia. These highly conserved mitochondrial *HSPs* promote cell survival, in otherwise lethal conditions, by acting as molecular chaperones that aid in the proper folding of nascent and stress-accumulated misfolded proteins, to avoid their accumulation (210). Remarkably, *HSPs* can counteract both the intrinsic and the extrinsic apoptotic pathways, by modulating a myriad of signaling pathways outside of the mitochondria, controlling the release of pro-apoptotic molecules from the mitochondria, and preventing the activation of caspases that consequently induce apoptosis (210). In particular, *HSP10* suppresses polyubiquitination of the *IGF-1R* (211), which likely prevents its proteosomal degradation (212) and leaves it bioavailable to contribute to PCOS pathogenesis (as previously described in **section 1.3.2**). In contrast to our findings, Jansen *et al.* (2004) found reduced expression of *DnaJ* (*Hsp40*) homolog, subfamily B, member 1 (*DNAJB1*) in the ovaries of women with PCOS (176). Nevertheless, a more recent study by Jones *et al.* (2011) postulated that *DNAJB1* may be common factor at the nexus of both the androgenic and insulin pathways that are frequently

dysfunctional in PCOS, and associated an intronic single nucleotide polymorphism in *DNAJB1* (rs1569198) with IR in patients with PCOS (213). In terms of functionality, *HSP40* is a co-chaperone that regulates ATP-dependent polypeptide binding by *HSP70* (214). Interestingly, *HSP70* levels were elevated in the ovaries of hyperandrogenic PCOS rats, and were postulated to decrease apoptosis and delay regression of follicular cysts, contributing to the PCOM (215). Elevated HSP70 was also found in the serum of non-obese patients with PCOS (216, 217), and was correlated with the regulatory T cell/T helper cell 17 imbalance, adding to the knowledge of the immunological etiology of PCOS (217).

7.2. Strengths and limitations

In many respects, the follicular numbers, and patterns of COS response in women with PCOS and POR seem to be at opposite ends of the same spectrum. Therefore, we postulated that these prominent infertility-related disorders might have similar molecular regulators. Indeed, the two prospective studies included in this thesis dissertation provided molecular evidence of several key molecules involved in PCOS pathogenesis that can lead to GC apoptosis, or impede proliferation and hormonal metabolism of the GCs, compromising the acquisition of oocyte competence, and thus reproductive outcomes of affected patients. Despite the promising nature of the transcriptomic findings from **Study II**, the DEGs we identified should be validated in a larger cohort, controlling for ethnicity along with other potentially confounding variables.

In addition to corroborating that women with PCOS have metabolic and endocrine abnormalities (171), PCOS patients showed altered expression of several genes involved in OXPHOS and mitochondrial function (169). Our study confirmed an altered expression in ER and mitochondrial genes of patients with IR-PCOS, with all significative genes presenting higher expression values in this cohort. Aberrant regulation of stress-mediating chaperones in both organelles indicated a disruption of the UPRs, suggesting the initiation of proapoptotic signaling. Furthermore, aberrant

chaperon activity might reinforce the negative effects of hyperinsulinemia in GCs, with *HSP10*, which favors survival of *IGF1R*, potentially driving the excessive androgen biogenesis in theca cells.

In **Study II**, oocyte maturation was achieved using hCG, GnRHa, or a combination of both. A study by Miller *et al.* (2015) (218) previously demonstrated that patients who receive hCG alone or as part of a double trigger (together with GnRHa) have higher levels of vascular endothelial growth factor and lower levels of pigment epithelium-derived growth factor. Others found upregulation of epidermal growth factor-like proteins (amphiregulin and epiregulin) and decreased gap junction protein Connexin 43 in women triggered with a combination of hCG and GnRHa compared to hCG alone (219), suggesting that different trigger methods might contribute to the differential gene expression patterns observed between the groups. However, it is noteworthy that none of the previous studies identified the genes implicated in our study to be affected by the medications used to induce oocyte maturation and ovulatory changes.

The main limitation of **Study III** may be the age difference between the PCOS and control patients. As recently reviewed, age-related ovarian alterations leads to the accumulation of ROS in oocytes, and the consequential oxidative stress negatively impacts reproductive outcomes (220) through GC apoptosis, chromosomal non-disjunction, failed implantation, and reduced embryo competence. Thus, although the overexpression of UPR-related genes may partially be due to age, the elevated number of recovered oocytes and serum estradiol in the IR-PCOS group may counteract this effect and highlight the efficacy of individualizing COS protocols. Another limitation is potentially the use of *GAPDH* gene expression used to normalize the expression of our selected genes. A recent report argued that β-actin may be a more appropriate reference gene, due to the enhanced expression of *GAPDH* in placental tissue of PCOS patients (221), however, due to the limited quantity of cDNA we obtained from the GCs, and the difficulty of rule out the effects of stress or different hormonal environments on *GAPDH* transcription, it was not possible to additionally evaluate the expression of β-actin in our samples. Further, we considered participants with a HOMA value of ≥2

to have IR-PCOS (80), however different cutoffs corresponding to the 75th or 90th percentile have been used for other populations (81).

7.3. Future perspectives

The international guidelines for PCOS management highlighted the need to refine individual diagnostic criteria to more accurately diagnose patients, reduce unnecessary testing, promote safer fertility management and suggested that clinicians shift their focus to improving the patients quality of life (through education, lifestyle modifications and emotional wellbeing) (35). By identifying specific molecules/mutations that contribute to mitochondrial dysfunction in PCOS pathogenesis, and ultimately, reproductive potential, our studies set a foundation for future research that aims to potentially reverse and/or restore the dysregulations through gene editing [e.g., of PCOS-related point mutations or deletions that only affect the ovaries (222)] or post-translational modifications (such as acetylation/deacetylation or phosphorylation/dephosphorylation that can be used to sequester relevant proteins to certain subcellular localizations; **Figure 7**).

Further, **Study II** highlighted that according to the transcriptomic profiles, our PCOS cohort was closer to the YNRs than YPRs. Future studies can consider using trained machine learning algorithms to identify whether there exist transcriptomically-defined subgroups between the extreme phenotypes of PCOS and POR. This innovative approach was recently utilized to stratify patients with endometrial-factor recurrent implantation failure into four subgroups (based on a pathology risk score) with the aim of being able to improve and/or personalize diagnostic and therapeutic strategies for patients with this heterogeneous disorder (223), and it would be interesting to translate this methodology to further characterize the heterogeneous profiles of patients with PCOS. Although, as described in section 1.2.1 herein, there are already four phenotypes of PCOS based on different combinations of clinical presentations, it could be beneficial to associate these phenotypes with specific molecular alterations to better understand the mechanisms that drive disease progression and find novel therapeutic targets for each subgroup of patients. Notably, several groups have recently

taken the first steps towards stratifying patients with PCOS by means of transcriptomics, with microarray analyses comparing the ovaries of women with PCOS or normoresponders (176), and the development of a four-variable model considering the free androgen index, 17-hydroxyprogesterone, AMH and waist circumference (224).

7.4. Clinical implications

Characterizing the differences between women with PCOS and those with POR/DOR can potentially reveal clinically relevant molecular mechanisms that promote follicular growth and biomarkers of aberrant follicle development. Our findings collectively suggested that hyperinsulinaemia-induced ER stress alters GC gene expression, leading to a dysfunctional follicular microenvironment that may have negative repercussions on oocyte maturation and competence in patients with PCOS. Thus, we propose that therapies aimed at increasing insulin sensitivity, such as metformin or IGF-1R inhibitors, might be key to improving their reproductive outcomes.

Metformin is a guanidine derivative, extracted from *Galega officinalis* (French lilac), that was used to treat diabetes-related symptoms in traditional herbal medicine since the middle ages (225). In 1957, its glucose-lowering activity was serendipitously re-discovered in the context of adult-onset type 2 diabetes mellitus; and metformin has prominently been used for this purpose for over 65 years now (225, 226). Due to the myriad of beneficial pleiotropic effects metformin provides (**Figure 9**), its application has been explored in the context of obesity, cancer, nonalcoholic fatty liver disease, and metabolic syndrome in addition to PCOS (225, 226).

Indeed, metformin has been prescribed for PCOS-related symptoms for almost three decades (227), and was tested in a plethora of PCOS-related clinical trials thus far (**Appendix 1**). Along with reducing the risk of OHSS (228), it has proved to effectively induce ovulation in non-obese women with PCOS and clomiphene-resistant women (227). Although the role of metformin in follicle development was postulated to be involved with the sensitization to FSH (206), additional preclinical research, ideally using a PCOS animal model, would be required to determine the effective doses and

appropriate duration of treatment to alleviate ER stress without compromising oocyte maturation and subsequent embryo and fetal development. Interestingly, Tso *et al.* (228) recently conducted a systematic review of thirteen randomized clinical trials involving metformin use during and after *in vitro* fertilization or intracytoplasmic sperm injection in women with PCOS, and argued there was no conclusive evidence for metformin improving birth rates because results were of poor quality, due to inconsistent, imprecise and potentially biased findings. Thus, further well-designed, multicenter, randomized clinical trials are required to reliably determine if metformin treatment has the potential to improve clinical pregnancy and live birth rates.

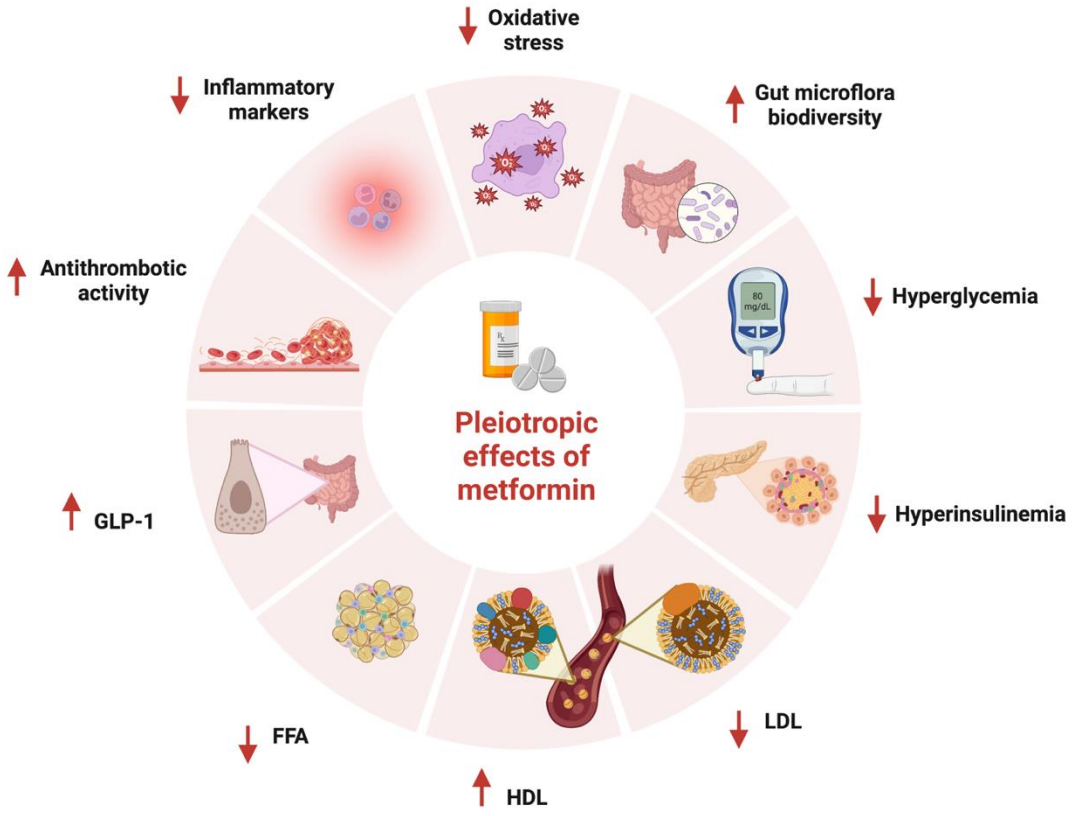

Figure 8: Pleiotropic effects of metformin. By increasing insulin sensitivity, metformin concomitantly reduces hyperglycemia, hyperinsulinemia, free fatty acids (FFA), inflammatory molecules and oxidative stress, along with the transport of cholesterol from the liver to other tissues [mediated by low-density lipoprotein (LDL) (229)]. On the other hand, metformin promotes the return of excess cholesterol from the tissues to the liver to be broken down [mediated by high-density lipoprotein (HDL) (229)], along with the biodiversity of gut microflora, the production of glucagon-like protein 1 (GLP-1; encoded by the proglucagon gene) in L cells of the small intestine and antithrombotic activity.

As recently reviewed by Tong *et al.* (2022) (62) several groups have previously tried to improve IR by modulating P13K/AKT signaling. Notably, Wu *et al.* (2018) (230) demonstrated that metformin and pioglitazone combination therapy efficiently regulated the AMPK/PI3K/JNK pathway to improve PCOS symptoms, by suppressing the high level of testosterone and reducing the proportion of cystic and primary follicles, while promoting antral follicle development, in a rat model. On the other hand, the reduction of IGF-1R density has enhanced insulin sensitivity in various cell types, including osteoblasts (231), breast cancer cells (232), preadipocyte cells (233), vascular smooth muscle cells (234), and Arg59Ter-mutated fibroblasts (235). Reduced IGF-1R might promote stress resistance via upregulation of FOXO-target genes (FOXO is upregulated in the absence of IGF-1 signaling) or inhibition of mechanistic target of rapamycin (mTOR) signaling (236). mTOR is an important regulator of cell growth and survival that integrates various environmental cues from growth factors, cellular stress, amino acids and intracellular ATP energy levels to potentiate responses such as mRNA translation or autophagy suppression (237, 238). In mice, mTOR inhibition during controlled ovarian stimulation significantly reduced GC proliferation but did not induce cell death; had no deleterious effects on the follicular reserve or preantral follicles; reduced the number of antral follicles and ovulated oocytes but not their developmental competence to the blastocyst stage (239). Interestingly, the addition of an mTOR inhibitor (rapamycin) during the *in vitro* maturation of donor human oocytes significantly decreased DNA damage in the oocytes and increased the number of high-quality embryos with normal karyotype produced following intracytoplasmic sperm injection (240)

CHAPTER 8: CONCLUSIONS

The conclusions that can be drawn from this PhD dissertation are the following:

1. Our bibliographic review (**Study I**) highlighted that the excessive reactive oxygen species and oxidative stress caused by mutations in the oxidative phosphorylation genes and/or transfer RNAs encoded by the mitochondria, together with the mitochondrial DNA copy numbers negatively correlated with insulin resistance, are likely driving metabolic dysfunction in the granulosa cells and oocytes of women with polycystic ovary syndrome (PCOS).
2. The granulosa cells of women with PCOS had differentially expressed genes related to DNA-damage induced apoptosis, cell cycle arrest and potentially transcriptional regulation of respiratory chain complexes, compared to those of young normoresponders. On the other hand, granulosa cells of women with PCOS exhibited differentially expressed genes related to excessive oxidative phosphorylation, mitochondrial dysfunction, and downregulated sirtuin signaling pathways, compared to those of young poor responders. Together, these findings demonstrated that PCOS-related follicular dysfunction is mediated by alterations in different molecular mechanisms, and the complexities of aberrant follicle development and oocyte development merit further investigation to be able to develop targeted diagnostic and therapeutic strategies.
3. Mitochondrial dysfunction activates the unfolded protein response (in the endoplasmic reticulum and the mitochondria). Indeed, **Study III** revealed that the granulosa cells of women with PCOS and insulin resistance activated the unfolded protein responses through IRE1-XBP1 signaling to reduce endoplasmic reticulum stress, and HSP10 and HSP40 to restore mitostasis, however the attempts were unsuccessful, and led to CHOP-mediated apoptosis of the granulosa cells, that can compromise follicle development.
4. In the future, therapies aimed at alleviating hyperinsulinaemia-induced endoplasmic reticulum or mitochondrial stress may restore granulosa cell gene expression, adequate follicular

development, and, ultimately, improve the developmental competence of oocytes from patients with polycystic ovarian syndrome.

CHAPTER 9: REFERENCES

1. Kaprara A, Huhtaniemi IT. The hypothalamus-pituitary-gonad axis: Tales of mice and men. *Metabolism: clinical and experimental* 2018;86:3-17.

2. Marshall JC, Kelch RP. Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. *New England Journal of Medicine* 1986;315:1459-68.

3. Kerr JB, Myers M, Anderson RA. The dynamics of the primordial follicle reserve. *Reproduction* 2013;146:R205-R15.

4. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrine reviews* 1996;17:121-55.

5. Gougeon A. Histologic definition of ovarian follicles and analysis of folliculogenesis disruptions due to gene mutations In: Adashi EY, ed. *Ovulation: Evolving and Scientific Clinical Concenpts* New York Springer, 2000:19-30.

6. Iwase A, Kiyono T, Takikawa S, Goto M, Nakamura T, Nagatomo Y *et al.* Establishment of a human nonluteinized granulosa cell line that transitions from the gonadotropin-independent to the gonadotropin-dependent status. *Endocrinology* 2012;153:2851-60.

7. Ortega I, Villanueva JA, Wong DH, Cress AB, Sokalska A, Stanley SD *et al.* Resveratrol potentiates effects of simvastatin on inhibition of rat ovarian theca-interstitial cells steroidogenesis. *Journal of ovarian research* 2014;7:1-8.

8. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends in Molecular Medicine* 2013;19:197-209.

9. Krisher R. The effect of oocyte quality on development. *Journal of animal science* 2004;82:E14-E23.

10. Gilchrist RB, Thompson JG. Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology* 2007;67:6-15.

11. Richani D, Gilchrist RB. The epidermal growth factor network: role in oocyte growth, maturation and developmental competence. *Human reproduction update* 2018;24:1-14.

12. Picton H, Briggs D, Gosden R. The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology* 1998;145:27-37.

13. Franciosi F, Conti M. Acquisition of oocyte competence to develop as an embryo: integrated nuclear and cytoplasmic events. *Human Reproduction Update* 2018;24:245-66.

14. Iager AE, Kocabas AM, Otu HH, Ruppel P, Langerveld A, Schnarr P *et al.* Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. *Fertility and sterility* 2013;99:745-52. e6.

15. Espey LL. An overview of 37 years of research on ovulation In: Adashi EY, ed. *Ovulation: Evolving and Scientific Clinical Concenpts* New York Springer, 2000:1-16.

16. Landim-Alvarenga F, Maziero R. Control of oocyte maturation. *Animal Reproduction* 2014;11:150-8.

17. Albertini DF, Combelles C, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 2001;121:647-53.

18. Gilbert I, Macaulay A, Robert C. Oocyte developmental competence and embryo quality: distinction and new perspectives. *Anim Reprod* 2015;12:397-407.

19. Ernst EH, Franks S, Hardy K, Villesen P, Lykke-Hartmann K. Granulosa cells from human primordial and primary follicles show differential global gene expression profiles. *Human Reproduction* 2018;10.1093/humrep/dey011.

20. Kidder GM, Vanderhyden BC. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. *Can J Physiol Pharmacol* 2010;88:399-413.

21. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz R. Criteria, prevalence, and phenotypes of polycystic ovary syndrome. *Fertility and sterility* 2016;106:6-15.

22. Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. *Endocrine reviews* 2016;37:467-520.

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

24. Broekmans F, Knauff E, Valkenburg O, Laven J, Eijkemans M, Fauser B. PCOS according to the Rotterdam consensus criteria: change in prevalence among WHO-II anovulation and association with metabolic factors. *BJOG: An International Journal of Obstetrics & Gynaecology* 2006;113:1210-7.

25. Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. *The journal of clinical endocrinology & metabolism* 2015;100:911-9.

26. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JSE, Legro RS *et al.* Polycystic ovary syndrome. *Nature Reviews Disease Primers* 2016;2:16057.

27. McCartney CR, Marshall JC. Polycystic Ovary Syndrome. *New England Journal of Medicine* 2016;375:54-64.

28. Shivaprakash G, Basu A, Kamath A, Shivaprakash P, Adhikari P, Up R *et al.* Acanthosis Nigricansin PCOS Patients and Its Relation with Type 2 Diabetes Mellitus and Body Mass at a Tertiary Care Hospital in Southern India. *J Clin Diagn Res* 2013;7:317-9.

29. Zawadzki J. Diagnostic criteria for polycystic ovary syndrome (a rational approach). *Polycystic ovary syndrome* 1992:377-84.

30. ESHRE TR, Group A-SPCW. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertility and sterility* 2004;81:19-25.

31. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W *et al.* Criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an androgen excess society guideline. *The Journal of Clinical Endocrinology & Metabolism* 2006;91:4237-45.

32. Mumusoglu S, Yildiz BO. Polycystic ovary syndrome phenotypes and prevalence: Differential impact of diagnostic criteria and clinical versus unselected population. *Current Opinion in Endocrine and Metabolic Research* 2020;12:66-71.

33. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R *et al.* Diagnosis and treatment of polycystic ovary syndrome: an Endocrine Society clinical practice guideline. *The Journal of clinical endocrinology and metabolism* 2013;98:4565-92.

34. Williams T, Mortada R, Porter S. Diagnosis and Treatment of Polycystic Ovary Syndrome. *Am Fam Physician* 2016;94:106-13.

35. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L *et al.* Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. *Human reproduction* 2018;33:1602-18.

36. Hoeger KM, Dokras A, Piltonen T. Update on PCOS: Consequences, Challenges, and Guiding Treatment. *The Journal of Clinical Endocrinology & Metabolism* 2021;106:e1071-e83.

37. Webber L, Stubbs S, Stark J, Trew G, Margara R, Hardy K *et al.* Formation and early development of follicles in the polycystic ovary. *The Lancet* 2003;362:1017-21.

38. Maciel GA, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ *et al.* Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism* 2004;89:5321-7.

39. Siristatidis CS, Maheshwari A, Vaidakis D, Bhattacharya S. In vitro maturation in subfertile women with polycystic ovarian syndrome undergoing assisted reproduction. *Cochrane Database of Systematic Reviews* 2018.

40. Mason HD, Willis DS, Beard RW, Winston R, Margara R, Franks S. Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. *The Journal of Clinical Endocrinology & Metabolism* 1994;79:1355-60.

41. Jonard S, Dewailly D. The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. *Human reproduction update* 2004;10:107-17.

42. Franks S, Stark J, Hardy K. Follicle dynamics and anovulation in polycystic ovary syndrome. *Human reproduction update* 2008;14:367-78.

43. Willis DS, Watson H, Mason HD, Galea R, Brincat M, Franks S. Premature response to luteinizing hormone of granulosa cells from anovulatory women with polycystic ovary syndrome: relevance to mechanism of anovulation. *The Journal of Clinical Endocrinology & Metabolism* 1998;83:3984-91.

44. Hillier SG. Gonadotropic control of ovarian follicular growth and development. *Molecular and Cellular Endocrinology* 2001;179:39-46.

45. McNatty K, Reader K, Smith P, Heath D, Juengel I. Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. *Society of Reproduction and Fertility (Supplement)* 2007;64:55-68.

46. Franks S, Mason H, Willis D. Follicular dynamics in the polycystic ovary syndrome. *Molecular and Cellular Endocrinology* 2000;163:49-52.

47. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. *Fertility and sterility* 2001;76:936-42.

48. Heijnen E, Eijkemans M, Hughes E, Laven J, Macklon N, 3, Fauser B. A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome. *Human reproduction update* 2006;12:13-21.

49. Homburg R. Pregnancy complications in PCOS. *Best Practice & Research Clinical Endocrinology & Metabolism* 2006;20:281-92.

50. Kollmann M, Martins W, Lima M, Craciunas L, Nastri C, Richardson A *et al.* Strategies for improving outcome of assisted reproduction in women with polycystic ovary syndrome: systematic review and meta-analysis. *Ultrasound in Obstetrics & Gynecology* 2016;48:709-18.

51. Weghofer A, Munne S, Chen S, Barad D, Gleicher N. Lack of association between polycystic ovary syndrome and embryonic aneuploidy. *Fertility and sterility* 2007;88:900-5.

52. Qiao J, Feng HL. Extra-and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. *Human reproduction update* 2011;17:17-33.

53. Li Y, Wang L, Xu J, Niu W, Shi H, Hu L *et al.* Higher chromosomal aberration rate in miscarried conceptus from polycystic ovary syndrome women undergoing assisted reproductive treatment. *Fertility and sterility* 2019;111:936-43. e2.

54. Yildiz BO. Diagnosis of hyperandrogenism: clinical criteria. *Best Practice & Research Clinical Endocrinology & Metabolism* 2006;20:167-76.

55. Kanbour SA, Dobs AS. Hyperandrogenism in Women with Polycystic Ovarian Syndrome: Pathophysiology and Controversies. *Androgens: Clinical Research and Therapeutics* 2022;3:22-30.

56. de Medeiros SF, Yamamoto MMW, Souto de Medeiros MA, Barbosa BB, Soares JM, Baracat EC. Changes in clinical and biochemical characteristics of polycystic ovary syndrome with advancing age. *Endocrine Connections* 2020;9:74-89.

57. Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M *et al.* The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. *Journal of Biological Chemistry* 2003;278:26380-90.

58. Nelson VL, Legro RS, Strauss III JF, McAllister JM. Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. *Molecular endocrinology* 1999;13:946-57.

59. Nelson VL, Qin K-n, Rosenfield RL, Wood JR, Penning TM, Legro RS *et al.* The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism* 2001;86:5925-33.

60. Liu K, Motan T, Claman P. No. 350-Hirsutism: Evaluation and Treatment. *Journal of Obstetrics and Gynaecology Canada* 2017;39:1054-68.

61. Dahan MH, Reaven G. Relationship among obesity, insulin resistance, and hyperinsulinemia in the polycystic ovary syndrome. *Endocrine* 2019;64:685-9.

62. Tong C, Wu Y, Zhang L, Yu Y. Insulin resistance, autophagy and apoptosis in patients with polycystic ovary syndrome: Association with PI3K signaling pathway. *Front Endocrinol (Lausanne)* 2022;13:1091147.

63. Christakou CD, Diamanti-Kandarakis E. Role of androgen excess on metabolic aberrations and cardiovascular risk in women with polycystic ovary syndrome. *Women's Health* 2008;4:583-94.

64. Rajendran S, Willoughby SR, Chan WPA, Liberts EA, Heresztyn T, Saha M *et al.* Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects. *Atherosclerosis* 2009;204:509-14.

65. Shahin L, Hyassat D, Batieha A, Khader Y, El-Khateeb M, Ajlouni K. Insulin Sensitivity Indices in Patients with Polycystic Ovary Syndrome with Different Body Mass Index Categories. *Curr Diabetes Rev* 2020;16:483-9.

66. Rojas J, Chávez M, Olivar L, Rojas M, Morillo J, Mejías J *et al.* Polycystic ovary syndrome, insulin resistance, and obesity: navigating the pathophysiologic labyrinth. *Int J Reprod Med* 2014;2014:719050.

67. Layegh P, Mousavi Z, Farrokh Tehrani D, Parizadeh SM, Khajedaluee M. Insulin resistance and endocrine-metabolic abnormalities in polycystic ovarian syndrome: Comparison between obese and non-obese PCOS patients. *Int J Reprod Biomed* 2016;14:263-70.

68. De Leo V, Musacchio MC, Cappelli V, Massaro MG, Morgante G, Petraglia F. Genetic, hormonal and metabolic aspects of PCOS: an update. *Reproductive biology and endocrinology : RB&E* 2016;14:38.

69. Duleba AJ, Spaczynski RZ, Olive DL. Insulin and insulin-like growth factor I stimulate the proliferation of human ovarian theca-interstitial cells. *Fertility and sterility* 1998;69:335-40.

70. Ebrahimi-Mamaghani M, Saghafi-Asl M, Pirouzpanah S, Aliasgharzadeh A, Aliashrafi S, Rezayi N *et al.* Association of insulin resistance with lipid profile, metabolic syndrome, and hormonal aberrations in overweight or obese women with polycystic ovary syndrome. *J Health Popul Nutr* 2015;33:157-67.

71. Glueck CJ, Goldenberg N. Characteristics of obesity in polycystic ovary syndrome: Etiology, treatment, and genetics. *Metabolism: clinical and experimental* 2019;92:108-20.

72. Willis D, Mason H, Gilling-Smith C, Franks S. Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. *The Journal of Clinical Endocrinology & Metabolism* 1996;81:302-9.

73. Jones MR, Brower MA, Xu N, Cui J, Mengesha E, Chen Y-DI *et al.* Systems Genetics Reveals the Functional Context of PCOS Loci and Identifies Genetic and Molecular Mechanisms of Disease Heterogeneity. *PLOS Genetics* 2015;11:e1005455.

74. Dumesic DA, Phan JD, Leung KL, Grogan TR, Ding X, Li X *et al.* Adipose Insulin Resistance in Normal-Weight Women With Polycystic Ovary Syndrome. *The Journal of clinical endocrinology and metabolism* 2019;104:2171-83.

75. Vrbíková J, Cibula D, Dvoráková K, Stanická S, Sindelka G, Hill M *et al.* Insulin sensitivity in women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism* 2004;89:2942-5.

76. Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR *et al.* Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PloS one* 2010;5:e10074.

77. Dandona P, Mohanty P, Ghanim H, Aljada A, Browne R, Hamouda W *et al.* The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. *The Journal of clinical endocrinology and metabolism* 2001;86:355-62.

78. Smits A, Marei WFA, De Neubourg D, Leroy JLMR. Diet normalization or caloric restriction as a preconception care strategy to improve metabolic health and oocyte quality in obese outbred mice. *Reproductive Biology and Endocrinology* 2021;19:166.

79. Gayoso-Diz P, Otero-González A, Rodriguez-Alvarez MX, Gude F, García F, De Francisco A *et al.* Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: effect of gender and age: EPIRCE cross-sectional study. *BMC Endocr Disord* 2013;13:47.

80. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412-9.

81. Lewandowski KC, Płusajska J, Horzelski W, Bieniek E, Lewiński A. Limitations of insulin resistance assessment in polycystic ovary syndrome. *Endocr Connect* 2018;7:403-12.

82. Serra D, Mera P, Malandrino MI, Mir JF, Herrero L. Mitochondrial fatty acid oxidation in obesity. *Antioxid Redox Signal* 2013;19:269-84.

83. Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM *et al.* Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. *PloS one* 2010;5:e15901.

84. Victor VM, Rocha M, Bañuls C, Alvarez A, de Pablo C, Sanchez-Serrano M *et al.* Induction of oxidative stress and human leukocyte/endothelial cell interactions in polycystic ovary syndrome patients with insulin resistance. *The Journal of clinical endocrinology and metabolism* 2011;96:3115-22.

85. Lee SH, Chung DJ, Lee HS, Kim TJ, Kim MH, Jeong HJ *et al.* Mitochondrial DNA copy number in peripheral blood in polycystic ovary syndrome. *Metabolism: clinical and experimental* 2011;60:1677-82.

86. Skov V, Glintborg D, Knudsen S, Jensen T, Kruse TA, Tan Q *et al.* Reduced expression of nuclear-encoded genes involved in mitochondrial oxidative metabolism in skeletal muscle of insulin-resistant women with polycystic ovary syndrome. *Diabetes* 2007;56:2349-55.

87. Wolstenholme DR. Animal Mitochondrial DNA: Structure and Evolution. In: Wolstenholme DR, Jeon KW, eds. *International Review of Cytology*. Vol. 141: Academic Press, 1992:173-216.

88. Taanman JW. The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et biophysica acta* 1999;1410:103-23.

89. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009;417:1-13.

90. Seli E. Mitochondrial DNA as a biomarker for in-vitro fertilization outcome. *Current opinion in obstetrics & gynecology* 2016;28:158-63.

91. Cozzolino M, Marin D, Sisti G. New Frontiers in IVF: mtDNA and autologous germline mitochondrial energy transfer. *Reproductive biology and endocrinology : RB&E* 2019;17:55.

92. Reddy TV, Govatati S, Deenadayal M, Sisinthy S, Bhanoori M. Impact of mitochondrial DNA copy number and displacement loop alterations on polycystic ovary syndrome risk in south Indian women. *Mitochondrion* 2019;44:35-40.

93. Zhuo G, Ding Y, Feng G, Yu L, Jiang Y. Analysis of mitochondrial DNA sequence variants in patients with polycystic ovary syndrome. *Archives of gynecology and obstetrics* 2012;286:653-9.

94. Ding Y, Xia BH, Zhang CJ, Zhuo GC. Mitochondrial tRNA(Leu(UUR)) C3275T, tRNA(Gln) T4363C and tRNA(Lys) A8343G mutations may be associated with PCOS and metabolic syndrome. *Gene* 2018;642:299-306.

95. Ding Y, Zhuo G, Zhang C. The Mitochondrial tRNALeu(UUR) A3302G Mutation may be Associated With Insulin Resistance in Woman With Polycystic Ovary Syndrome. *Reprod Sci* 2016;23:228-33.

96. Ding Y, Zhuo G, Zhang C, Leng J. Point mutation in mitochondrial tRNA gene is associated with polycystic ovary syndrome and insulin resistance. *Mol Med Rep* 2016;13:3169-72.

97. Ding Y, Xia BH, Zhang CJ, Zhuo GC. Mutations in mitochondrial tRNA genes may be related to insulin resistance in women with polycystic ovary syndrome. *Am J Transl Res* 2017;9:2984-96.

98. Cozzolino M, Seli E. Mitochondrial function in women with polycystic ovary syndrome. *Current Opinion in Obstetrics and Gynecology* 2020;32.

99. Victor VM, Rocha M, Bañuls C, Sanchez-Serrano M, Sola E, Gomez M *et al.* Mitochondrial complex I impairment in leukocytes from polycystic ovary syndrome patients with insulin resistance. *The Journal of clinical endocrinology and metabolism* 2009;94:3505-12.

100. Jeng JY, Yeh TS, Lee JW, Lin SH, Fong TH, Hsieh RH. Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. *Journal of cellular biochemistry* 2008;103:347-57.

101. Hütter E, Unterluggauer H, Garedew A, Jansen-Dürr P, Gnaiger E. High-resolution respirometry--a modern tool in aging research. *Exp Gerontol* 2006;41:103-9.

102. Yowe DL, Ames BN. Quantitation of age-related mitochondrial DNA deletions in rat tissues shows that their pattern of accumulation differs from that of humans. *Gene* 1998;209:23-30.

103. Ibáñez L, Potau N, Zampolli M, Riqué S, Saenger P, Carrascosa A. Hyperinsulinemia and decreased insulin-like growth factor-binding protein-1 are common features in prepubertal and pubertal girls with a history of premature pubarche. *The Journal of clinical endocrinology and metabolism* 1997;82:2283-8.

104. Lewis JG, Borowski KK, Shand BI, George PM, Scott RS. Plasma sex hormone-binding globulin, corticosteroid-binding globulin, cortisol, and free cortisol levels in outpatients attending a lipid disorders clinic: a cross-sectional study of 1137 subjects. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 2010;42:274-9.

105. Ou XH, Li S, Wang ZB, Li M, Quan S, Xing F *et al.* Maternal insulin resistance causes oxidative stress and mitochondrial dysfunction in mouse oocytes. *Human reproduction (Oxford, England)* 2012;27:2130-45.

106. Hu M, Zhang Y, Guo X, Jia W, Liu G, Zhang J *et al.* Hyperandrogenism and insulin resistance induce gravid uterine defects in association with mitochondrial dysfunction and aberrant reactive oxygen species production. *Am J Physiol Endocrinol Metab* 2019;316:E794-e809.

107. Sabuncu T, Vural H, Harma M, Harma M. Oxidative stress in polycystic ovary syndrome and its contribution to the risk of cardiovascular disease. *Clin Biochem* 2001;34:407-13.

108. González F, Rote NS, Minium J, Kirwan JP. Reactive oxygen species-induced oxidative stress in the development of insulin resistance and hyperandrogenism in polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism* 2006;91:336-40.

109. Murri M, Luque-Ramírez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. *Hum Reprod Update* 2013;19:268-88.

110. Siddiqui S, Mateen S, Ahmad R, Moin S. A brief insight into the etiology, genetics, and immunology of polycystic ovarian syndrome (PCOS). *Journal of assisted reproduction and genetics* 2022;39:2439-73.

111. Hennet M, Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. *International Journal of Developmental Biology* 2013;56:819-31.

112. Bianchi L, Gagliardi A, Landi C, Focarelli R, De Leo V, Luddi A *et al.* Protein pathways working in human follicular fluid: the future for tailored IVF? *Expert reviews in molecular medicine* 2016;18:e9.

113. Angelucci S, Ciavardelli D, Di Giuseppe F, Eleuterio E, Sulpizio M, Tiboni GM *et al.* Proteome analysis of human follicular fluid. *Biochimica et biophysica acta* 2006;1764:1775-85.

114. Twigt J, Steegers-Theunissen RP, Bezstarosti K, Demmers JA. Proteomic analysis of the microenvironment of developing oocytes. *Proteomics* 2012;12:1463-71.

115. Nuñez-Calonge R, Cortés S, Gutierrez Gonzalez LM, Kireev R, Vara E, Ortega L *et al.* Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors. *Reproductive biomedicine online* 2016;32:446-56.

116. Fenkci V, Fenkci S, Yilmazer M, Serteser M. Decreased total antioxidant status and increased oxidative stress in women with polycystic ovary syndrome may contribute to the risk of cardiovascular disease. *Fertility and sterility* 2003;80:123-7.

117. Palacio JR, Iborra A, Ulcova-Gallova Z, Badia R, Martínez P. The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients. *Clin Exp Immunol* 2006;144:217-22.

118. Dinger Y, Akcay T, Erdem T, Ilker Saygili E, Gundogdu S. DNA damage, DNA susceptibility to oxidation and glutathione level in women with polycystic ovary syndrome. *Scand J Clin Lab Invest* 2005;65:721-8.

119. Insenser M, Martínez-García MA, Montes R, San-Millán JL, Escobar-Morreale HF. Proteomic analysis of plasma in the polycystic ovary syndrome identifies novel markers involved in iron metabolism, acute-phase response, and inflammation. *The Journal of clinical endocrinology and metabolism* 2010;95:3863-70.

120. Adashi EY. Endocrinology of the ovary. *Human reproduction (Oxford, England)* 1994;9:815-27.

121. Uyar A, Torrealday S, Seli E. Cumulus and granulosa cell markers of oocyte and embryo quality. *Fertility and sterility* 2013;99:979-97.

122. Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *The Journal of clinical endocrinology and metabolism* 2001;86:1318-23.

123. Lai Q, Xiang W, Li Q, Zhang H, Li Y, Zhu G *et al.* Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. *Front Med* 2018;12:518-24.

124. Bañuls C, Rovira-Llopis S, Martinez de Marañon A, Veses S, Jover A, Gomez M *et al.* Metabolic syndrome enhances endoplasmic reticulum, oxidative stress and leukocyte-endothelium interactions in PCOS. *Metabolism: clinical and experimental* 2017;71:153-62.

125. Zhang J, Xu Y, Liu H, Pan Z. MicroRNAs in ovarian follicular atresia and granulosa cell apoptosis. *Reproductive biology and endocrinology : RB&E* 2019;17:9.

126. Yi HS, Chang JY, Shong M. The mitochondrial unfolded protein response and mitohormesis: a perspective on metabolic diseases. *J Mol Endocrinol* 2018;61:R91-r105.

127. Fiorese CJ, Haynes CM. Integrating the UPR(mt) into the mitochondrial maintenance network. *Crit Rev Biochem Mol Biol* 2017;52:304-13.

128. Nargund AM, Pellegrino MW, Fiorese CJ, Baker BM, Haynes CM. Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science* 2012;337:587-90.

129. Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, Ron D. Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J Cell Sci* 2004;117:4055-66.

130. Schulz AM, Haynes CM. UPR(mt)-mediated cytoprotection and organismal aging. *Biochimica et biophysica acta* 2015;1847:1448-56.

131. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. *Annu Rev Biochem* 2007;76:723-49.

132. Wang T, Babayev E, Jiang Z, Li G, Zhang M, Esencan E *et al.* Mitochondrial unfolded protein response gene Clpp is required to maintain ovarian follicular reserve during aging, for oocyte competence, and development of pre-implantation embryos. *Aging Cell* 2018;17:e12784.

133. Shpilka T, Haynes CM. The mitochondrial UPR: mechanisms, physiological functions and implications in ageing. *Nature Reviews Molecular Cell Biology* 2018;19:109-20.

134. Seli E, Wang T, Horvath TL. Mitochondrial unfolded protein response: a stress response with implications for fertility and reproductive aging. *Fertility and sterility* 2019;111:197-204.

135. Rath E, Berger E, Messlik A, Nunes T, Liu B, Kim SC *et al.* Induction of dsRNA-activated protein kinase links mitochondrial unfolded protein response to the pathogenesis of intestinal inflammation. *Gut* 2012;61:1269-78.

136. Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G *et al.* Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature* 2013;497:451-7.

137. Münch C. The different axes of the mammalian mitochondrial unfolded protein response. *BMC Biology* 2018;16:81.

138. Tremblay BP, Haynes CM. *Mitochondrial distress call moves to the cytosol to trigger a response to stress*. In: Nature Publishing Group UK London, 2020.

139. Teske BF, Fusakio ME, Zhou D, Shan J, McClintick JN, Kilberg MS *et al.* CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell* 2013;24:2477-90.

140. Xie X, Shu R, Yu C, Fu Z, Li Z. Mammalian AKT, the Emerging Roles on Mitochondrial Function in Diseases. *Aging Dis* 2022;13:157-74.

141. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ *et al.* SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab* 2012;15:675-90.

142. Fasano C, Disciglio V, Bertora S, Lepore Signorile M, Simone C. FOXO3a from the Nucleus to the Mitochondria: A Round Trip in Cellular Stress Response. *Cells* 2019;8:1110.

143. Tseng AH, Shieh SS, Wang DL. SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative damage. *Free Radic Biol Med* 2013;63:222-34.

144. Anderson RM, Barger JL, Edwards MG, Braun KH, O'Connor CE, Prolla TA *et al.* Dynamic regulation of PGC-1alpha localization and turnover implicates mitochondrial adaptation in calorie restriction and the stress response. *Aging Cell* 2008;7:101-11.

145. De Vitto H, Bode AM, Dong Z. The PGC-1/ERR network and its role in precision oncology. *npj Precision Oncology* 2019;3:9.

146. Tang BL. Sirt1 and the Mitochondria. *Mol Cells* 2016;39:87-95.

147. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *The Journal of biological chemistry* 2001;276:9817-24.

148. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011;334:1081-6.

149. Babayev E, Wang T, Szigeti-Buck K, Lowther K, Taylor HS, Horvath T *et al.* Reproductive aging is associated with changes in oocyte mitochondrial dynamics, function, and mtDNA quantity. *Maturitas* 2016;93:121-30.

150. Takahashi N, Harada M, Hirota Y, Nose E, Azhary JM, Koike H *et al.* Activation of Endoplasmic Reticulum Stress in Granulosa Cells from Patients with Polycystic Ovary Syndrome Contributes to Ovarian Fibrosis. *Sci Rep* 2017;7:10824.

151. Takahashi N, Harada M, Hirota Y, Zhao L, Yoshino O, Urata Y *et al.* A potential role of endoplasmic reticulum stress in development of ovarian hyperstimulation syndrome. *Mol Cell Endocrinol* 2016;428:161-9.

152. Babayev E, Lalioti MD, Favero F, Seli E. Cross-Talk Between FSH and Endoplasmic Reticulum Stress: A Mutually Suppressive Relationship. *Reprod Sci* 2016;23:352-64.

153. Lee J, Ozcan U. Unfolded protein response signaling and metabolic diseases. *The Journal of biological chemistry* 2014;289:1203-11.

154. Jovaisaite V, Mouchiroud L, Auwerx J. The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. *J Exp Biol* 2014;217:137-43.

155. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA *et al.* Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. *Nature* 2000;403:98-103.

156. Harada M, Nose E, Takahashi N, Hirota Y, Hirata T, Yoshino O *et al.* Evidence of the activation of unfolded protein response in granulosa and cumulus cells during follicular growth and maturation. *Gynecol Endocrinol* 2015;31:783-7.

157. Azhary JMK, Harada M, Takahashi N, Nose E, Kunitomi C, Koike H *et al.* Endoplasmic Reticulum Stress Activated by Androgen Enhances Apoptosis of Granulosa Cells via Induction of Death Receptor 5 in PCOS. *Endocrinology* 2019;160:119-32.

158. Hillary RF, FitzGerald U. A lifetime of stress: ATF6 in development and homeostasis. *Journal of Biomedical Science* 2018;25:48.

159. Adams CJ, Kopp MC, Larburu N, Nowak PR, Ali MMU. Structure and Molecular Mechanism of ER Stress Signaling by the Unfolded Protein Response Signal Activator IRE1. *Frontiers in Molecular Biosciences* 2019;6.

160. Sriburi R, Jackowski S, Mori K, Brewer JW. XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. *J Cell Biol* 2004;167:35-41.

161. Meusser B, Hirsch C, Jarosch E, Sommer T. ERAD: the long road to destruction. *Nature cell biology* 2005;7:766-72.

162. Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P *et al.* DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic acids research* 2006;34:D668-72.

163. Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Human reproduction (Oxford, England)* 2004;19:41-7.

164. Alviggi C, Andersen CY, Buehler K, Conforti A, De Placido G, Esteves SC *et al.* A new more detailed stratification of low responders to ovarian stimulation: from a poor ovarian response to a low prognosis concept. *Fertility and sterility* 2016;105:1452-3.

165. Cozzolino M, Cruz M, Patel A, Patel J, Pacheco A, Garcia-Velasco JA. Serum and follicular fluid Stem Cell Factor assay in IVF poor responder and normal responder patients: a predictive biomarker of oocyte retrieval. *Archives of gynecology and obstetrics* 2019;300:447-54.

166. Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J *et al.* GENCODE reference annotation for the human and mouse genomes. *Nucleic acids research* 2019;47:D766-d73.

167. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc* 2016;11:1650-67.

168. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.

169. Cozzolino M, Herraiz S, Titus S, Roberts L, Romeu M, Peinado I *et al.* Transcriptomic landscape of granulosa cells and peripheral blood mononuclear cells in women with PCOS compared to young poor responders and women with normal response. *Human Reproduction* 2022;37:1274-86.

170. Cozzolino M, Herraiz S, Cakiroglu Y, Garcia-Velasco JA, Tiras B, Pacheco A *et al.* Distress response in granulosa cells of women affected by PCOS with or without insulin resistance. *Endocrine* 2023;79:200-7.

171. Azziz R. How polycystic ovary syndrome came into its own. *F&S Science* 2021;2:2-10.

172. Dapas M, Dunaif A. Deconstructing a Syndrome: Genomic Insights Into PCOS Causal Mechanisms and Classification. *Endocrine Reviews* 2022;43:927-65.

173. Wang J, Zhou Q, Ding J, Yin T, Ye P, Zhang Y. The Conceivable Functions of Protein Ubiquitination and Deubiquitination in Reproduction. *Frontiers in Physiology* 2022;13.

174. Zou J, Chang SC, Marjanovic J, Majerus PW. MTMR9 increases MTMR6 enzyme activity, stability, and role in apoptosis. *The Journal of biological chemistry* 2009;284:2064-71.

175. Zou J, Majerus PW, Wilson DB, Schrade A, Chang S-C, Wilson MP. The role of myotubularin-related phosphatases in the control of autophagy and programmed cell death. *Adv Biol Regul* 2012;52:282-9.

176. Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C *et al.* Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. *Molecular Endocrinology* 2004;18:3050-63.

177. Habara O, Logan CY, Kanai-Azuma M, Nusse R, Takase HM. Self-activation of Wnt signaling in pre-granulosa cells is required for ovarian folliculogenesis. *bioRxiv* 2020;10.1101/2020.10.20.346304:2020.10.20.346304.

178. Sanchez AM, Viganò P, Quattrone F, Pagliardini L, Papaleo E, Candiani M *et al.* The WNT/β-catenin signaling pathway and expression of survival promoting genes in luteinized granulosa cells: endometriosis as a paradigm for a dysregulated apoptosis pathway. *Fertility and sterility* 2014;101:1688-96.

179. Zhang GC, Yu XN, Sun JL, Xiong J, Yang YJ, Jiang XM *et al.* UBE2M promotes cell proliferation via the β-catenin/cyclin D1 signaling in hepatocellular carcinoma. *Aging (Albany NY)* 2020;12:2373-92.

180. Statello L, Guo C-J, Chen L-L, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. *Nature Reviews Molecular Cell Biology* 2021;22:96-118.

181. Rackham O, Shearwood A-MJ, Mercer TR, Davies SM, Mattick JS, Filipovska A. Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins. *Rna* 2011;17:2085-93.

182. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* 2012;24:981-90.

183. Kuşçu NK, Var A. Oxidative stress but not endothelial dysfunction exists in non-obese, young group of patients with polycystic ovary syndrome. *Acta Obstet Gynecol Scand* 2009;88:612-7.

184. Zhao H, Zhao Y, Li T, Li M, Li J, Li R *et al.* Metabolism alteration in follicular niche: The nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. *Free Radical Biology and Medicine* 2015;86:295-307.

185. Tatone C, Di Emidio G, Vitti M, Di Carlo M, Santini S, D'Alessandro AM *et al.* Sirtuin functions in female fertility: possible role in oxidative stress and aging. *Oxidative medicine and cellular longevity* 2015;2015.

186. Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S *et al.* Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Human reproduction update* 2018;24:267-89.

187. Pacella-Ince L, Zander-Fox D, Lane M. Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age. *Human reproduction* 2014;29:1490-9.

188. Sreerangaraja Urs DB, Wu W-H, Komrskova K, Postlerova P, Lin Y-F, Tzeng C-R *et al.* Mitochondrial function in modulating human granulosa cell steroidogenesis and female fertility. *International journal of molecular sciences* 2020;21:3592.

189. Monteagudo-Sánchez A, Hernandez Mora JR, Simon C, Burton A, Tenorio J, Lapunzina P *et al.* The role of ZFP57 and additional KRAB-zinc finger proteins in the maintenance of human imprinted methylation and multi-locus imprinting disturbances. *Nucleic acids research* 2020;48:11394-407.

190. Moon SW, Mo HY, Choi EJ, Yoo NJ, Lee SH. Mutational Alterations of DNA Methylation-related Genes CTCF, ZFP57, and ATF7IP Genes in Colon Cancers. *Appl Immunohistochem Mol Morphol* 2022;30:e16-e20.

191. Concha CF, Sir PT, Recabarren SE, Pérez BF. [Epigenetics of polycystic ovary syndrome]. *Rev Med Chil* 2017;145:907-15.

192. Lee SW, Kim HK, Naidansuren P, Ham KA, Choi HS, Ahn HY *et al.* Peroxidasin is essential for endothelial cell survival and growth signaling by sulfilimine crosslink-dependent matrix assembly. *Faseb j* 2020;34:10228-41.

193. Woodruff TK, Shea LD. The role of the extracellular matrix in ovarian follicle development. *Reprod Sci* 2007;14:6-10.

194. Velazquez ME, Millan AL, Rojo M, Abruzzese GA, Cocucci SE, Iglesias Molli AE *et al.* Telomere Length Differently Associated to Obesity and Hyperandrogenism in Women With Polycystic Ovary Syndrome. *Front Endocrinol (Lausanne)* 2021;12:604215.

195. Pölönen J, Pinola P, Ronkainen J, Blakemore AI, Buxton JL, Tapanainen JS *et al.* Polycystic ovary syndrome and leukocyte telomere length: cross-sectional and longitudinal changes. *European Journal of Endocrinology* 2022;187:651-61.

196. Gilleron J, Bouget G, Ivanov S, Meziat C, Ceppo F, Vergoni B *et al.* Rab4b Deficiency in T Cells Promotes Adipose Treg/Th17 Imbalance, Adipose Tissue Dysfunction, and Insulin Resistance. *Cell Rep* 2018;25:3329-41.e5.

197. Park H-J, Park J-Y, Kim J-W, Yang S-G, Jung J-M, Kim M-J *et al.* Melatonin improves the meiotic maturation of porcine oocytes by reducing endoplasmic reticulum stress during in vitro maturation. *Journal of Pineal Research* 2018;64:e12458.

198. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (ΔΨm), and embryo development. *Molecular endocrinology* 2012;26:562-73.

199. Cree LM, Hammond ER, Shelling AN, Berg MC, Peek JC, Green MP. Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. *Human Reproduction* 2015;30:1410-20.

200. Liu J, Luo L-F, Wang D-L, Wang W-x, Zhu J-l, Li Y-c *et al.* Cadmium induces ovarian granulosa cell damage by activating PERK-eIF2α-ATF4 through endoplasmic reticulum stress. *Biology of Reproduction* 2019;100:292-9.

201. Vašíčková K, Moráň L, Gurín D, Vaňhara P. Alleviation of endoplasmic reticulum stress by tauroursodeoxycholic acid delays senescence of mouse ovarian surface epithelium. *Cell and tissue research* 2018;374:643-52.

202. Zhang Z, Zhang L, Zhou L, Lei Y, Zhang Y, Huang C. Redox signaling and unfolded protein response coordinate cell fate decisions under ER stress. *Redox Biology* 2019;25:101047.

203. Kim JH, Lee E, Friedline RH, Suk S, Jung DY, Dagdeviren S *et al.* Endoplasmic reticulum chaperone GRP78 regulates macrophage function and insulin resistance in diet-induced obesity. *The FASEB Journal* 2018;32:2292-304.

204. Lee AS. Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nature Reviews Cancer* 2014;14:263-76.

205. Haynes CM, Petrova K, Benedetti C, Yang Y, Ron D. ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. *Developmental cell* 2007;13:467-80.

206. Harborne L, Fleming R, Lyall H, Norman J, Sattar N. Descriptive review of the evidence for the use of metformin in polycystic ovary syndrome. *The Lancet* 2003;361:1894-901.

207. Brenjian S, Moini A, Yamini N, Kashani L, Faridmojtahedi M, Bahramrezaie M *et al.* Resveratrol treatment in patients with polycystic ovary syndrome decreased pro-inflammatory and endoplasmic reticulum stress markers. *American Journal of Reproductive Immunology* 2020;83:e13186.

208. Azhary JMK, Harada M, Kunitomi C, Kusamoto A, Takahashi N, Nose E *et al.* Androgens Increase Accumulation of Advanced Glycation End Products in Granulosa Cells by Activating ER Stress in PCOS. *Endocrinology* 2020;161.

209. Jin J, Ma Y, Tong X, Yang W, Dai Y, Pan Y *et al.* Metformin inhibits testosterone-induced endoplasmic reticulum stress in ovarian granulosa cells via inactivation of p38 MAPK. *Human Reproduction* 2020;35:1145-58.

210. Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C. Heat shock proteins: essential proteins for apoptosis regulation. *Journal of cellular and molecular medicine* 2008;12:743-61.

211. Shan YX, Yang TL, Mestril R, Wang PH. Hsp10 and Hsp60 suppress ubiquitination of insulin-like growth factor-1 receptor and augment insulin-like growth factor-1 receptor signaling in cardiac muscle: implications on decreased myocardial protection in diabetic cardiomyopathy. *The Journal of biological chemistry* 2003;278:45492-8.

212. Sehat B, Andersson S, Vasilcanu R, Girnita L, Larsson O. Role of ubiquitination in IGF-1 receptor signaling and degradation. *PloS one* 2007;2:e340.

213. Jones MR, Chua A, Chen YD, Li X, Krauss RM, Rotter JI *et al.* Harnessing expression data to identify novel candidate genes in polycystic ovary syndrome. *PloS one* 2011;6:e20120.

214. Fan CY, Lee S, Cyr DM. Mechanisms for regulation of Hsp70 function by Hsp40. *Cell Stress Chaperones* 2003;8:309-16.

215. Wu G, Hu X, Ding J, Yang J. Abnormal expression of HSP70 may contribute to PCOS pathology. *Journal of Ovarian Research* 2019;12:74.

216. Gao H, Meng J, Xu M, Zhang S, Ghose B, Liu J *et al.* Serum heat shock protein 70 concentration in relation to polycystic ovary syndrome in a non-obese Chinese population. *PloS one* 2013;8:e67727.

217. Yang Y, Xia J, Yang Z, Wu G, Yang J. The abnormal level of HSP70 is related to Treg/Th17 imbalance in PCOS patients. *J Ovarian Res* 2021;14:155.

218. Miller I, Chuderland D, Ron-El R, Shalgi R, Ben-Ami I. GnRH agonist triggering modulates PEDF to VEGF ratio inversely to hCG in granulosa cells. *The Journal of Clinical Endocrinology & Metabolism* 2015;100:E1428-E36.

219. Haas J, Ophir L, Barzilay E, Machtinger R, Yung Y, Orvieto R *et al.* Standard human chorionic gonadotropin versus double trigger for final oocyte maturation results in different granulosa cells gene expressions: a pilot study. *Fertility and sterility* 2016;106:653-9. e1.

220. Wang L, Tang J, Wang L, Tan F, Song H, Zhou J *et al.* Oxidative stress in oocyte aging and female reproduction. *Journal of Cellular Physiology* 2021;236:7966-83.

221. Panagodimou E, Koika V, Markatos F, Kaponis A, Adonakis G, Georgopoulos NA *et al.* Expression stability of ACTB, 18S, and GAPDH in human placental tissues from subjects with PCOS and controls: GAPDH expression is increased in PCOS. *Hormones* 2022:1-5.

222. Finsterer J. Mitochondrial Dysfunction in Polycystic Ovary Syndrome. *Reprod Sci* 2022;10.1007/s43032-022-01100-z.

223. Josefa María SR. *Pathological endometrial function characterization in the mid-secretory phase in infertile patients*. Doctoral thesis. Department of Pediatrics, Obstetrics, and Gyneacology, Universitat de Valencia. Valencia, Spain. 2023.

224. Deshmukh H, Papageorgiou M, Kilpatrick ES, Atkin SL, Sathyapalan T. Development of a novel risk prediction and risk stratification score for polycystic ovary syndrome. *Clin Endocrinol (Oxf)* 2019;90:162-9.

225. Drzewoski J, Hanefeld M. The Current and Potential Therapeutic Use of Metformin—The Good Old Drug. *Pharmaceuticals* 2021;14:122.

226. Zhou J, Massey S, Story D, Li L. Metformin: An Old Drug with New Applications. *International journal of molecular sciences* 2018;19.

227. Johnson NP. Metformin use in women with polycystic ovary syndrome. *Ann Transl Med* 2014;2:56.

228. Tso LO, Costello MF, Albuquerque LET, Andriolo RB, Macedo CR. Metformin treatment before and during IVF or ICSI in women with polycystic ovary syndrome. *Cochrane Database of Systematic Reviews* 2020.

229. Lee C-K, Liao C-W, Meng S-W, Wu W-K, Chiang J-Y, Wu M-S. Lipids and Lipoproteins in Health and Disease: Focus on Targeting Atherosclerosis. *Biomedicines* 2021;9:985.

230. Wu Y, Li P, Zhang D, Sun Y. Metformin and pioglitazone combination therapy ameliorate polycystic ovary syndrome through AMPK/PI3K/JNK pathway. *Exp Ther Med* 2018;15:2120-7.

231. Fulzele K, DiGirolamo DJ, Liu Z, Xu J, Messina JL, Clemens TL. Disruption of the insulin-like growth factor type 1 receptor in osteoblasts enhances insulin signaling and action. *Journal of Biological Chemistry* 2007;282:25649-58.

232. Zhang H, Pelzer AM, Kiang DT, Yee D. Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin. *Cancer research* 2007;67:391-7.

233. Entingh-Pearsall A, Kahn CR. Differential Roles of the Insulin and Insulin-like Growth Factor-I (IGF-I) Receptors in Response to Insulin and IGF-I*[boxs]. *Journal of Biological Chemistry* 2004;279:38016-24.

234. Engberding N, San Martín A, Martin-Garrido A, Koga M, Pounkova L, Lyons E *et al.* Insulin-like growth factor-1 receptor expression masks the antiinflammatory and glucose uptake capacity of insulin in vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* 2009;29:408-15.

235. Raile K, Klammt J, Schneider A, Keller A, Laue S, Smith R *et al.* Clinical and Functional Characteristics of the Human Arg59Ter Insulin-Like Growth Factor I Receptor (IGF1R) Mutation: Implications for a Gene Dosage Effect of the Human IGF1R. *The Journal of Clinical Endocrinology & Metabolism* 2006;91:2264-71.

236. Junnila RK, List EO, Berryman DE, Murrey JW, Kopchick JJ. The GH/IGF-1 axis in ageing and longevity. *Nat Rev Endocrinol* 2013;9:366-76.

237. Wolfson R, Chantranupong L, Sabatini D. *mTOR signalling pathway* In, https://www.cellsignal.com/contents/science-pathway-research-pi3k-akt-signaling-resources/mtor-signaling-pathway/pathways-mtor-signaling. Cell Signalling Technology 2008.

238. Xie J, Wang X, Proud CG. mTOR inhibitors in cancer therapy. *F1000Research* 2016;5:2078.

239. Yu J, Yaba A, Kasiman C, Thomson T, Johnson J. mTOR controls ovarian follicle growth by regulating granulosa cell proliferation. *PloS one* 2011;6:e21415.

240. Yang Q, Xi Q, Wang M, Liu J, Li Z, Hu J *et al.* Rapamycin improves the developmental competence of human oocytes by alleviating DNA damage during IVM. *Hum Reprod Open* 2022;2022:hoac050.

CHAPTER 10: APPENDICE

APPENDIX 1: Complete list of PCOS-related drug trials (Phase 0-4)

DRUG	DRUG NAME	PHASE	STATUS	COUNT
DB00331	Metformin	0	Active Not Recruiting	1
DB09126	Chorionic Gonadotropin (Human)	0	Completed	1
DB00882	Clomifene	0		1
DB04839	Cyproterone acetate	0		1
DB06785	Ganirelix	0		1
DB00644	Gonadorelin	0		1
DB01006	Letrozole	0		2
DB00331	Metformin	0		5
DB06710	Methyltestosterone	0		1
DB01132	Pioglitazone	0		4
DB00421	Spironolactone	0		1
DB00624	Testosterone	0		1
DB00945	Acetylsalicylic acid	0	Not Yet Recruiting	1
DB00378	Dydrogesterone	0		1
DB13956	Estradiol valerate	0		1
DB00097	Choriogonadotropin alfa	0	Recruiting	1
DB01285	Corticotropin	0		1
DB00783	Estradiol	0		3
DB00499	Flutamide	0		1
DB00331	Metformin	0		2
DB00717	Norethisterone	0		1
DB00396	Progesterone	0		2
DB00421	Spironolactone	0		3
DB01284	Tetracosactide	0		1
DB00158	Folic acid	0	Unknown Status	1
DB11196	Inositol	0		1
DB13178	Inositol	0		1
DB00331	Metformin	0		2
DB00097	Choriogonadotropin alfa	0	Withdrawn	1
DB01234	Dexamethasone	0		1
DB00741	Hydrocortisone	0		2
DB00007	Leuprolide	0		1
DB01284	Tetracosactide	0		1
DB00499	Flutamide	1	Active Not Recruiting	1
DB00331	Metformin	1	Completed	1
DB00396	Progesterone	1		1
DB01006	Letrozole	1	Not Yet Recruiting	1
DB00644	Gonadorelin	1	Recruiting	1
DB00331	Metformin	1		1
DB08893	Mirabegron	1		1

DB00396	Progesterone	1		1
DB13961	Fish oil	1	Suspended	1
DB00331	Metformin	1		1
DB01167	Itraconazole	1	Terminated	1
DB01399	Salsalate	1		1
DB00882	Clomifene	1	Unknown Status	1
DB01395	Drospirenone	1		1
DB00783	Estradiol	1		1
DB00977	Ethinylestradiol	1		1
DB13981	Nomegestrol acetate	1		1
DB08907	Canagliflozin	1 / 2	Completed	1
DB00331	Metformin	1 / 2		1
DB00882	Clomifene	1 / 2	Not Yet Recruiting	1
DB11094	Vitamin D	1 / 2		1
DB15726	Human Umbilical Cord Mesenchymal Stem Cells	1 / 2	Recruiting	1
DB00331	Metformin	1 / 2		1
DB01261	Sitagliptin	1 / 2		1
DB00158	Folic acid	1 / 2	Unknown Status	1
DB15823	Lactobacillus acidophilus	1 / 2		1
DB16540	Lactobacillus delbrueckii bulgaricus	1 / 2		1
DB08842	Acetylcarnitine	2	Completed	1
DB06151	Acetylcysteine	2		1
DB00026	Anakinra	2		1
DB01076	Atorvastatin	2		1
DB00882	Clomifene	2		1
DB04839	Cyproterone acetate	2		1
DB11979	Elagolix	2		1
DB00977	Ethinylestradiol	2		1
DB01276	Exenatide	2		1
DB00007	Leuprolide	2		1
DB00583	Levocarnitine	2		2
DB15048	Licogliflozin	2		1
DB00331	Metformin	2		8
DB01083	Orlistat	2		1
DB01132	Pioglitazone	2		2
DB02709	Resveratrol	2		1
DB00421	Spironolactone	2		1
DB11094	Vitamin D	2		1
DB00882	Clomifene	2	Recruiting	1
DB00499	Flutamide	2		1
DB13178	Inositol	2		1
DB00331	Metformin	2		2
DB00806	Pentoxifylline	2		1

DB01132	Pioglitazone	2		1
DB01399	Salsalate	2		1
DB00421	Spironolactone	2		1
DB00169	Cholecalciferol	2	Suspended	1
DB11692	Pavinetant	2	Terminated	1
DB09126	Chorionic Gonadotropin (Human)	2	Unknown Status	1
DB00882	Clomifene	2		3
DB04839	Cyproterone acetate	2		1
DB00977	Ethinylestradiol	2		1
DB00066	Follitropin	2		1
DB09024	Follitropin	2		1
DB01050	Ibuprofen	2		1
DB13178	Inositol	2		1
DB01006	Letrozole	2		1
DB00331	Metformin	2		1
DB00094	Urofollitropin	2		1
DB01276	Exenatide	2 / 3	Active Not Recruiting	1
DB00097	Choriogonadotropin alfa	2 / 3	Completed	1
DB00882	Clomifene	2 / 3		1
DB15350	D-chiro-Inositol	2 / 3		1
DB09038	Empagliflozin	2 / 3		1
DB13178	Inositol	2 / 3		1
DB01006	Letrozole	2 / 3		1
DB00331	Metformin	2 / 3		3
DB00396	Progesterone	2 / 3		1
DB00421	Spironolactone	2 / 3		1
DB16447	Thymoquinone	2 / 3		1
DB11340	Ubiquinol	2 / 3		1
DB00331	Metformin	2 / 3	Not Yet Recruiting	1
DB13928	Semaglutide	2 / 3		1
DB13928	Semaglutide	2 / 3	Recruiting	1
DB00331	Metformin	2 / 3	Unknown Status	1
DB06292	Dapagliflozin	3	Active Not Recruiting	1
DB00630	Alendronic acid	3	Completed	1
DB13100	Biguanide	3		1
DB00050	Cetrorelix	3		3
DB00169	Cholecalciferol	3		1
DB00097	Choriogonadotropin alfa	3		3
DB09126	Chorionic Gonadotropin (Human)	3		3
DB00882	Clomifene	3		4
DB06292	Dapagliflozin	3		1
DB06699	Degarelix	3		1
DB01234	Dexamethasone	3		1

DB00254	Doxycycline	3		1
DB01395	Drospirenone	3		3
DB00977	Ethinylestradiol	3		3
DB01276	Exenatide	3		1
DB00066	Follitropin	3		1
DB09024	Follitropin	3		1
DB00644	Gonadorelin	3		3
DB00030	Insulin human	3		1
DB01006	Letrozole	3		3
DB06655	Liraglutide	3		1
DB00331	Metformin	3		16
DB00191	Phentermine	3		1
DB00481	Raloxifene	3		1
DB06335	Saxagliptin	3		1
DB00641	Simvastatin	3		1
DB00273	Topiramate	3		1
DB06825	Triptorelin	3		1
DB00050	Cetrorelix	3	Not Yet Recruiting	1
DB00882	Clomifene	3		1
DB00644	Gonadorelin	3		1
DB00603	Medroxyprogesterone acetate	3		1
DB09066	Corifollitropin alfa	3	Recruiting	1
DB00304	Desogestrel	3		1
DB00066	Follitropin	3		1
DB01006	Letrozole	3		1
DB00331	Metformin	3		1
DB00331	Metformin	3	Terminated	1
DB01200	Bromocriptine	3	Unknown Status	1
DB00882	Clomifene	3		1
DB01395	Drospirenone	3		1
DB00977	Ethinylestradiol	3		1
DB00158	Folic acid	3		1
DB13178	Inositol	3		1
DB00882	Clomifene	3	Withdrawn	1
DB00331	Metformin	3		1
DB11638	Artenimol	4	Active Not Recruiting	1
DB00331	Metformin	4		2
DB01132	Pioglitazone	4		1
DB06203	Alogliptin	4	Completed	1
DB08907	Canagliflozin	4		1
DB00169	Cholecalciferol	4		3
DB00882	Clomifene	4		10
DB04839	Cyproterone acetate	4		2

DB00151	Cysteine	4		1
DB01395	Drospirenone	4		4
DB09045	Dulaglutide	4		1
DB01175	Escitalopram	4		1
DB13956	Estradiol valerate	4		1
DB00977	Ethinylestradiol	4		6
DB01276	Exenatide	4		2
DB00158	Folic acid	4		4
DB00066	Follitropin	4		1
DB09024	Follitropin	4		1
DB00644	Gonadorelin	4		1
DB13178	Inositol	4		1
DB00030	Insulin human	4		1
DB11629	Laropiprant	4		1
DB01006	Letrozole	4		3
DB00007	Leuprolide	4		1
DB00583	Levocarnitine	4		1
DB11256	Levomefolic acid	4		1
DB06655	Liraglutide	4		9
DB00032	Menotropins	4		2
DB00331	Metformin	4		30
DB09242	Moxonidine	4		1
DB00627	Niacin	4		1
DB09389	Norgestrel	4		1
DB01132	Pioglitazone	4		2
DB00396	Progesterone	4		1
DB01656	Roflumilast	4		2
DB00412	Rosiglitazone	4		1
DB13928	Semaglutide	4		1
DB01105	Sibutramine	4		1
DB00641	Simvastatin	4		1
DB01261	Sitagliptin	4		3
DB00675	Tamoxifen	4		1
DB11094	Vitamin D	4		4
DB09145	Water	4		1
DB00331	Metformin	4	Enrolling by Invitation	1
DB00050	Cetrorelix	4	Recruiting	2
DB09126	Chorionic Gonadotropin (Human)	4		2
DB00882	Clomifene	4		1
DB01395	Drospirenone	4		1
DB09038	Empagliflozin	4		1
DB00977	Ethinylestradiol	4		2
DB00294	Etonogestrel	4		1

DB01006	Letrozole	4		1
DB08882	Linagliptin	4		1
DB00331	Metformin	4		3
DB06825	Triptorelin	4		2
DB00169	Cholecalciferol	4	Terminated	1
DB00882	Clomifene	4		1
DB00783	Estradiol	4		1
DB13178	Inositol	4		1
DB00007	Leuprolide	4		1
DB00032	Menotropins	4		1
DB00331	Metformin	4		3
DB01132	Pioglitazone	4		1
DB11094	Vitamin D	4		1
DB00284	Acarbose	4	Unknown Status	1
DB00050	Cetrorelix	4		1
DB09126	Chorionic Gonadotropin (Human)	4		1
DB00882	Clomifene	4		4
DB04839	Cyproterone acetate	4		3
DB01395	Drospirenone	4		1
DB00977	Ethinylestradiol	4		3
DB01276	Exenatide	4		3
DB00158	Folic acid	4		1
DB06785	Ganirelix	4		1
DB00644	Gonadorelin	4		1
DB00007	Leuprolide	4		1
DB06655	Liraglutide	4		1
DB00331	Metformin	4		10
DB01083	Orlistat	4		2
DB00860	Prednisolone	4		1
DB01105	Sibutramine	4		1
DB00273	Topiramate	4		1
DB06825	Triptorelin	4		1
DB11094	Vitamin D	4		1
DB09126	Chorionic Gonadotropin (Human)	4	Withdrawn	1
DB00882	Clomifene	4		2
DB13956	Estradiol valerate	4		1
DB01006	Letrozole	4		1
DB00603	Medroxyprogesterone acetate	4		1
DB00331	Metformin	4		2
DB00396	Progesterone	4		1
DB00050	Cetrorelix	N/A	Active Not Recruiting	1
DB00097	Choriogonadotropin alfa	N/A		1
DB09126	Chorionic Gonadotropin (Human)	N/A		1

DB04839	Cyproterone acetate	N/A		1
DB00783	Estradiol	N/A		2
DB00977	Ethinylestradiol	N/A		1
DB00644	Gonadorelin	N/A		1
DB00367	Levonorgestrel	N/A		1
DB00032	Menotropins	N/A		1
DB00396	Progesterone	N/A		1
DB06825	Triptorelin	N/A		1
DB00284	Acarbose	N/A	Completed	2
DB01076	Atorvastatin	N/A		1
DB00248	Cabergoline	N/A		1
DB01373	Calcium	N/A		1
DB00097	Choriogonadotropin alfa	N/A		1
DB09126	Chorionic Gonadotropin (Human)	N/A		1
DB04272	Citric acid	N/A		1
DB00882	Clomifene	N/A		11
DB04839	Cyproterone acetate	N/A		1
DB00151	Cysteine	N/A		1
DB01234	Dexamethasone	N/A		1
DB09341	Dextrose, unspecified form	N/A		1
DB01119	Diazoxide	N/A		1
DB01395	Drospirenone	N/A		2
DB00783	Estradiol	N/A		2
DB13956	Estradiol valerate	N/A		1
DB00977	Ethinylestradiol	N/A		3
DB00499	Flutamide	N/A		1
DB00158	Folic acid	N/A		2
DB09024	Follitropin	N/A		2
DB00644	Gonadorelin	N/A		1
DB11196	Inositol	N/A		1
DB13178	Inositol	N/A		2
DB01006	Letrozole	N/A		5
DB00583	Levocarnitine	N/A		1
DB00166	Lipoic acid	N/A		1
DB00603	Medroxyprogesterone acetate	N/A		1
DB00032	Menotropins	N/A		1
DB00331	Metformin	N/A		24
DB00393	Nimodipine	N/A		1
DB00727	Nitroglycerin	N/A		1
DB09389	Norgestrel	N/A		1
DB01132	Pioglitazone	N/A		1
DB00396	Progesterone	N/A		5
DB00863	Ranitidine	N/A		1

DB00412	Rosiglitazone	N/A		1
DB00641	Simvastatin	N/A		1
DB00421	Spironolactone	N/A		2
DB00624	Testosterone	N/A		1
DB01284	Tetracosactide	N/A		1
DB09270	Ubidecarenone	N/A		1
DB11094	Vitamin D	N/A		2
DB01128	Bicalutamide	N/A	Not Yet Recruiting	1
DB00169	Cholecalciferol	N/A		1
DB00882	Clomifene	N/A		1
DB04839	Cyproterone acetate	N/A		1
DB00977	Ethinylestradiol	N/A		1
DB01006	Letrozole	N/A		1
DB00331	Metformin	N/A		1
DB00125	Arginine	N/A	Recruiting	1
DB00097	Choriogonadotropin alfa	N/A		1
DB04839	Cyproterone acetate	N/A		1
DB01234	Dexamethasone	N/A		2
DB01395	Drospirenone	N/A		1
DB00977	Ethinylestradiol	N/A		1
DB13178	Inositol	N/A		1
DB00331	Metformin	N/A		4
DB01708	Prasterone	N/A		1
DB00421	Spironolactone	N/A		1
DB01284	Tetracosactide	N/A		1
DB11094	Vitamin D	N/A		1
DB00331	Metformin	N/A	Suspended	1
DB00882	Clomifene	N/A	Terminated	2
DB00783	Estradiol	N/A		2
DB01039	Fenofibrate	N/A		1
DB00499	Flutamide	N/A		2
DB00603	Medroxyprogesterone acetate	N/A		2
DB00331	Metformin	N/A		1
DB01132	Pioglitazone	N/A		1
DB00396	Progesterone	N/A		2
DB00481	Raloxifene	N/A		1
DB04115	Berberine	N/A	Unknown Status	2
DB00882	Clomifene	N/A		4
DB04839	Cyproterone acetate	N/A		1
DB06699	Degarelix	N/A		1
DB01395	Drospirenone	N/A		1
DB00783	Estradiol	N/A		1
DB00977	Ethinylestradiol	N/A		1

DB00644	Gonadorelin	N/A		1
DB01006	Letrozole	N/A		5
DB00583	Levocarnitine	N/A		1
DB00166	Lipoic acid	N/A		1
DB01065	Melatonin	N/A		1
DB00331	Metformin	N/A		12
DB00641	Simvastatin	N/A		1
DB06825	Triptorelin	N/A		1
DB00030	Insulin human	N/A	Withdrawn	1
DB00052	Somatotropin	N/A		1

Data was exported from the DrugBank database (162) as of January 5, 2023. The count column represents the number of drug trials for each drug. Each count has a hyperlink to a DrugBank webpage that indicates the ClincalTrials.gov identifier, title and purpose of the related drug trials.